Reproductive Biology and Controlled Reproductive Development of Captive Cobia (Rachycentron canadum)

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Reproductive Biology and Controlled Reproductive Development of Captive Cobia (*Rachycentron canadum*)

Thesis by

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Dedicated to the little people in my life; Alice, Jack and Will.

Love you guys…

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This thesis represents not just my own work, but the collective effort of colleagues and collaborators that I gratefully acknowledge.

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I would like to acknowledge the Department of Agriculture and Fisheries for giving me the opportunity to conduct this research as part of my employment with the department. A big thank you to all of the staff at the Bribie Island Research Centre (BIRC), an excellent facility, but it's the people that make it such an amazing place. I would like to thank Trevor Borchert, David Nixon, Hazra Thaggard and Tania Lymar specifically for their technical assistance, dedication and hard work to make cobia production a reality. Thanks also to Ido Bar for assistance with lab techniques both at BIRC and the university and for compiling the thesis. Thank you to Dave Mayer for his assistance with statistical analysis and to Warwick Nash for supporting this endeavour and providing feedback on manuscripts.

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Abstract

Cobia aquaculture began in Australia in 2007; however, expansion of commercial production has been limited, due in part to low and inconsistent supply of seed stock for on-growing. This study aimed to address the constraints of reproductive performance of cobia in captive conditions and investigate strategies to improve the efficiencies of broodstock management in commercial and research facilities.

In a study evaluating the growth of three cohorts of captive reared cobia, to determine the existence and extent of sexually dimorphic growth, intersex development in cobia was identified and recorded for the first time. There was no sexually dimorphic growth in the first two cohorts of fish. In contrast, females from third cohort were significantly larger than males once mean body weight exceeded 2 kg. It is proposed that the observed variations in growth and gonad malformations observed in cohorts 1 and 2 were the result of exposure to endocrine disruptors, the type and source of which remains unknown.

In order to quantify the ovarian development of cobia, there was a need to develop an accurate method of assessing and quantifying ovarian maturation in the presence of asynchronous development. A simple, commercially applicable methodology was devised that used the proportional distribution of different oocyte stages to describe the developmental state of the ovary.

Two trials were conducted to examine the effect of repeated injections of luteinising hormone releasing hormone analogue (LHRHa) and human chorionic gonadotropin (hCG) on stimulating vitellogenesis, supporting ovarian maturation and levels of circulating 17β-estradiol (E2). Ovarian development was highly variable within treatments and those fish that initiated vitellogenesis continued to develop regardless of treatment with exogenous hormones. There was no change in E2 concentrations as a result of LHRHa injections and limited evidence to suggest a change in plasma E2 in response to hCG injection. While

hormonal therapy is effective in inducing spawning in cobia, the results suggest that hormone therapy is not an effective approach to initiating or supporting early stage ovarian development in cobia.

Two cohorts of cobia broodstock were assessed to examine ovarian development and circulating E2 in response to photothermal manipulation. In each study, broodstock were subjected to either a temporally compressed regime or an ambient regime. In the first study ovarian development was generally limited, irrespective of the phototherm regime and there was no significant difference in development between treatments. At the completion of the second trial there was no significant difference in ovarian development between the compressed and ambient phototherm; however, fish in the compressed phototherm were found to develop earlier in the season than those in ambient conditions. Fish in the first trial showed sporadic development in which ovarian samples contained low numbers of late stage oocytes amongst a large percentage of previtellogenic oocytes, possibly due to exposure to endocrine disruption in the early life history of the cohort.

Two cohorts of captive reared cobia were progressively examined as pre- and post-pubescent fish to examine the suitability of identifying gender by analysing the androgen 11-ketotestosterone (11KT) in blood and fin clip samples. The gender of individual cobia could be identified by analysing plasma 11KT between the months of October and March, provided the mean population weight was 2 kg or above. The measurement of 11KT concentrations in fin clip samples did not provide an accurate indication of plasma 11KT and as such, was not suitable for predicting gender in cobia. Overall the relative cost, infrastructure and equipment required to conduct steroid analysis limits the application of this methodology in commercial cobia production in comparison to the traditional method of gonadal biopsy.

Declaration of Originality

The work presented in this study does not contain any material which has been previously published or written by any person other than the candidate, except where due and proper reference has been given in the text. All experimental work described was conducted and analysed by Luke Dutney unless otherwise stated.

Relative contributions of the respective authors in joint publications:

- Dutney, L., Elizur, A. and Lee, P. (submitted Dec 12, 2015, under review). **Analysis of sexually dimorphic growth in captive reared cobia (*Rachycentron canadum*) and the occurrence of intersex individuals**. Aquaculture. LD participated in the design of the study and drafted the manuscript. AE provided feedback on the design of the study, assisted with data collection and assisted in drafting the manuscript. PL provided feedback on the design of the study, assisted with data collection and analysis and assisted in drafting the manuscript.
- Dutney, L., Elizur, A. and Lee, P. (to be submitted to Aquaculture). **Hormonal manipulation strategies to enhance reproductive development in cobia (*Rachycentron canadum*).** LD participated in the design of the study and drafted the manuscript. AE provided feedback on the design of the study, assisted with data collection and assisted in drafting the manuscript. PL provided feedback on the design of the study, assisted with data collection and analysis and assisted in drafting the manuscript.
- Dutney, L., Elizur, A. and Lee, P. (to be submitted to Aquaculture). **The influence of photothermal manipulation on reproductive development of captive cobia (*Rachycentron canadum*).** LD participated in the design of the study and drafted the manuscript. AE provided feedback on the design of the study, assisted with data

collection and assisted in drafting the manuscript. PL provided feedback on the design of the study, assisted with data collection and analysis and assisted in drafting the manuscript.

- Dutney, L., Elizur, A. and Lee, P. **Identification of gender in captive reared cobia (*Rachycentron canadum*) using 11-Ketotestosterone analysis.** LD participated in the design of the study and drafted the manuscript. AE provided feedback on the design of the study, assisted with data collection and assisted in drafting the manuscript. PL provided feedback on the design of the study, assisted with data collection and analysis and assisted in drafting the manuscript.

I hereby declare that the content of the above statement is accurate:

Signature: Date: April 1, 2016

Research Ethics

This project was conducted at the Department of Agriculture and Fisheries (DAF) Bribie Island Research Centre. As such, a multi-agency animal ethics committee (AEC) approval between DAF and the University of the Sunshine Coast (USC) was required. For the purposes of AEC application and approval, the project operated under the title of "***Towards the development of an Australian cobia aquaculture industry***".

DAF AEC approval number: **CA 2011/10/548**

USC AEC approval number: **AN/A/12/63**

All progress and final report obligations for both agencies were met during and at the completion of experimental work.

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Dutney, L., Elizur, A. and Lee, P. 2017. Analysis of sexually dimorphic growth in captive reared cobia (*Rachycentron canadum*) and the occurrence of intersex individuals. *Aquaculture,* **468**: 348-355. http://dx.doi.org/10.1016/j.aquaculture.2016.09.044

Chapter 1: General Introduction

1.1 Cobia background

Cobia, *Rachycentron canadum*, is a large benthopelagic fish species, endemic to all tropical and subtropical waters across the globe with the exception of the eastern Pacific (Shaffer and Nakamura 1989). It belongs to the order Perciformes and is the only species within the family Rachycentridae. Cobia can attain a length of up to 2 metres and exceed 60 kilograms (Franks et al. 1999). They have an elongated fusiform body and broad, flattened heads. The body is smooth with small embedded scales. Body coloration varies from a dark brown dorsal surface grading to a prominently white ventral surface. During juvenile and adolescent stages, a prominent white stripe runs the entire length of the mid-section, which becomes less obvious as the fish ages (FAO 2016). Cobia are opportunistic carnivores, feeding on cephalopods, fish and crustaceans (Salini et al. 1994).

Cobia is a highly prized sport fish in Australia and the USA. They rarely occur in large numbers and as such are not heavily exploited by commercial fisheries. The commercial catch of cobia from Australian waters is relatively small at less than 30 metric tonnes per annum (van der Velde et al. 2010).

1.1.1 Cobia aquaculture

Several biological attributes make cobia an exceptional candidate for aquaculture including: growth rates that exceed 5 kg per year, adaptability to commercially available aquafeeds, excellent palatability and temperature and salinity tolerance (Holt et al. 2007b, Shiau 2007, Weirich et al. 2007). Commercial cobia aquaculture began in Taiwan in the late 1990s and has since been adopted by several nations through the Asia-Pacific, USA and South America (Liao et al. 2007, Benetti et al. 2008a, Nhu et al. 2011, Sampaio et

al. 2011). Global production is dominated by China, which produces approximately 39,600 of the global annual production of 43,400 metric tonnes (FAO 2013). Although China produces the majority of cobia globally, the most detailed information on large scale commercial production of cobia comes from Taiwan.

Cobia production in Taiwan involves several different phases (Figure 1.1). The production cycle begins with the selection and transfer of mature broodstock from ocean cages to spawning ponds (400-600 m^2; 1.5 m deep). Approximately 100 brood fish are maintained in ponds and are allowed to spawn spontaneously. Fertilised eggs are collected and then transferred to extensive pond systems where they hatch and are raised for approximately 20 days. Two nursery stages are then incorporated into the production cycle before the fish are moved to nursery sea cages. Fish are moved to grow out cages when they are 600-1000 g and reach 6-10 kg in 6-8 months in this final production stage before being sent to market (Liao et al. 2004).

Cobia production in Vietnam has increased rapidly in recent years to exceed 1000 T in 2012 (FAO 2013). The production model used is a similar production model to that of Taiwan, however more intensive hatchery and juvenile production methods are used (Nhu et al. 2011). Pilot scale production in the Caribbean using submersible seacages stocked with fingerlings produced in a land-based hatchery has also shown promising results (Benetti et al. 2010), with 980 T produced in 2013 (FAO 2013).

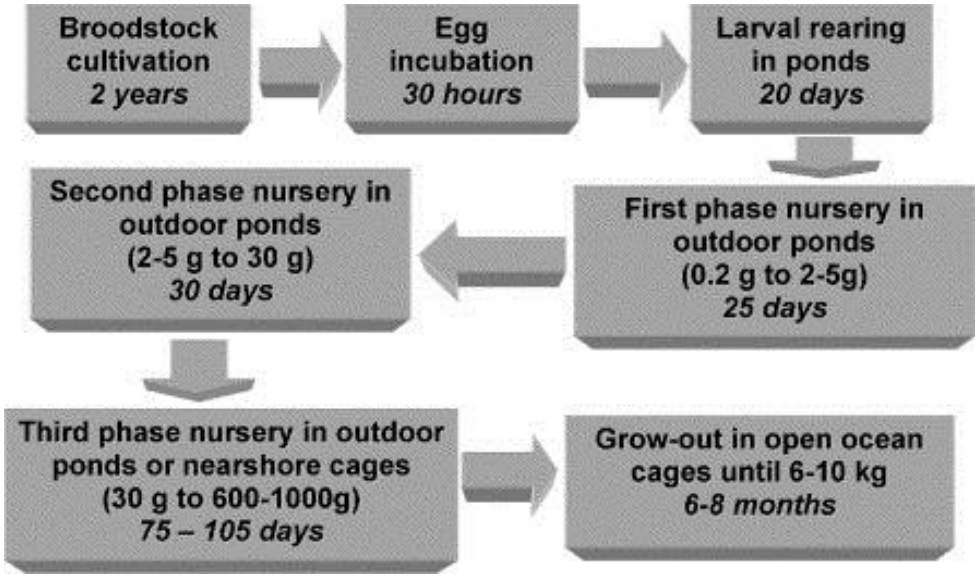

Figure 1.1 Flowchart of cobia aquaculture in Taiwan (Liao et al. 2004).

1.1.2 Cobia aquaculture in Australia

Cobia research and development began in Australia in 2007, focusing largely on introducing cobia as an alternative and off season crop for prawn farms in Queensland (Dutney and Palmer 2008). Trials conducted at the Bribie Island Research Centre (BIRC) demonstrated the feasibility of juvenile production of cobia using hatchery infrastructure and techniques similar to those used to produce other marine and estuarine species at the site. The broodstock management and juvenile production strategy used at BIRC is described in Figure 1.2. As part of a collaborative research partnership, juvenile cobia were supplied to commercial prawn farms to test the commercial viability of cobia production in prawn grow-out ponds. These trials where the first to demonstrate the technical feasibility of cobia grow-out production in prawn ponds and provided evidence of higher productivity yields in tropical localities (Dutney et al. 2010).

Despite the excellent potential and demonstrated feasibility of cobia production, the development of commercial cobia aquaculture in Australia has been relatively limited, due in part to low or inconsistent supply of seed stock for grow out.

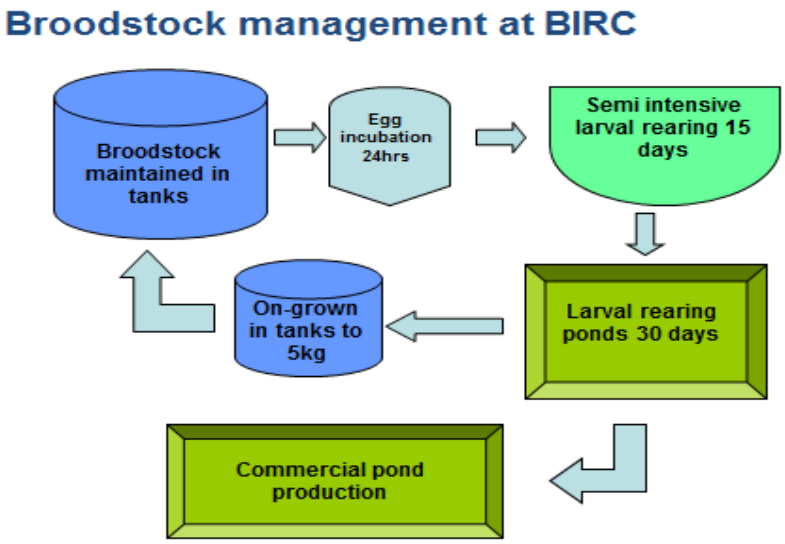

Figure 1.2 The broodstock management and juvenile production strategy used at the Bribie Island Research Centre (BIRC).

1.2 Teleost reproduction

1.2.1 The ovary

The ovary of teleost fish is most commonly a hollow paired cylindrical organ, attached to the body cavity on either side of the dorsal mesentery. The ovarian cavity is continuous with the oviduct which opens into the ovipore at the urogenital papilla (Zohar 1989). The ovary consists of two particular cell types; somatic cells and germinal line cells, along with vascular and nervous tissue. Somatic cells form the ovarian structures, such as ovarian capsule, supportive tissue and ovarian follicles (Kagawa 2013). Germinal line cells consist of oogonia and oocytes, which are surrounded by the follicular layer referred to as granulosa cell layer, which is responsible for the synthesis of steroid converting enzymes (Nagahama et al. 1994). The thecal cell layer, that forms the outer layer of the follicular envelope of developing oocytes, contains fibroblasts, collagen fibres, blood vessels and steroidogenic "special thecal cells" (Figure 1.3) (Nagahama 1983).

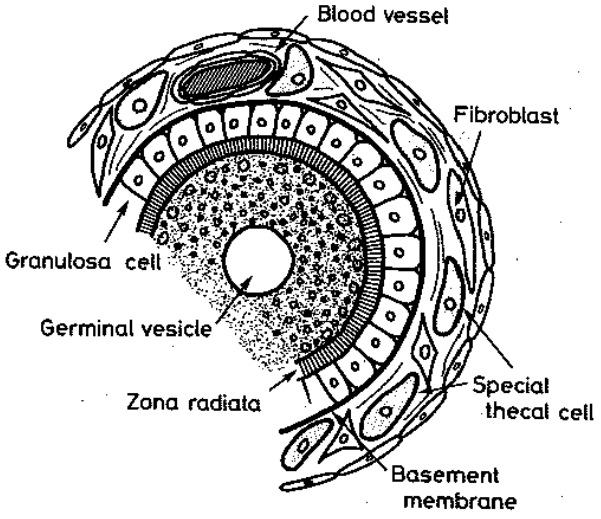

Figure 1.3 Diagrammatic representation of the follicle layer of an early vitellogenic ovary in a salmonid (Nagahama 1983).

Three main types of ovarian development are generally observed in fish and have been described in detail by Wallace and Selman (1981):

- Synchronous: all oocytes develop and ovulate in unison and all the eggs are spawned in a single event.
- Group synchronous: two or more distinct groups of oocytes at different developmental stages are present concurrently and fish are capable of multiple distinct spawning events associated with seasonal or lunar cycles.
- Asynchronous: oocytes at all stages of development and ovulation can occur continually or over a protracted period.

1.2.2 The hypothalamus-pituitary-gonadal-axis

The hypothalamus-pituitary-gonadal-axis (HPG) plays a central role in the reproductive function of fish including oogenesis and reproductive behaviour (Figure 1.4). The HPG axis activates in response to environmental, social and endogenous stimuli. The hypothalamic neurons synthesise and release gonadotropin releasing hormone (GnRH),

which in turn regulates the gonadotropic pituitary cells to synthesise and release the gonadotropins; luteinising hormone (LH) and follicle stimulating hormone (FSH). The release of gonadotropins is inhibited by the release of dopamine and gonadotropin inhibiting hormones (GnIH). The synthesis of sex steroid hormones by the gonads is then stimulated by FSH and LH to elicit and regulate gonadal development (Mylonas et al. 2010).

In female fish, 17β-estradiol (E2), plays a major role in oocyte growth with increasing concentration of plasma E2 associated with early development, before a shift to increased levels of 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) associated with oocyte maturation (Nagahama 1994). The androgen 11-ketotestosterone (11KT) plays a major role in spermatogenesis (Nagahama 1994, Devlin and Nagahama 2002). Both testosterone (T) and 11KT tend to increase steadily during the quiescent period, with a rapid increase associated with the peak of the reproductive cycle (Mylonas et al. 2013). However; 11KT often occurs at significantly higher levels than T during the peak of the cycle, most likely as a function of the role of T in the synthesis of 11KT (Scott et al. 1980b). Maturation of male fish is also controlled by 17,20βP, evidenced by a steroidogenic shift from 11KT to increased levels of 17,20βP with the onset of spermiation (Nagahama 1999).

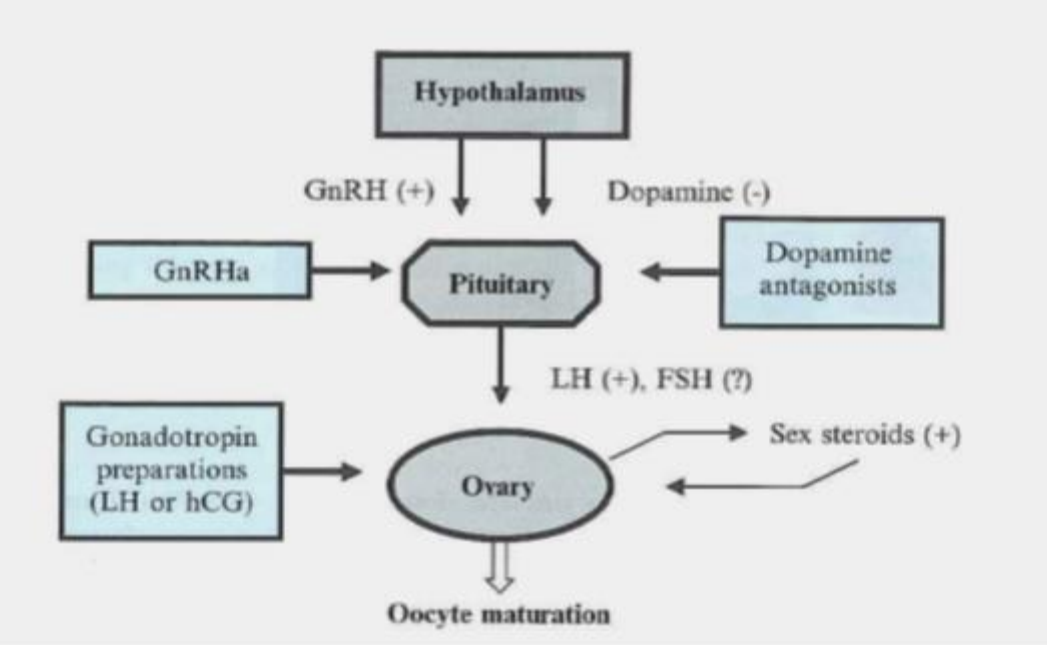

Figure 1.4 Schematic diagram of the hypothalamus-pituitary-gonad axis and effecting gonadotropins releasing hormones .
The hypothalamus-pituitary-gonad axis (in grey) and the level at which gonadotropins releasing hormones (GnRH), dopamine, luteinising hormone (LH), follicle stimulating hormone (FSH) and sex steroids act. The exogenous hormone treatments (discussed in the following section) are shaded in blue: gonadotropin releasing hormone analogue (GnRHa), luteinising hormone (LH) and human chorionic gonadotropin (hCG), and their points of influence are shaded grey (Mylonas and Zohar 2007).

1.2.3 Oogenesis

Oogenesis refers to the process by which primordial germ cells develop to form a mature ovum, capable of being fertilised to form a viable embryo (Figure 1.5). The early stages of oogenesis involve the proliferation of oogonia via successive mitotic divisions. Oogonia then enter meiosis, which is halted at the diplotene stage of the first meiotic prophase. At this point they are referred to as primary oocytes (Zohar 1989). Oocytes then undergo a period of rapid growth, primarily due to the process of yolk accumulation, referred to as vitellogenesis. Thecal cells are stimulated by FSH or LH to produce testosterone which is then converted to 17β-estradiol (E2), in the presence of aromatase enzymes, in the granulosa (Nagahama et al. 1994). In response to increased circulating concentrations of E2, the liver synthesises and releases the glycolipophosphoprotein, vitellogenin, into the

plasma. Vitellogenins are sequestered by the developing oocytes through receptor mediated endocytosis to form the yolk protein of the oocyte, which becomes the nutritional reserve for the developing embryo (Lubzens et al. 2010). The relationship between E2 and vitellogenic development facilitates the measurement of circulating E2 as an effective indicator of ovarian development in a number of fish species (Scott et al. 1980a, King and Pankhurst 2003, Scott et al. 2013).

Following vitellogenesis, there is a shift in steroid production in the gonad, from E2 to the maturation inducing hormone (MIH) 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP), at which point the oocyte enters the oocyte maturation phase (Nagahama 1999). Oocyte maturation involves several processes to facilitate ovulation and subsequent fertilisation and is characterised by the reduction or stoppage of endocytosis, germinal vesicle migration and breakdown, and the resumption of meiosis. Within the cytoplasm the yolk globules fuse and the lipid droplets coalesce. The oocyte increases in size and becomes transparent as it undergoes hydration. During ovulation the oocyte is expelled from the surrounding follicular cell layers and into the ovarian lumen. Following the second stage of meiotic division, the eggs are ready to be fertilised (Zohar 1989, Lubzens et al. 2010). In oviparous fish the eggs are then spawned and fertilised externally.

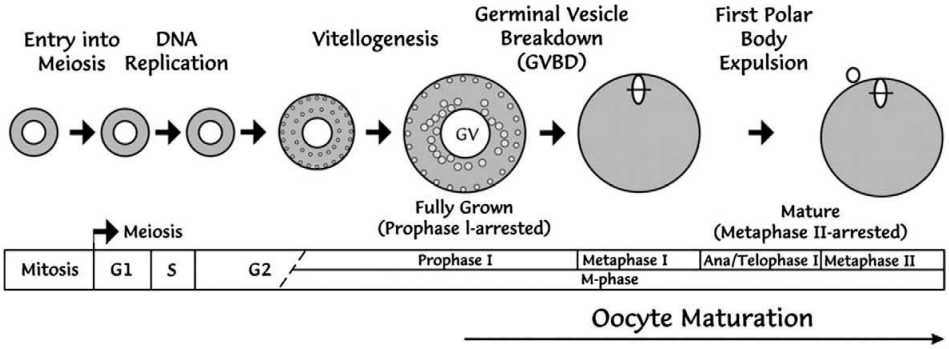

Figure 1.5 Oocyte development from primary oocytes to vitellogenesis and mature oocytes (Suwa and Yamashita 2007).

1.3 Control of reproduction in captive broodstock

Gaining control of the reproductive process of a species is a prerequisite for the development of a sustainable aquaculture industry. The reliance upon wild caught seed through the collection of juveniles or capture of gravid broodstock can be unpredictable and unreliable and ultimately is not sustainable. In order for genuine commercialisation and sustainable industrialisation of a species to occur, the life cycle must be closed in captivity to provide a regular supply of high quality seed stock for the on-growing of marketable product. This also enables selective breeding to improve production efficiency through genetic improvements in traits such as growth, survival, appearance and product quality.

1.3.1 Reproductive dysfunction in captive fish

As with many wild animals, a majority of fish species held in captivity experience reproductive dysfunction to some extent (Mylonas and Zohar 2007). Reproductive dysfunction in some fish species may be as simple as reduced spermiation in male fish, but in female fish, it is generally more problematic (Mylonas and Zohar 2007). Zohar and Mylonas (2001) described three categories of reproductive dysfunction in female fish:

- An absence of, or only partial, vitellogenic development; insufficient to induce ovulation and spawning using exogenous hormones.
- Failure of post-vitellogenic oocytes to undergo oocyte maturation and ovulation.
- Absence of spawning at the end of the reproductive cycle.

Whilst captivity and the associated human presence itself can be a stressor that may inhibit gonadal maturation, captive reared fish also often miss critical environmental and/or social cues responsible for initiating gonad maturation and spawning (Mylonas et

al. 2010). The environmental parameters required for some species to spawn often cannot be replicated in captivity. Many salmonid species, for example, partake in mass spawning migrations in which they are exposed to vast changes in hydrology, salinity, temperature, water depth and substrate. Certain tropical species are reliant on rainfall and floods to stimulate and synchronise spawning (Lam 1983). However, in many cases the reproductive dysfunctions of captive finfish broodstock have been overcome through manipulation of the environment, or the use of exogenous hormones.

1.3.2 External influences on reproductive development

Genetics, diet, environmental parameters and pollutants can all result in impaired reproduction and gonad malformation in both farmed and wild fish. Inbreeding depression has been linked to impaired reproductive performance in some salmonid species, demonstrated by a reduction in gonadosomatic index, fecundity and survival of eggs (Su et al. 1996, Gallardo et al. 2004). The occurrence of intersex gonads in wild fish populations has been associated with a wide range of environmental pollutants (Jobling et al. 2002), broadly grouped as endocrine disrupting chemicals (EDC). These compounds are capable of interfering with the action and/or synthesis of endogenous steroid hormones. The result is an imbalance of male and female hormones which may manifest as reproductive abnormalities such as increased levels of circulating vitellogenin in male fish, reduced reproductive performance and intersex fish (Purdom et al. 1994, Bergman et al. 2013, Kroon et al. 2015). The impacts of EDCs are heightened at critical developmental stages driven by circulating steroidal hormones, such as sexual differentiation and puberty, and the impacts are often irreversible (Bergman et al. 2013). Endocrine disrupting chemicals that have an estrogenic effect have been found in the effluent from waste water treatment facilities and other anthropogenic influences. Such

compounds include synthetic estrogens, such as ethynyl estradiol, used in birth control pills and compounds associated with pesticides such as organochlorine and organophosphate (Sumpter 1999, Senthilkumaran 2015). Exposure to potential EDCs may also occur via other paths. Soy bean meal has been demonstrated as a suitable alternative to fish meal for a number of marine species, including cobia (Suarez et al. 2013). A potential drawback of the use of such plant-based proteins is the presence of estrogen-mimicking compounds, also known as phytoestrogens. In particular, the isoflavone genistein has been demonstrated to have a feminising effect and an associated impact on reproductive performance of a number on rainbow trout, *Oncorhynchus mykiss* (Bennetau-Pelissero et al. 2001). The presence of genistein has also been associated with increased incidence of intersex in channel catfish, *Ictalurus punctatus* (Green and Kelly 2009) and reduction of secondary sex characteristics in Japanese medaka, *Oryzias latipes* (Kiparissis et al. 2003).

The sex determining mechanism in some fish species are influenced by environmental parameters, which can result in skewed sex ratios within a population. Variations in larval rearing temperature have been shown to affect sex ratios in sea bass, *Dicentrachus labrax*, channel catfish, *Paralichthys olivaceus*, and Nile tilapia, *Oreochromis niloticus* (Baroiller et al. 1995, Patiño et al. 1996, Pavlidis et al. 2000).

1.3.3 Environmental control and manipulation

Most fishes, in their natural environment, display some degree of seasonality in reproductive activity. Fish endemic to temperate and cold water environments are exposed to pronounced temperature and photoperiod fluctuations through the seasons; whereas those in the tropics experience lower variability in the annual phototherm. Coordinating the reproductive cycle with seasonal changes in environmental parameters

ensures that spawning coincides with the conditions most suited for the growth and survival of juveniles (Bromage et al. 2001). The higher amplitude of variation in temperate environs necessitates an acute response to environmental stimuli and greater synchrony in a spawning population to ensure that the short period of suitable conditions for larval survival are exploited (Pankhurst and Porter 2003). Photoperiod is capable of providing an unambiguous date signal and is considered to be the principle environmental determinant to stimulate reproductive development in temperate species; while temperature has a role in cueing the precise timing of maturation and spawning (Bromage, Porter et al. 2001). The importance of photoperiod as a determinant of reproductive development is most clearly illustrated in salmonids where the entire reproductive cycle can be entrained by manipulation of photoperiod alone (Pankhurst and Porter 2003).

Due to the limited variation in the annual photoperiod and temperature in the tropics, the environmental cues associated with reproductive development are more subtle. Whilst both photoperiod and temperature remain as likely proximate cues for gonad development, it is likely that temperature has a greater influence than photoperiod (Zohar 1989, Pankhurst and Porter 2003, Stieglitz et al. 2012). Spawning activity in the tropics may also coincide with periods of increased ecosystem productivity following high rainfall or shifts in oceanic currents (Bromage et al. 2001, van der Velde et al. 2010). Such cues may be less predictable than photoperiod changes, but signal an acute environmental change indicating appropriate conditions for reproduction (Pankhurst and Porter 2003). Other environmental factors that may influence reproductive development in both temperate and tropical regions include; broodstock nutrition, the lunar cycle, suitable substrate, social interaction and pheromonal influence (Figure 1.6) (Pankhurst and Porter 2003).

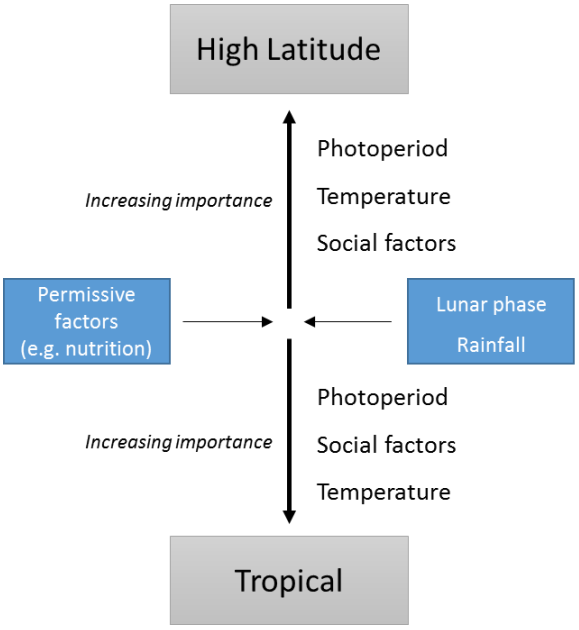

Figure 1.6 The relationship between latitude and proximate factors that influence reproductive activity (Pankhurst and Porter 2003).

Photothermal manipulation has been used to improve broodstock management by extending the production seasons and facilitating the out-of-season spawning of a large range of fish species. This allows for regular seed supply for on growing and enables producers to meet the market requirement for annual supply of product. The spawning season of Atlantic salmon (*Salmo salar*) is extended by implementing an accelerated photoperiod (Taranger et al. 1998), while exposure to low water temperature prior to ovulation will further advance and synchronise spawning (Taranger et al. 1999). Other examples photothermal manipulation to improve reproductive outputs include; out of season larval supply of striped trumpeter (*Latris lineata*) and gilthead sea bream (*Sparus aurata*) being facilitated by exposing broodstock to a compressed phototherm regime (Zohar et al. 1995, Morehead et al. 2000). Precise control of gonadal recrudescence and year-round spawning in southern flounder (*Paralichthys lethostigma*) is also achieved by manipulation of photoperiod and temperature (Watanabe et al. 2006).

1.3.4 Hormonal manipulation

The most common reproductive dysfunction in captive reared fish occurs when the animals undergo vitellogenesis as normal, but fail to undergo final oocyte maturation (FOM) and ovulation, and therefore spawning (Mylonas et al. 2010). As such, the majority of research on hormonal manipulation has focused on this aspect of reproductive development (Zohar and Mylonas 2001). A variety of hormones that act on different sites in the hypolthalamus-pituitary-gonadal-axis have been used to induce ovulation and spawning (Figure 1.4). Even in those species that spawn spontaneously in captivity, hormone induction affords a level of control to provide regular seed production, as well as facilitating more controlled and regulated spawning time that is essential for selective breeding.

The failure of female fish to undergo FOM and ovulation in captivity has been attributed primarily to the dysfunctional release of LH. There is evidence to suggest that in these fish, LH is synthesised and stored in the pituitary but is not released (Mylonas et al. 2010). Reproductive development is arrested at the end of vitellogenesis due to the failure to release LH, suggesting that the problem is not due to pituitary response but related to the control of the pituitary by the hypothalamus (Mylonas et al. 1998, Steven et al. 1999, Mylonas and Zohar 2001). The early application of hormonal therapies to address this issue involved the injection of ground pituitaries or pituitary extracts from reproductively mature fish. This method provides an increase in gonadotropins, primarily LH, to the donor fish, sufficient to stimulate FOM (Mylonas et al. 2010). Although demonstrating success in some studies, the use of fresh or extracted pituitary tissue has a number of drawbacks including variability in LH content and potential disease transmission. Another gonadotropin successfully used in spawning induction of

a multitude of fish species is human chorionic gonadotropin (hCG). As with other gonadotropins, hCG acts at the level of the gonads to stimulate the release of sex steroids, without the drawbacks associated with pituitary extracts. However, repeated use of hCG can cause an immune response in the fish limiting its efficacy in long term applications (Mylonas and Zohar 2001, Mylonas and Zohar 2007).

A variety of synthetic gonadotropin releasing hormone analogues (GnRHa) that were developed commercially for application in human medicine, are also implemented in the hormonal therapy of a range of captive finfish broodstock (Mylonas et al. 2010). The use of synthetic GnRHa offers a number of advantages over gonadotropins by eliminating any biosecurity risk, as well as providing a therapeutic agent that acts at a higher level of the HPG to stimulate the release of the endogenous gonadotropins FSH and LH (Figure 1.4). Gonadotropin releasing hormone analogue therapies are generally the most utilised method for stimulating final oocyte maturation and spawning in captive fishes (Mylonas and Zohar 2001). In order to increase the duration of GnRHa bioavailability in circulation, a variety of GnRHa delivery systems have been developed which provide for sustained release of the active agent. These include cholesterol-, ethyl vinyl acetate- (EVAc) based pellets, biodegradable polyanhydride microspheres and osmotic pumps (Kanemaru et al. 2012, Lee et al. 1986, Mylonas et al. 1995, Mylonas and Zohar 2000).

Commercial products that incorporate GnRHa with a dopamine antagonist have been shown in some studies to improve efficacy over GnRHa alone. This is proposed to be due to inhibition of down regulation of the pituitary by circulating dopamines (Peter et al. 1988). The benefits of dopamine antagonists are more evident in freshwater species than marine species (Zohar et al. 1995).

Given that most fish undergo vitellogenesis in captivity there is limited literature pertaining to the use of hormonal therapies to stimulate vitellogenesis. In most cases the

use of GnRHa has not been effective in stimulating vitellogenesis and therefore oocyte maturation and spawning (Mylonas and Zohar 2000). In general, for GnRHa to be effective in stimulating the early stages of oogenesis, vitellogenesis must have already commenced. Chronic administration of GnRHa was shown to enhance, rather than initiate, vitellogenesis in milk fish (*Chanos chanos*) (Lee et al. 1986) and studies conducted on the Pacific herring (*Clupeaharengus pallasi*) demonstrated a response in vitellogenic oocytes to GnRHa; however, there was no evidence of an effect on previtellogenic oocytes (Carolsfeld et al. 1988). One of the few studies to report successful induction of vitellogenesis with GnRHa was conducted on red sea bream (*Pagrus major*) (Matsuyama et al. 1995). There remains limited explanation for the relative inability of GnRHa to initiate vitellogenesis, largely due to the limited knowledge of the functions of the brain and pituitary during this stage of gonadal development (Mylonas and Zohar 2007). In contrast to GnRHa, the use of gonadotropins has been generally more effective for the induction of vitellogenesis. Repeated weekly doses of salmon and carp pituitary extract over several months has been shown to effectively induce vitellogenesis in the Japanese and European eel (Suetake et al. 2002, Palstra et al. 2010). Successful induction of vitellogenesis in Mekong catfish has been realised by administering multiple daily injections of hCG at low doses. Maturation and ovulation is then induced using two stage injections with higher doses of hCG (Cacot et al. 2002).

1.4 Overview of cobia aquaculture research

1.4.1 Reproductive biology of cobia

Wild cobia are multiple spawners with a protracted spawning season that extends from mid-spring through to mid-autumn with peaks in late spring and early autumn. Batch

fecundity of wild cobia is estimated at 249 eggs/g of spawner body weight, with a spawning estimated to occur every 5-12 days through the season (Brown-Peterson et al. 2001, van der Velde et al. 2010). Studies on wild caught cobia in tropical regions have noted that peaks in spawning activity of cobia coincide with the influx of freshwater resulting from monsoonal periods (Brown-Peterson et al. 2001, van der Velde et al. 2010).

Asynchronous and group synchronous gonadal development have been reported in wild cobia, consistent with cobia being a multiple spawner (Biesiot et al. 1994, Brown-Peterson et al. 2001). These studies found that although cobia have a protracted spawning season, a portion of fish were spent and regressed early in the summer, while others remained in spawning condition through to early autumn.

1.4.2 Larval rearing

The first decade of this century saw a large research effort focused on intensifying larval and grow out production of cobia in order to obtain more predictable and consistent production (Benetti et al. 2007, Holt et al. 2007b, Schwarz et al. 2007, Weirich et al. 2007). Intensification of cobia larviculture has seen a shift in production technique from the reliance on natural phytoplankton blooms provided by extensive pond systems, to semi-intensive tank based culture systems. Stocking densities, environmental requirements, live food enrichment, feeding regimes and weaning strategies for larval cobia production have been refined and optimised (Faulk and Holt 2005, Faulk and Holt 2006, Hitzfelder et al. 2006, Faulk et al. 2007a, Faulk et al. 2007b, Webb Jr et al. 2007). The University of Miami demonstrated further improvements in larval rearing methodologies, with reported survival rates from hatch to weaned juveniles of above 25% (Benetti et al. 2008a, Benetti et al. 2008b).

1.4.3 Nutrition

Limited research has been conducted on the specific nutritional requirements of cobia. This is due in part to the moderate success of producing cobia using existing manufactured diets designed for other carnivorous marine fish such as sea bass and grouper (Fraser and Davies 2009). Cobia have been successfully produced on diets with a wide range of protein and lipid levels and protein to energy ratios (Chen and Liao 2007). A review conducted by Fraser and Davies (2009) suggests that whilst protein and lipid levels have been optimised, there is limited information available on cobia's specific amino acid, essential fatty acid, vitamin or mineral requirements. Optimal dietary protein and energy specifications for cobia can be derived through the bio-energetic factorial approach developed by Van Tien et al. (2016). Cobia nutrition is an area that needs further research and refinement to ensure the sustainable and most optimal development of the species.

1.4.4 Health Management

As with many cultured species, cobia are susceptible to viral, bacterial and parasitic disease at each of the culture stages (Liao et al. 2004, McLean et al. 2008). Cobia are a warm water species and as such become stressed and are generally more disease susceptible when water temperatures fall below 20 °C (Liao et al. 2007, Nhu et al. 2011).

1.4.5 Maturation and spawning manipulation of captive cobia

Cobia broodstock have been successfully brought to maturity using a wide variety of aquaculture systems and management techniques. Methods used for maturation of broodstock include the capture of wild fish in spawning condition, the selection of suitable fish from ocean growout cages, holding fish in spawning ponds and maintaining

fish in environmentally controlled tank systems (Holt et al. 2007b, Liao et al. 2007).Traditional methods of broodstock management in Taiwan involve the transfer of mature fish from farm cages to spawning ponds, where they are held in ambient conditions. Broodstock cobia spawn spontaneously through the warmer months with peak production occurring in spring and autumn (Liao et al. 2004). As interest in cobia production increased in other parts of the world, efforts have focused on more controlled and regular production from broodstock.

Early attempts at inducing spawning in cobia with hCG met with limited success; however they demonstrated that it was possible (Caylor et al. 1994). Cobia were successfully induced to spawn using GnRHa implants in the USA in 2001 (Arnold et al. 2002) and reports of volitional spawning of cobia held in tanks under ambient conditions soon followed (Weirich et al. 2007). More consistent volitional spawning was achieved by using environmentally controlled recirculating aquaculture systems to condition broodstock (Holt et al. 2007b). A combination of volitional spawning and hormone induction is used by producers in Vietnam, with increasing preference for the latter due to the advantage of better forward planning (Nhu et al. 2011). As with other cultured finfish species, captive bred broodstock cobia have been shown to be more likely to spawn spontaneously and to do so more frequently than wild caught fish (Holt et al. 2007a, Benetti et al. 2008a).

Assessment of the reproductive development of cobia broodstock over several spawning seasons at BIRC has found highly variable gonadal development across a number of mature sized fish. There have also been irregular and generally poor results from hormone induction trials. The reproductive dysfunctions observed in cobia at BIRC are similar to those described for other species by Zohar and Mylonas (2001) and fall predominantly into two categories. In some cases there is an absence of or only partial

vitellogenic development which is insufficient to induce ovulation and spawning using exogenous hormones. Alternatively, post vitellogenic oocytes may fail to undergo final oocyte maturation and ovulation, with a subsequent failure to spawn. Whilst successful hormone inductions resulted in spawnings producing large numbers of high quality eggs and larvae, they involved a low number/proportion of the broodstock. The majority of captive broodstock at BIRC did not reach a developmental stage suitable for induction or did not to respond to hormone induction and failed to undergo final maturation and spawning.

Although studies conducted on wild populations of cobia by Biesiot et al. (1994) and Brown-Peterson et al. (2001) were unable to follow the development of individual broodstock over a spawning season, these studies demonstrate inherent variability in the reproductive state of individuals within cobia populations. A similar level of variability is evident in captive fish at BIRC and the extent of this may be exacerbated as a function of a captive environment. In addition, for the commercial hatchery production of cobia, the impact of variation in broodstock reproductive state is pertinent due to impacts on predictability, efficiency and regularity of production from captive reared fish.

Cobia have been successfully spawned across the globe using a variety of systems and techniques, however there remains a scarcity of information available that recognises the development of individuals in a broodstock population or their relative contribution to a spawning event. Studies published on cobia broodstock tend to be production-focused and lack both replication and a structured approach to identifying physiological responses by broodstock. Benetti et al. (2008a) report successful repeated volitional spawning from cobia held in environmental controlled recirculating systems. Whilst demonstrating an effective system, the study did not analyse development of individuals or offer suggestion on the acute stimuli to initiate spawning.

On the basis of a single comparative trial, Stieglitz et al. (2012), proposed that exposure to sustained high temperatures was sufficient to provide out of season volitional spawning in cobia. This was demonstrated by comparing the spawning frequency of two population groups of cobia, each maintained on different temperature cycles. Due to the nature of mass spawning events, there was no measure of the number of individuals contributing to the spawn, or their relative contributions to the spawning event. Whilst this study was able to support the premise that temperature may be an important factor in the spawning of cobia, it did not address the inconsistency of development, nor provide for a structured and traceable breeding program.

Australian aquaculture is confronted with several developmental impediments: limited suitable site availability, high land costs, high labour cost and strict environmental regulation. As such, commercial operations are required to produce a top quality product, farmed with the utmost efficiency, in order for the business to remain economically viable. Cobia broodstock present particular management issues for a hatchery, as their rapid growth rate and large size may only allow broodstock to be held for spawning for 2-3 years. Beyond this point, production efficiencies are compromised as a function of fish size. Hatcheries therefore, need to have effective broodstock management practices and well developed breeding strategies in order to manage a high turnover of broodstock. Well established Australian aquaculture industries such as Atlantic salmon have flourished due, in part, to a solid understanding of the reproductive biology of the fish. This has allowed the incorporation of breeding programs, genetic selection and other novel techniques such as inducing triploidy, sex inversion and single sex culture to improve production efficiency of the species (Benfey 2001, Lee 2004). Existing methods for managing cobia broodstock do not provide the necessary high level control, regularity and predictability required to maintain commercial viability in the long term.

1.5 The present study

In order to develop the framework for a viable Australian cobia aquaculture industry, there is a need to develop more robust and reliable techniques for cobia seed production. The overarching aim of this study was to address some of the constraints of reproductive performance of cobia in captive conditions, to provide commercially applicable strategies to improve the production efficiencies and economics of cobia production.

There is a need to develop a better understanding of the reproductive physiology of cobia to identify the triggers and cues that stimulate gonadal development in cobia. The current research project investigated how environmental control and manipulation as well as exogenous hormone therapy influences both early and late stage ovarian development in cobia. To accurately assess the influence of environmental and hormonal manipulation on ovarian development of cobia there was a need to develop an accurate method of measuring ovarian development in the presence of asynchronous development.

Other aspects of broodstock management, which help maximise productivity and efficiency, were also considered. This study aimed to progressively track and compare the growth of male and female cobia from an early age, under controlled conditions, to determine the existence and extent of sexually dimorphic growth and evaluate its potential commercial significance. The final aspect of the study was to investigate the feasibility of using steroid analysis as a means of identifying gender in cobia, to assist with improved broodstock management efficiency.

1.6 References

Arnold, C. R., J. B. Kaiser and G. J. Holt (2002). "Spawning of cobia *Rachycentron canadum* in captivity." Journal of the World Aquaculture Society **33**(2): 205-208.

Baroiller, J. F., D. Chourrout, A. Fostier and B. Jalabert (1995). "Temperature and sex chromosomes govern sex ratios of the mouthbrooding Cichlid fish *Oreochromis niloticus*." Journal of Experimental Zoology **273**(3): 216-223.

Benetti, D. D., B. O'Hanlon, J. A. Rivera, A. W. Welch, C. Maxey and M. R. Orhun (2010). "Growth rates of cobia (*Rachycentron canadum*) cultured in open ocean submerged cages in the Caribbean." Aquaculture **302**(3-4): 195-201.

Benetti, D. D., M. R. Orhun, B. Sardenberg, B. O'Hanlon, A. Welch, R. Hoenig, I. Zink, J. A. Rivera, B. Denlinger, D. Bacoat, K. Palmer and F. Cavalin (2008a). "Advances in hatchery and grow-out technology of cobia *Rachycentron canadum* (Linnaeus)." Aquaculture Research **39**(7): 701-711.

Benetti, D. D., M. R. Orhun, I. Zink, F. Cavalin, B. Sardenberg, K. Palmer, B. Denlinger, D. Bacoat and B. O'Hanlon (2007). Aquaculture of cobia (*Rachycentron canadum*) in the Americas and the Caribbean. Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan**:** 57-78.

Benetti, D. D., B. Sardenberg, A. Welch, R. Hoenig, M. R. Orhun and I. Zink (2008b). "Intensive larval husbandry and fingerling production of cobia *Rachycentron canadum*." Aquaculture **281**(1-4): 22-27.

Benfey, T. J. (2001). "Use of sterile triploid Atlantic salmon (*Salmo salar* L.) for aquaculture in New Brunswick, Canada." ICES Journal of Marine Science: Journal du Conseil **58**(2): 525-529.

Bennetau-Pelissero, C., F. Le Menn, B. Davail-Cuisset, C. Helou, B. Breton B, B. Bennetau, G. Corraze and S. J. Kaushik (2001). "Effect of genistein-enriched diets on the endocrine process of gametogenesis and on reproduction efficiency of the rainbow trout *Oncorhynchus mykiss*." General and Comparative Endocrinology **121**(2): 173-187.

Bergman, A., J. Heindel, S. Jobling, K. A. Kidd and R. T. Zoellar (2013). State of the Science of Endocrine Disrupting Chemicals - 2012. Switzerland, United Nations Environmental Programme and the World Health Organisation.

Biesiot, P. M., R. E. Caylor and J. S. Franks (1994). "Biochemical and histological changes during ovarian development of cobia, *Rachycentron canadum*, from the northern Gulf of Mexico." Fishery Bulletin **92**: 686-696.

Bromage, N., M. Porter and C. Randall (2001). "The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin." Aquaculture **197**(1-4): 63-98.

Brown-Peterson, N. J., R. M. Overstreet, J. M. Lotz, J. S. Franks and K. M. Burns (2001). "Reproductive biology of cobia, *Rachycentron canadum*, from coastal waters of the United States." Fishery Bulletin **99**: 15-28.

Cacot, P., M. Legendre, T. Q. Dan, L. T. Tung, P. T. Liem, C. Mariojouls and J. Lazard (2002). "Induced ovulation of *Pangasius bocourti* (Sauvage, 1880) with a progressive hCG treatment." Aquaculture **213**(1–4): 199-206.

Carolsfeld, J., N. M. Sherwood, H. Kreiberg and S. A. Sower (1988). "Induced sexual maturation of herring using GnRH 'quick-release' cholesterol pellets." Aquaculture **70**(1–2): 169-181.

Caylor, R. E., P. M. Biesiot and J. S. Franks (1994). "Culture of cobia (*Rachycentron canadum*): cryopreservation of sperm and induced spawning." Aquaculture **125**(1-2): 81-92.

Chen, H. Y. and I. C. Liao (2007). Nutritional research and feed development in cobia: Status and prospects. Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University**:** 89-96.

Devlin, R. H. and Y. Nagahama (2002). "Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences." Aquaculture **208**(3–4): 191-364.

Dutney, L., P. Lee and P. Palmer (2010). Developing cobia aquaculture in Queensland through collaborative research. Australasian Aquaculture 2010 International Conference and Trade Show. Hobart, Tasmania.

Dutney, L. and P. Palmer (2008). Farmed cobia making progress at BIARC. Queensland Aquaculture News, Queensland Government Department of Primary Industries and Fisheries**:** 6-7.

FAO (2013). Fisheries Statistical Database: Aquaculture production 1950-2010. www.fao.org/fishery/statistics/software/fishstat/en.

FAO. (2016). "Cultured aquatic species information programme *Rachycentron canadum* (Linnaeus, 1766) " Retrieved 16/02/2016, from http://www.fao.org/fishery/culturedspecies/Rachycentron_canadum/en.

Faulk, C. K., A. D. Benninghoff and G. J. Holt (2007a). "Ontogeny of the gastrointestinal tract and selected digestive enzymes in cobia *Rachycentron canadum* (L.)." Journal of Fish Biology **70**(2): 567-583.

Faulk, C. K. and G. J. Holt (2005). "Advances in rearing cobia *Rachycentron canadum* larvae in recirculating aquaculture systems: Live prey enrichment and greenwater culture." Aquaculture **249**(1-4): 231-243.

Faulk, C. K. and G. J. Holt (2006). "Responses of cobia *Rachycentron canadum* larvae to abrupt or gradual changes in salinity." Aquaculture **254**(1-4): 275-283.

Faulk, C. K., J. B. Kaiser and G. J. Holt (2007b). "Growth and survival of larval and juvenile cobia *Rachycentron canadum* in a recirculating raceway system." Aquaculture **270**(1-4): 149-157.

Franks, J. S., J. R. Warren and M. V. Buchanan (1999). "Age and growth of cobia, *Rachycentron canadum*, from the north eastern Gulf of Mexico." Fishery Bulletin **97**: 459-471.

Fraser, T. W. K. and S. J. Davies (2009). "Nutritional requirements of cobia, *Rachycentron canadum* (Linnaeus): a review." Aquaculture Research **40**(11): 1219-1234.

Gallardo, J. A., X. García, J. P. Lhorente and R. Neira (2004). "Inbreeding and inbreeding depression of female reproductive traits in two populations of Coho salmon selected using BLUP predictors of breeding values." Aquaculture **234**(1–4): 111-122.

Green, C. C. and A. M. Kelly (2009). "Effects of the estrogen mimic genistein as a dietary component on sex differentiation and ethoxyresorufin-O-deethylase (EROD) activity in channel catfish (*Ictalurus punctatus*)." Fish Physiology and Biochemistry **35**(3): 377-384.

Hitzfelder, G. M., G. J. Holt, J. M. Fox and D. A. McKee (2006). "The effect of rearing density on growth and survival of cobia, *Rachycentron canadum*, larvae in a closed recirculating aquaculture system." Journal of the World Aquaculture Society **37**(2): 204-209.

Holt, J. G., C. K. Faulk and M. H. Schwarz (2007a). "A review of the larviculture of cobia *Rachycentron canadum*, a warm water marine fish." Aquaculture **268**(1-4): 181-187.

Holt, J. G., J. Kaiser and C. Faulk (2007b). Advances in cobia research in Texas. Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University**:** 45-56.

Jobling, S., S. Coey, J. G. Whitmore, D. E. Kime, K. J. W. Van Look, B. G. McAllister, N. Beresford, A. C. Henshaw, G. Brighty, C. R. Tyler and J. P. Sumpter (2002). "Wild intersex roach (*Rutilus rutilus*) have reduced fertility." Biology of Reproduction **67**(2): 515-524.

Kagawa, H. (2013). "Oogenesis in Teleost Fish." Aqua-bioscience monographs ABSM **6**(1): 99-127.

Kanemaru, T., Nakamura, M., Murata, R., Kuroki, K., Horie, H., Uchida, K., Senthilkumaran, B. and Kagawa, H. (2012). "Induction of sexual maturation of the female honeycomb grouper, *Epinephelus merra*, in the non-breeding season by modulating environmental factors with GnRH analogue implantation." Aquaculture **358–359**: 85-91.

King, H. R. and N. W. Pankhurst (2003). "Ovarian growth and plasma sex steroid and vitellogenin profiles during vitellogenesis in Tasmanian female Atlantic salmon (*Salmo salar*)." Aquaculture **219**(1–4): 797-813.

Kiparissis, Y., G. C. Balch, T. L. Metcalfe and C. D. Metcalfe (2003). Effects of the Isoflavones Genistein and Equol on the Gonadal Development of Japanese Medaka (*Oryzias latipes*), National Institute of Environmental Health Sciences. National Institutes of Health. Department of Health, Education and Welfare**:** 1158.

Kroon, F. J., S. E. Hook, D. Jones, S. Metcalfe, B. Henderson, R. Smith, M. S. J. Warne, R. D. Turner, A. McKeown and D. A. Westcott (2015). "Altered transcription levels of endocrine associated genes in two fisheries species collected from the Great Barrier Reef catchment and lagoon." Marine Environmental Research **104**(0): 51-61.

Lam, T. J. (1983). Environmental influences on gonadal activity in fish. Fish Physiology. D. J. R. W.S. Hoar and E. M. Donaldson, Academic Press. **Volume 9, Part B:** 65-116.

Lee, C. S., C. S. Tamaru and C. D. Kelley (1986). "Technique for making chronic-release LHRH-a and 17α-methyltestosterone pellets for intramuscular implantation in fishes." Aquaculture **59**(2): 161-168.

Lee, P. S. (2004). Control of sex inversion in female Atlantic salmon (*Salmo salar*). PhD, University of Tasmania.

Liao, I. C., T.-S. Huang, W.-S. Tsai, C.-M. Hsueh, S.-L. Chang and E. M. LeaÃ±o (2004). "Cobia culture in Taiwan: current status and problems." Aquaculture **237**(1-4): 155-165.

Liao, I. C., E. M. Leano, C. T. Hsu and C. C. Ku (2007). Marine cage culture of cobia in Taiwan. Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University**:** 131-145.

Lubzens, E., G. Young, J. Bobe and J. CerdÃ (2010). "Oogenesis in teleosts: How fish eggs are formed." General and Comparative Endocrinology **165**(3): 367-389.

Matsuyama, M., H. Takeuchi, M. Kashiwagi, K. Hirose and H. Kagawa (1995). "Induced gonadal development and spawning of immature red sea bream (*Pagrus major*) with LHRH-a administration in different ways during winter season." Fisheries Science **61**(3): 472-477.

McLean, E., G. Salze and S. R. Craig (2008). "Parasites, diseases and deformities of cobia." Ribarstvo **66**(1): 1-16.

Morehead, D. T., A. J. Ritar and N. W. Pankhurst (2000). "Effect of consecutive 9- or 12-month photothermal cycles and handling on sex steroid levels, oocyte development, and reproductive performance in female striped trumpeter *Latris lineata* (Latrididae)." Aquaculture **189**(3-4): 293-305.

Mylonas, C. C., A. Fostier and S. Zanuy (2010). "Broodstock management and hormonal manipulations of fish reproduction." General and Comparative Endocrinology **165**(3): 516-534.

Mylonas, C. C., N. Mitrizakis, M. Papadaki and I. Sigelaki (2013). "Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity I. Description of the annual reproductive cycle." Aquaculture **414–415**(0): 309-317.

Mylonas, C. C., Y. Tabata, R. Langer and Y. Zohar (1995). "Preparation and evaluation of polyanhydride microspheres containing gonadotropin-releasing hormone (GnRH), for inducing ovulation and spermiation in fish." Journal of Controlled Release **35**(1): 23-34.

Mylonas, C. C., L. C. Woods Iii, P. Thomas and Y. Zohar (1998). "Endocrine profiles of female striped bass (*Morone saxatilis*) in captivity, during postvitellogenesis and induction of final oocyte maturation via controlled-release GnRHa-delivery systems." General and Comparative Endocrinology **110**(3): 276-289.

Mylonas, C. C. and Y. Zohar (2000). "Use of GnRHa-delivery systems for the control of reproduction in fish." Reviews in Fish Biology and Fisheries **10**(4): 463-491.

Mylonas, C. C. and Y. Zohar (2001). "Endocrine regulation and artificial induction of oocyte maturation and spermiation in basses of the genus *Morone*." Aquaculture **202**(3–4): 205-220.

Mylonas, C. C. and Y. Zohar (2007). Promoting oocyte maturation, ovulation and spawning in farmed fish. The fish oocyte: from basic studies to biotechnical applications. P. J. Babin, J. CerdÃ and E. Lubzens. Dordrecht, Springer**:** 437-474.

Nagahama, Y. (1983). The functional morphology of teleost gonads. Fish Physiology. W. S. Hoar, D. J. Randall and E. M. Donaldson. New York, Academic Press. **IXA:** 223-275.

Nagahama, Y. (1994). "Endocrine regulation of gametogenesis in fish." Int J Dev Biol **38**(2): 217-229.

Nagahama, Y. (1999). Gonadal steroid hormones: major regulators of gonadal sex differentiation and gametogenesis in fish. Reproductive physiology of fish: Proceedings of the 6th international symposium on the reproductive biology of fish. B. Norberg, O. S. Kjesbu, G. L. Taranger, E. Andersson and S. O. Stefansson. Bergen, University of Bergen**:** 211-222.

Nagahama, Y., M. Yoshikuni, M. Yamashita and M. Tanaka (1994). Regulation of oocyte maturation in fish. Fish Physiology. Vol. XIII: Molecular endocrinology of fish. A. P. Farrel and D. J. Randall. San Diego, California, Academic press**:** 393-439.

Nhu, V. C., H. Q. Nguyen, T. L. Le, M. T. Tran, P. Sorgeloos, K. Dierckens, H. Reinertsen, E. KjÃ¸rsvik and N. Svennevig (2011). "Cobia *Rachycentron canadum* aquaculture in Vietnam: Recent developments and prospects." Aquaculture **315**(1-2): 20-25.

Palstra, A. P., D. Schnabel, M. C. Nieveen, H. P. Spaink and G. E. E. J. M. van den Thillart (2010). "Temporal expression of hepatic estrogen receptor 1, vitellogenin1 and vitellogenin2 in European silver eels." General and Comparative Endocrinology **166**(1): 1-11.

Pankhurst, N. W. and M. Porter (2003). "Cold and dark or warm and light: variations on the theme of environmental control of reproduction." Fish Physiology and Biochemistry **28**: 385-389.

Patiño, R., N. C. Parker, J. E. Schoore, C. Uguz, K. B. Davis, B. A. Simco, C. A. Strüssmann and C. A. Goudie (1996). "Sex differentiation of channel catfish gonads: Normal development and effects of temperature." Journal of Experimental Zoology **276**(3): 209-218.

Pavlidis, M., G. Koumoundouros, A. Sterioti, S. Somarakis, P. Divanach and M. Kentouri (2000). "Evidence of temperature-dependent sex determination in the European sea bass (*Dicentrarchus labrax* L.)." Journal of Experimental Zoology **287**(3): 225-232.

Peter, R. E., H.-R. Lin and G. Van Der Kraak (1988). "Induced ovulation and spawning of cultured freshwater fish in China: Advances in application of GnRH analogues and dopamine antagonists." Aquaculture **74**(1–2): 1-10.

Purdom, C. E., P. A. Hardiman, V. V. J. Bye, N. C. Eno, C. R. Tyler and J. P. Sumpter (1994). "Estrogenic effects of effluents from sewage treatment works." Chemistry and Ecology **8**(4): 275-285.

Salini, J. P., S. J. Blaber and D. T. Brewer (1994). "Diets of trawled predatory fish of the Gulf of Carpentaria, Australia, with particular reference to predation on prawns." Australian Journal of Marine and Freshwater Research **45**: 397-411.

Sampaio, L. A., C. B. Moreira, K. C. Miranda-Filho and A. N. Rombenso (2011). "Culture of cobia *Rachycentron canadum* (L) in near-shore cages off the Brazilian coast." Aquaculture Research **42**(6): 832-834.

Schwarz, M. H., E. McLean and S. R. Craig (2007). Research experience with cobia: Larval rearing, juvenile nutrition and general physiology. Cobia Aquaculture: Research,

Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University**:** 1-18.

Scott, A. P., V. J. Bye and S. M. Baynes (1980a). "Seasonal variations in sex steroids of female rainbow trout (*Salmo gairdneri* Richardson)." Journal of Fish Biology **17**(5): 587-592.

Scott, A. P., V. J. Bye, S. M. Baynes and J. R. C. Springate (1980b). "Seasonal variations in plasma concentrations of 11-ketotestosterone and testosterone in male rainbow trout, *Salmo gairdnerii* Richardson." Journal of Fish Biology **17**(5): 495-505.

Scott, A. P., T. Hill, N. Beresford, T. J. Runnalls, J. P. Sumpter and T. Ellis (2013). "Out-of-season production of 17,20β-dihydroxypregn-4-en-3-one in the roach *Rutilus rutilus*." Journal of Fish Biology **83**(2): 233-249.

Senthilkumaran, B. (2015). "Pesticide- and sex steroid analogue-induced endocrine disruption differentially targets hypothalamo–hypophyseal–gonadal system during gametogenesis in teleosts – A review." General and Comparative Endocrinology **219**: 136-142.

Shaffer, R. V. and E. V. Nakamura (1989). Synopsis of biological data on the cobia *Rachycentron canadum* (Piscies: Rachycentridae). U. D. o. Commerce. Washington DC USA, FAO Fisheries synopsis **153:** 21.

Shiau, C.-Y. (2007). Biochemical composition and utilization of cultured cobia (*Rachycentron canadum*). Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University**:** 147-156.

Steven, C., Y. Gothilf, M. C. Holland, J. Stubblefield, C. C. Mylonas and Y. Zohar (1999). Differential expression of the three GnRH genes in wild and captive striped bass, *Morone saxatilis* in response to natural and hormonally induced maturation. Proceedings of the 6th international symposium on the reproductive biology of fish. B. Norberg, O. S. Kjesbu, G. L. Taranger, E. Andersson and S. O. Stefansson. Bergen, University of Bergen.

Stieglitz, J. D., D. D. Benetti, R. H. Hoenig, B. Sardenberg, A. W. Welch and S. Miralao (2012). "Environmentally conditioned, year-round volitional spawning of cobia (*Rachycentron canadum*) in broodstock maturation systems." Aquaculture Research **43**(10): 1557-1566.

Su, G.-S., L.-E. Liljedahl and G. A. E. Gall (1996). "Effects of inbreeding on growth and reproductive traits in rainbow trout (*Oncorhynchus mykiss*)." Aquaculture **142**(3–4): 139-148.

Suarez, J. A., C. Tudela, D. Davis, Z. Daugherty, M. Taynor, L. Glass, R. Hoenig, A. Buentello and D. D. Benetti (2013). "Replacement of fish meal by a novel non-GM variety of soybean meal in cobia, *Rachycentron canadum*: Ingredient nutrient digestibility and growth performance." Aquaculture **416–417**(0): 328-333.

Suetake, H., K. Okubo, N. Sato, Y. Yoshiura, Y. Suzuki and K. Aida (2002). "Differential expression of two gonadotropin (GTH) β subunit genes during ovarian maturation induced by repeated injection of salmon GTH in the Japanese eel *Anguilla japonica*." Fisheries Science **68**(2): 290-298.

Sumpter, J. (1999). Endocrine disrupting chemicals in the aquatic environment. Reproductive physiology of fish: Proceedings of the 6th international symposium on the reproductive biology of fish. B. Norberg, O. S. Kjesbu, G. L. Taranger, E. Andersson and S. O. Stefansson. Bergen, University of Bergen**:** 349-355.

Taranger, G. L., C. Haux, S. O. Stefansson, B. Björn Thrandur, B. T. Walther and T. Hansen (1998). "Abrupt changes in photoperiod affect age at maturity, timing of ovulation and plasma testosterone and oestradiol-17β profiles in Atlantic salmon, *Salmo salar*." Aquaculture **162**(1–2): 85-98.

Taranger, G. L., S. O. Stefansson, F. Oppedal, E. Andersson, T. Hansen and B. Norberg (1999). Photoperiod and temperature affects gonadal development and spawning time in Atlantic salmon (*Salmo salar* L.). Proceedings of VIth International Symposium on Reproductive Physiology of Fish. G. L. Taranger, B. Norberg, S. O. Stefansson et al. Bergen, University of Bergen.

van der Velde, T., S. Griffiths and G. Fry (2010). "Reproductive biology of the commercially and recreationally important cobia (*Rachycentron canadum*) in northeastern Australia." Fisheries Science **76**(1): 33-43.

Van Tien, N., Chinh, D.T, Huong, T.T., Phuong, T.H., Irvin, S. and Glencross, B (2016). "Development of a nutritional model to define the energy and protein requirements of cobia, *Rachycentron canadum*. Aquaculture **463:** 193-200.

Watanabe, W. O., C. A. Woolridge and H. V. Daniels (2006). "Progress toward year-round spawning of southern flounder broodstock by manipulation of photoperiod and temperature." Journal of the World Aquaculture Society **37**(3): 256-272.

Webb Jr, K. A., G. M. Hitzfelder, C. K. Faulk and G. J. Holt (2007). "Growth of juvenile cobia, *Rachycentron canadum*, at three different densities in a recirculating aquaculture system." Aquaculture **264**(1-4): 223-227.

Weirich, C. R., A. D. Stokes, T. I. J. Smith, W. E. Jenkins, M. R. Denson, J. R. Tomasso, J. Chappel and D. Burnside (2007). Cobia aquaculture research in South Carolina, USA: Captive reproduction, pond nursery production, and selected environmental requirements of juveniles. Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University**:** 19-44.

Zohar, Y. (1989). Fish reproduction: its physiology and artificial manipulation. Fish culture in warm water systems: problems and trends. M. Shilo and S. Sarig. Boca Raton, Florida, CRC Press**:** 65-119.

Zohar, Y., M. Harel, S. Hassin and A. Tandler (1995). Gilthead sea bream (*Saprus aurata*). Broodstock management and egg and larval quality. N. Bromage and R. Roberts. Oxford, Blackwell Science**:** 94-117.

Zohar, Y. and C. C. Mylonas (2001). "Endocrine manipulations of spawning in cultured fish: from hormones to genes." Aquaculture **197**(1-4): 99-136.

Chapter 2: Analysis of sexually dimorphic growth in captive reared cobia (*Rachycentron canadum*) and the occurrence of intersex individuals.

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Analysis of sexually dimorphic growth in captive reared cobia (*Rachycentron canadum*) and the occurrence of intersex individuals.

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2.1 Abstract

The growth of three cohorts of captive reared cobia was progressively measured to determine the existence and extent of sexually dimorphic growth in cobia. Approximately 100 fish from each cohort were individually identified and regularly weighed until the average weight of the fish was approximately five kilograms. The sex of individuals was determined through gonadal observations at the conclusion of each trial and the gender fitted retrospectively to the growth data set. Intersex gonads were observed in the first two cohorts of cobia, with 16.9% incidence in cohort 1 and 6.8% in cohort 2. Cobia is considered a gonochoristic species. This was the first reported observation of intersex gonads in cobia and the first reported occurrence of intersex gonads from a gonochoristic fish species from Australian waters. Only one fish out of the 182 examined in the third cohort was identified as intersex. There was no sexually dimorphic growth in cobia when there was a high incidence of the intersex anomaly, relative to endemic levels, as seen in the first two cohorts of fish. In the relative absence of the intersex condition, as observed in the third cohort, female cobia were significantly larger than males from 2 kg onwards. The weight of female cobia was almost 30% more than that of males at 17 months of age when average weight of the cohort was 4.6 kg. It is likely that the first two cobia cohorts were exposed to endocrine disruption in some form, and the possible sources are discussed.

Key words

Cobia, *Rachycentron canadum*, sexual dimorphism, growth, intersex.

Highlights

- The first reported incidence of intersex fish occurring in cobia and in gonochoristic fish in Australian waters.

- The occurrence of the intersex condition impacted on the growth dynamics of phenotypic male and female fish.
- In the relative absence of the intersex condition, female cobia grew significantly faster than males.

2.2 Introduction

Sexually dimorphic growth has been observed in a number of commercially significant fish species. Males grow faster than females in some catfish, tilapia and salmonid species (Nævdal et al. 1983 , Goudie et al. 1994, Toguyeni et al. 1997, Bonnet et al. 1999), whereas females have been shown to grow faster in *Mugil cephalus*, European sea bass, *Dicentrarchus labrax*, some anguilliforms and flat fish (Panfili et al. 1994, Imsland et al. 1997, Ibañez Aguirre et al. 1999, Saillant et al. 2001), to name a few. The evolutionary basis for sexually dimorphic growth in fish is based on a trade-off between mortality, growth rate and reproductive success (Roff 1983). Males tend to be larger than females in populations where there is competition between males for reproductive success, in which case physical size is an advantage. Conversely males tend to be smaller in mass spawning populations as male size has less influence on reproductive success (Parker 1992). Size differences between sexes can also arise due to varying age at maturity and differing energy investment in the physiological process of gonad development and maturation (Thorpe 1994).

Sexually dimorphic growth can be exploited to offer rapid and significant economic gains through single sex culture (Piferrer 2001). Productivity gains have been demonstrated in tilapia and catfish aquaculture by the culture of monosex populations (Goudie et al. 1994, Beardmore et al. 2001). The occurrence of sexually dimorphic growth can also impact broodstock management. If broodstock are allocated prior to sexual maturity, and selection is based on growth, the resultant population will be heavily biased towards a single sex. Therefore, in order to ensure a gender balance, a large group of randomly selected fish must be held until their sex can be positively identified.

Cobia, *Rachycentron canadum*, is a large benthopelagic species that is endemic to all tropical and subtropical waters across the globe with the exception of the eastern Pacific (Shaffer and Nakamura 1989). Some of the biological attributes that make cobia an exceptional candidate for aquaculture include; growth rates that exceed 5 kg per year, adaptability to commercially available aquafeeds, excellent palatability, and wide temperature and salinity tolerance (Holt et al. 2007b, Shiau 2007, Weirich et al. 2007). Commercial cobia aquaculture began in Taiwan in the late 1990's and has since been adopted by several nations through the Asian-Pacific, the USA and South America (Liao et al. 2007, Benetti et al. 2008, Nhu et al. 2011, Sampaio et al. 2011). Global production is dominated by China, which produces approximately 39,600 of the global annual production of 43,000 metric tonnes (FAO 2013).

There are conflicting reports in the literature regarding sexually dimorphic growth in wild cobia stocks. Franks et al. (1999) examined the growth increments of sectioned otoliths to develop age-length relationships of Gulf of Texas stocks. The analysis of von Bertalanffy growth models showed that females grew significantly faster than males. Furthermore, analysis of length-frequency distribution indicated that females achieved a greater theoretical asymptotic length than males. Conversely, Fry and Griffiths (2010) examined the age-length of cobia from the east coast of Australia, and using similar analyses, found no statistical difference in growth between genders. Anecdotal evidence from aquaculture facilities both in Australia and internationally suggests that female cobia grow faster than males in a captive environment (Daniel Benetti pers. comm, Bribie Island Research Centre unpublished data); however this has never been quantified or documented.

Cobia are considered a gonochoristic species, (Shaffer and Nakamura 1989), however during the course of this study a substantial number of intersex individuals were

identified. Genetics, diet, environmental parameters and pollutants can result in impaired reproduction and gonad malformation in aquaculture and wild fish stocks. The occurrence of intersex fish in wild fish populations has been associated with environmental pollutants (Jobling et al. 2002). Endocrine disrupting chemicals (EDC) originating from waste water treatment facilities and other anthropogenic influences are known to interfere with the synthesis and action of endogenous hormones resulting in reproductive abnormalities (Purdom et al. 1994, Bergman et al. 2013). The possibility of EDCs and other potential mechanisms that may interfere with the reproductive development of cobia in the current study are discussed.

This study aimed to progressively track and compare the growth of male and female cobia from an early age, under controlled conditions, to determine if there was significant sexually dimorphic growth. Sexually dimorphic growth was indeed observed; the point of divergence in growth and its potential commercial significance are evaluated.

2.3 Methods

2.3.1 Juvenile fish

Juvenile cobia used in the study were produced from broodstock held and maintained at the Bribie Island Research Centre (BIRC). Larvae were grown in semi-intensive green-water tank systems for two weeks post hatch and then transferred to extensive pond production for weaning and on-growing to approximately 10 g. A population of approximately 200 juvenile fish was randomly selected to be allocated as future broodstock and maintained in a flow through nursery system.

The study followed three cohorts of fish. The fish used in cohort 1 originated from a mass spawning on 19/01/2012 involving up to three wild caught females, one captive reared

female and four wild caught males. Cohort 2 originated from a mass spawning of two wild caught females and three wild caught males on 21/10/2012; of which one male and one female potentially also contributed to cohort 1. Wild caught fish were obtained from a similar area off the coast of southern Queensland. Cohort 3 originated from a single captive-reared female and three captive-reared males on 27/11/2013. The female fish contributing to cohort 3 was of the same origin as those fish used in cohort 1. The relative contribution of each of the brood fish was not determined. All spawning events occurred following hormone induction of both male and female brood fish.

The fish used in each of the trials were randomly selected from retained population of each of the three cohorts and individually identified using T bar tags. Once the fish reached sexual maturity, the gender of each individual was identified via gonadal biopsy and later confirmed by post mortem examination. This information was then fitted to the weight data collected at each time point to complete the data set (Figure 2.1).

2.3.2 Cohort 1

One hundred fish were tagged at the beginning of the trial for cohort 1 and weighed every two months over the 11-month trial period. The average weight of fish was approximately 350 g when the trial commenced on 06/09/2012. The fish were initially grown in a single 30,000 L tank fitted with an independent recirculating aquaculture system (RAS). On 20/03/13 the population was split evenly between two 30,000 L tanks fitted with identical RAS, where they were maintained until the completion of the trial. Due to low level mortality and lost or broken tags, only 71 of the fish in cohort 1 were used in the final assessment of growth and for most mortem analysis of gonads.

2.3.3 Cohort 2

One hundred fish with an average weight of 202 g were tagged when the trial commenced on 27/02/13. Weight samples were taken monthly for the first four months and every second month for the remainder of the 14-month trial. The fish were maintained in a single 10,000 L circular tank fitted with flow-through seawater. In order to maintain suitable water temperature through the winter period, the fish were transferred to a single 10,000 L tank that was part of a 50,000 L RAS on 27/5/13 at an average weight of 627 g. The population was split and returned to two 10,000 L flow through systems on 18/09/2012, where they remained until the completion of the trial. Due to low level mortality and lost or broken tags, only 88 of the fish in cohort 2 were used in the final assessment of growth and for most mortem analysis of gonads.

2.3.4 Cohort 3

Cohort 3 was weighed monthly over an eight-month period. The average weight of fish from cohort 3 was 820 g on 21/8/2013, at the commencement of the trial. From a population of 123 fish, 75 fish were tagged to provide growth data and observations of gonad morphology for cohort 3. An additional 107 fish from the same origin as the cohort 3 fish were euthanased and the gonads examined macroscopically to determine the level of intersex at the commencement of the trial. This provided 75 fish for growth data analysis and 182 fish to determine the incidence of intersex in the cohort.

The trial fish were maintained in two 10,000 L tanks that were part of a 50,000 L RAS, until 28/10/15. They were then transferred and split evenly between four 10,000 L circular tanks fitted with flow-through seawater.

2.3.5 Fish Husbandry

The growing conditions remained similar for each tank of fish within each cohort after each population split. The maximum stocking densities were 2.6 kg/m^3, 17.2 kg/m^3 and 15 kg/m^3 in cohorts 1, 2 and 3 respectively. Temperature, salinity, dissolved oxygen and pH were measured and recorded daily using YSI professional plus multiparameter meter. Total ammonia nitrogen (TAN) was measured weekly when stocking densities exceeded 5 kg/m^3 in recirculating systems. Fish were fed to satiation twice daily for five days per week, and once daily for two days per week on commercially available marine fish diets (approx. 50% protein, 14% lipid).

2.3.6 Data analysis

The growth data were analysed by ANOVA at each sample point, using Genstat 16th Edition. A chi-squared test was used to determine if the sex ratio differed significantly from the expected 1:1 ratio.

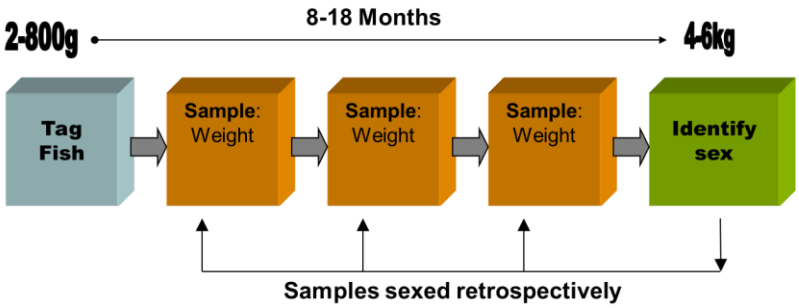

Figure 2.1 The sampling model used to examine sexually dimorphic growth in cobia. Fish were individually identified, weighed periodically and the sex of individuals identified at the conclusion of the trial.

2.4 Results

2.4.1 Water quality

All water quality parameters were maintained within normal operating levels for cobia production at BIRC (Table 2.1).

Table 2.1 Range of water quality parameters for cohorts 1, 2 and 3

Cohort	Temp (°C)	DO (ppm)	Salinity (ppt)	pH	TAN (ppm)
1	18.8-28.8	>4.5	26.0-36.1	7.4-8.1	<0.6
2	19.4-27.5	>4.5	26.5-35.3	7.5-8.2	<0.5
3	20.9-29.4	>4.8	29.2-36.5	8.0-8.4	<0.6

2.4.2 Reproductive abnormalities

Several individuals in cohorts 1 and 2 were identified as potential intersex fish, with both sperm and oocytes observed within individual gonadal biopsy samples. Post mortem examination of the fish confirmed the presence of both ovarian and testis tissue in these individuals (Figure 2.2). Intersex gonads were also identified in fish that originated from the same spawns as cohorts 1 and 2 but were not part of the current study. Intersex and normal gonads were first distinguished macroscopically; when the fish were approximately 400 g (Figure 2.3). Intersex gonads comprised discrete ovarian tissue located anteriorly, abutting testis tissue located posteriorly (Figures 2.2, 2.3 and 2.4). The proportions of each tissue varied between intersex individuals, independent of the size of the gonad (Figure 2.4). Histological examination of the intersex gonads confirmed there was a distinct junction between ovary and testis sections containing connective tissue and a mixing of gonad tissues (Figure 2.5). There was no evidence of mixing of tissues beyond the junction area. Individuals showed varying stages of gonadal

development, in most cases both tissues appeared capable of producing gametes. Active sperm and developing oocytes were observed in some intersex gonads; however gamete quality was not examined. Both ovarian and testis sections of the intersex gonad were generally misshapen, being asymmetrical within and between each side of the gonad and lobulated rather than a smooth cylindrical shape (Figures 2.3 and 2.4). The gonads of several single sex fish, both male and female, were also misshapen to some extent (Figure 2.6).

Post mortem examination confirmed the incidence of intersex fish was 17% in cohort 1 and 7% in cohort 2 (Table 2.2). Excluding these fish from an analysis of sex ratios (female: male) showed them to be significantly skewed towards female at 1.8:1 (X^2=4.90, 1d.f., p=0.03) and 2.4: 1 (X^2 =14.10,1d.f., p<0.001) in cohort 1 and 2 respectively. Only one of the 182 fish examined in cohort 3 was identified as intersex (0.5%) and the sex ratio did not differ significantly from 1:1, at 1.1: 1 (X^2 =0.45, 1d.f, p=0.50).

Table 2.2 Sex ratios of cobia in each cohort

Cohort	Total	Female (%)	Male (%)	Intersex (%)
1	71	38 (53.5)	21 (29.6)	12 (16.9%)
2	88	58 (65.9)	24 (27.3)	6 (6.8%)
3	182	95 (52.2)	86 (47.3)	1 (0.5%)

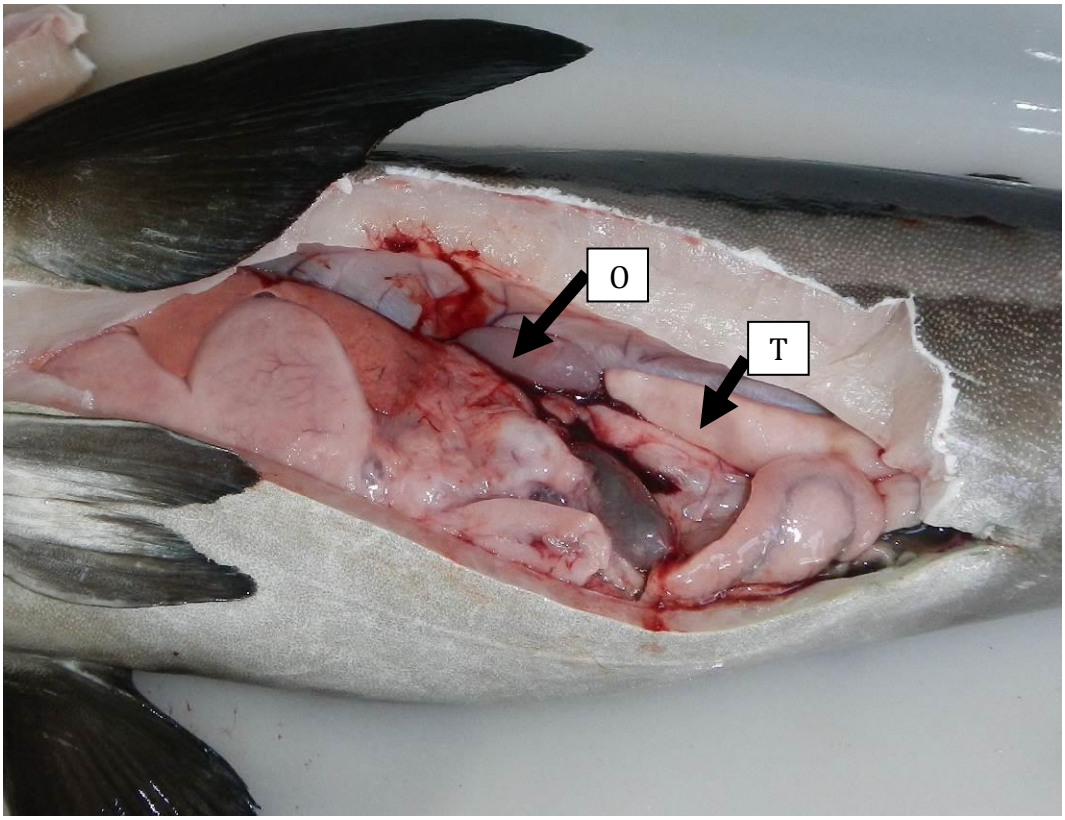

Figure 2.2 Cobia peritoneal cavity showing the orientation of the intersex gonad *in situ*. In all intersex fish the anterior section was ovary (O) and posterior section is testis (T).

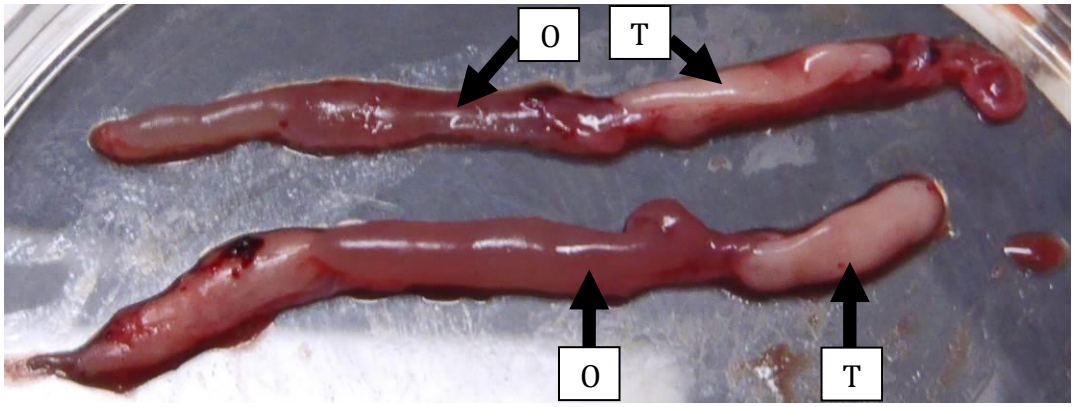

Figure 2.3 Intersex gonad from 400 g cobia. Intersex gonads were identifiable when ovarian (O) and testis (T) could be determined macroscopically.

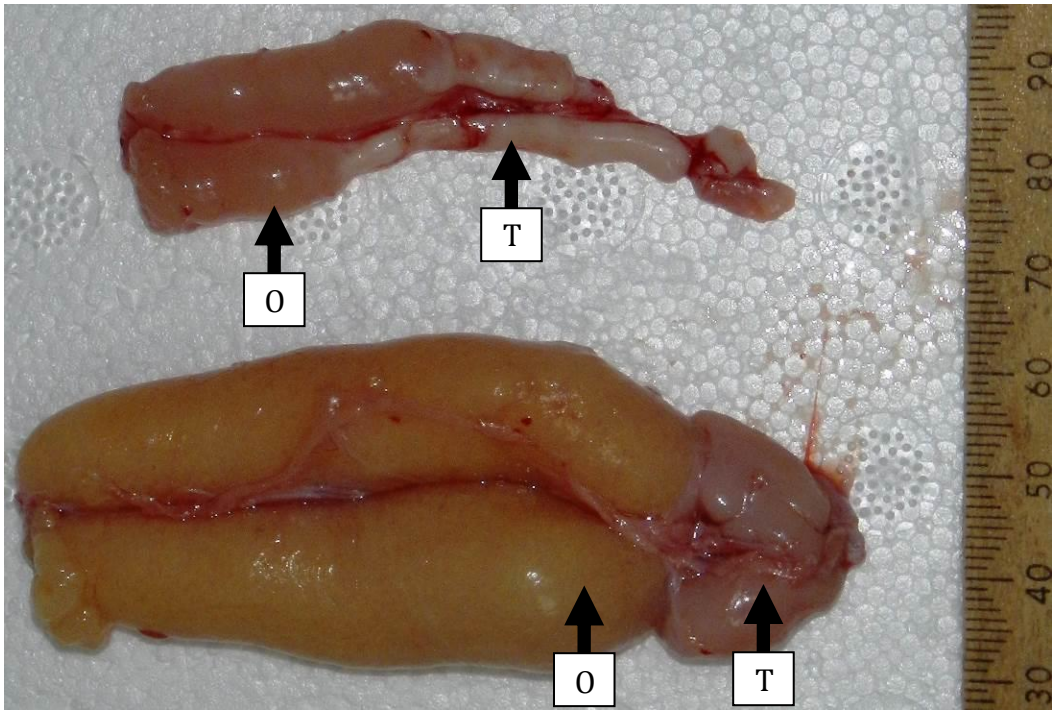

Figure 2.4 Intersex gonads of cobia showing varying proportions of ovarian (O) and testicular tissue (T).

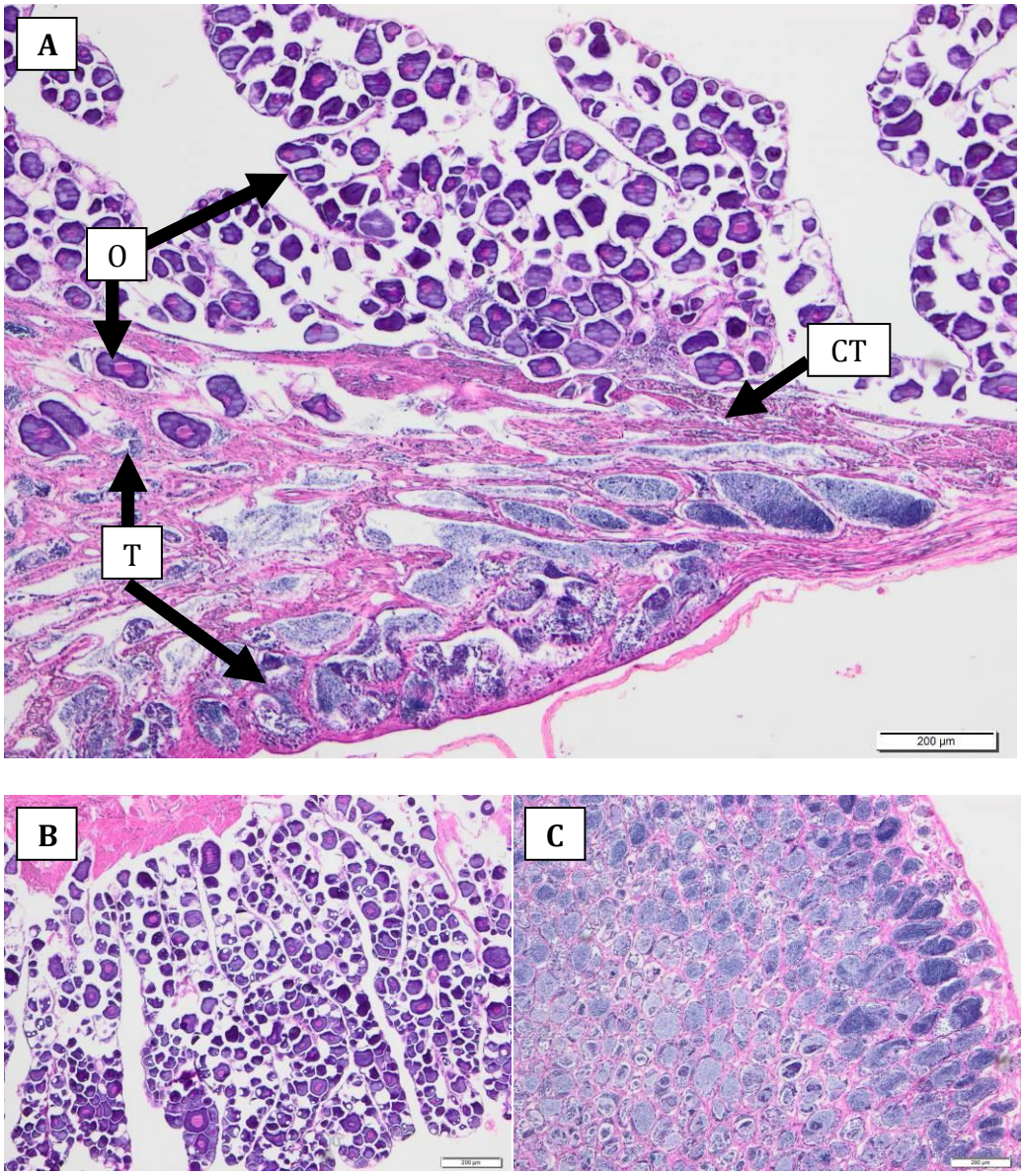

Figure 2.5 Histological sections of an intersex cobia gonad.
A - The junction of the testis and ovary, showing connective tissue (CT) and a mixture of ovarian (O) and testis (T) material, with minimal mixing distal to the junction. **B** - Anterior section of the gonad showing ovarian tissue. **C** - A section of the posterior gonad showing only testis material. Scale bar = 200µm.

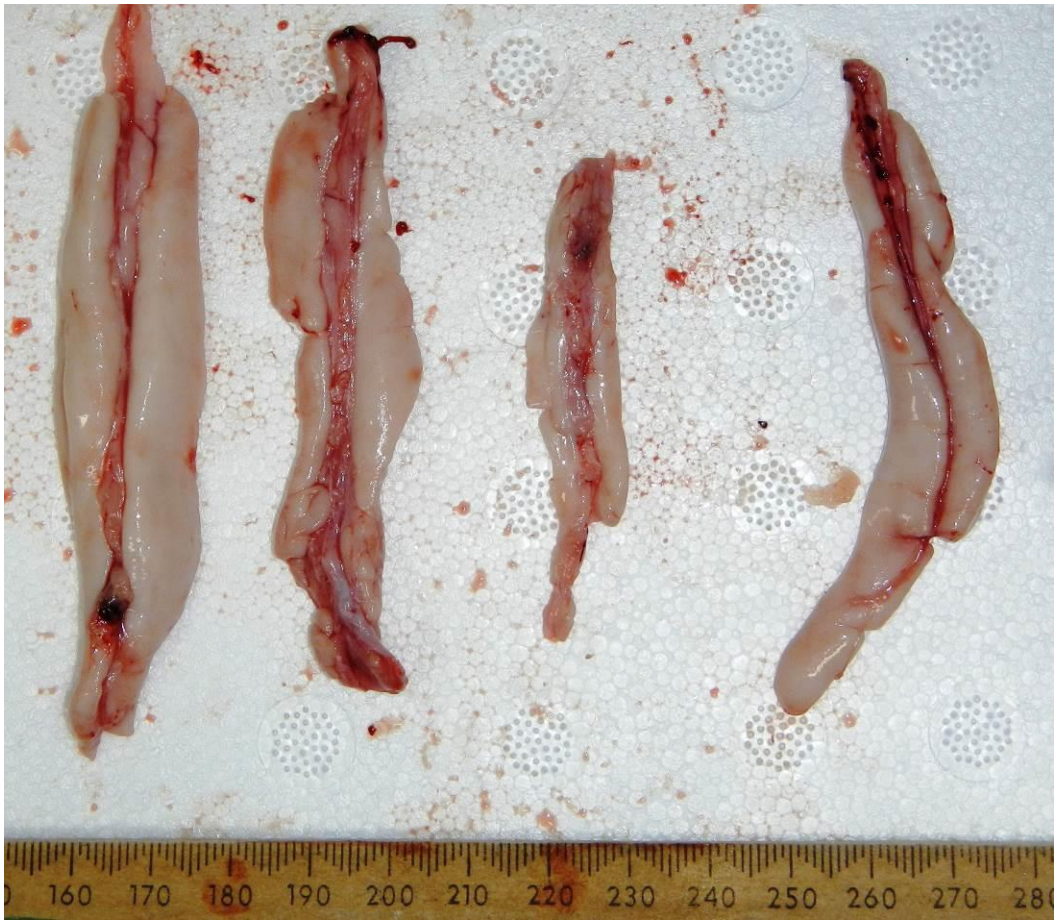

Figure 2.6 Gonads from male cobia showing regular shaped testes on the left and three misshapen testes to the right.

2.4.3 Growth

The average weight of fish in each cohort was approximately 5 kg at the conclusion of the sampling period. There was no significant difference between the weight of male and female cobia at any sample point in cohort 1 or 2 (Figure 2.7). There was no significant difference between intersex fish and single sex fish in cohort 1. Intersex fish were significantly smaller than male and female fish in cohort 2 ($p<0.05$).

Female fish in cohort 3 were significantly larger than males at the December sample point, approximately one year post hatch, with average weight of 2.20 kg (±0.90 SE) compared with male fish at 1.95kg (±0.76 SE) ($p=0.04$). The difference in weight between

the sexes increased as the trial continued (Figure 2.7). The average weight of females was 29.7% higher than males at an average weight of 5.28 kg (±0.23 SE) compared to 4.07 kg (±0.17 SE) in males at the conclusion of the trial ($p<0.01$).

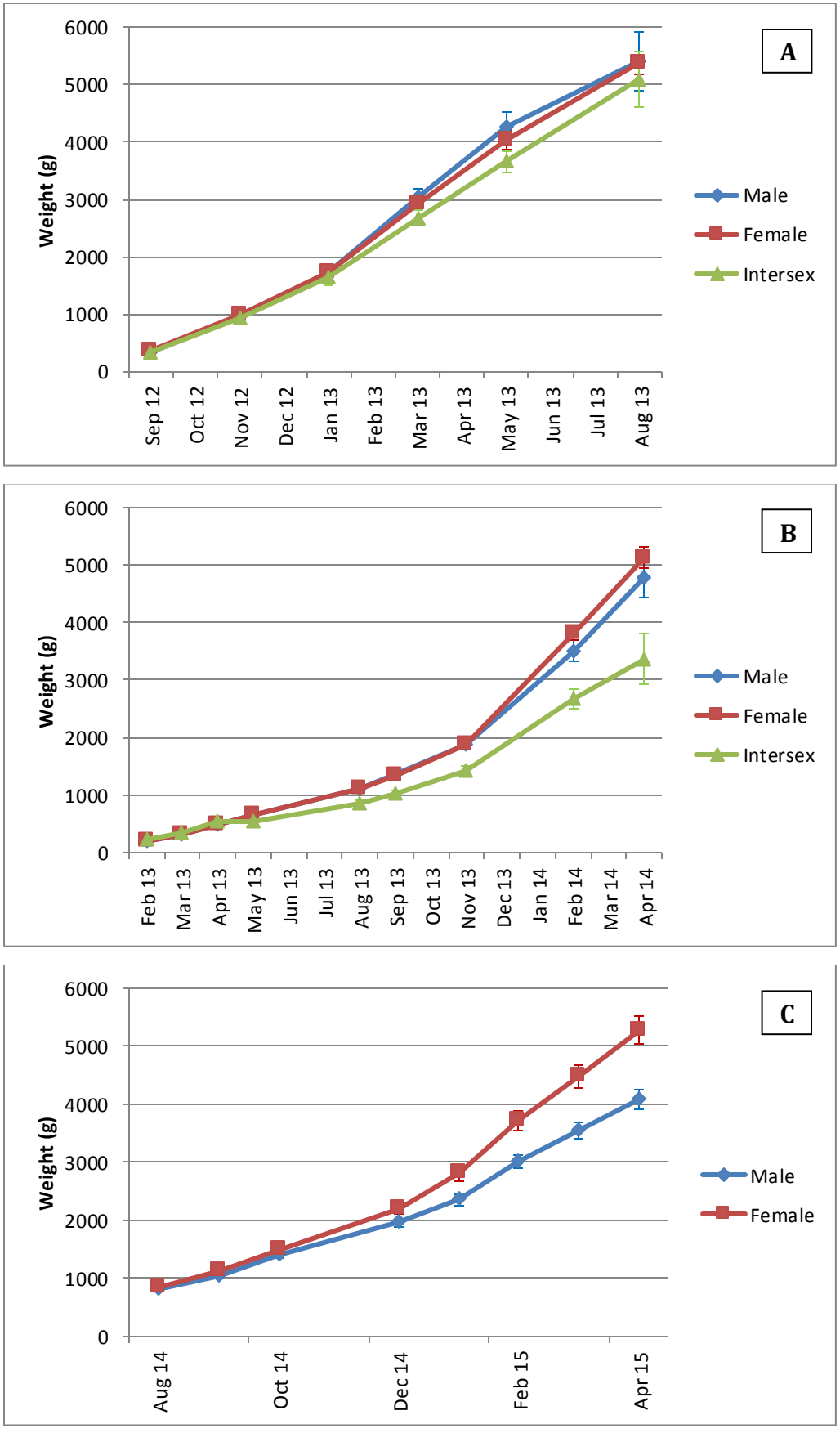

Figure 2.7 The growth trajectories of cobia in cohort 1 (**A**), cohort 2 (**B**) and cohort 3 (**C**).

2.5 Discussion

This is the first documented study examining the progressive growth of individual cobia to identify and determine the extent of sexually dimorphic growth in this species. The occurrence of intersex individuals in cobia, which is a gonochoristic species, has not previously been reported, despite numerous studies involved in cobia reproduction. The occurrence of the intersex condition has impacted the growth dynamics of cobia, evidenced by a lack of sexually dimorphic growth in cohort 1 and 2, which had a relatively high incidence of intersex fish. The current study demonstrated that captive-reared cobia exhibit sexually dimorphic growth in the relative absence of the intersex anomaly, with females growing faster than males. This supports the findings of Franks et al. (1999) that female cobia grew faster than males in Gulf of Texas wild stock, and is in contrast to the findings of the Fry and Griffiths (2010) study conducted on wild cobia on the east coast of Australia. The analysis of sexually dimorphic growth in wild populations of cobia based is on single data points for individual fish to provide length frequency distributions. Although there may be differences in growth dynamics between wild and captive populations, more accurate results of growth dynamics can be obtained from captive populations as the fish are of known age and individuals can be sampled multiple times over a trial period.

The existence of sexual growth dimorphism in cobia has implications for both broodstock management and optimal production strategies. Growth rate is a strongly heritable trait in most aquaculture species and many aquaculture hatcheries base broodstock selection, wholly or partly, on larger size, and therefore greater growth rate. Based on the evidence of the current study, cobia broodstock selection can be based on size until approximately 2 kg, with a low risk of skewing sex ratios in the selected broodstock. After this point

selecting broodstock based solely on weight could result in a sex ratio biased toward female fish. This can be particularly problematic in a fast-growing species such as cobia, where broodstock numbers may be limited by the availability of infrastructure. The allocation of cobia broodstock over 2 kg should be based on positive identification of gender through gonad biopsy or by the development of methods to assess gender specific sex steroids such as 17ß-estradiol, 11-ketotestosterone, or DNA markers.

The current study demonstrated a difference of 29.7% in growth between female and male cobia at harvestable size, suggesting that pursuing monosex culture of cobia could make significant productivity and economic gains. Due to the number of fish involved and the absence of external sexual dimorphism in cobia, visual selection of female fish for commercial aquaculture is not practical. As for other fish species, the production of single-sex populations is most efficiently achieved through broodstock manipulation. The production of monosex populations has been successful in a number of species; however, it is often a complicated task that first requires the identification of the sex determining mechanism of the species. The review by Piferrer (2001) describes a variety of methods used on species with various sex determining mechanisms to produce single-sex populations. The development of such techniques would also be advantageous in the protection of genetically improved stocks of cobia.

The findings of the current study support the suggestion of Parker (1992) that male fish tend to be smaller than females in mass spawning populations as there is limited reproductive advantage associated with size. This is further supported by evidence that wild cobia stocks form offshore spawning aggregations (Brown-Peterson et al. 2001). Roff (1983) suggests that the growth of some male fish is reduced as a function of reduced feeding effort. By foraging less the individuals are less exposed to predation risk. Female fecundity, however, is directly related to size, providing a reproductive advantage in mass

spawning populations associated with size, which outweighs the risk associated with predation (Roff 1983). The increased growth rate of female sea bass, *Dicentrarchus labrax*, and dab, *Limanda limanda*, compared with males, has been attributed to a larger digestive capacity facilitating increased food intake and therefore growth (Lozán 1992, Saillant et al. 2001). Although not examined in the current study, female cobia may have similar attributes that contribute to their increased growth. Aggressive behaviour within or between sexes in competing for resources is unlikely to have contributed to growth differences in the current study as fish were fed to satiety.

Significantly skewed sex ratios were associated with those cohorts with a high incidence of intersex fish in this study. In the absence of the intersex anomaly, sex ratios were not skewed significantly from the expected 1:1. There are contrasting reports of significantly skewed sex ratios occurring in wild cobia populations. Thompson et al. (1992) found a male-biased population 2.1:1 whereas a concurrent study conducted by Franks et al. (1999) reported a female biased sex ratio of 2.7:1. Each study was conducted using substantial sample size of 682 and 1005 fish by Thompson et al. (1992) and Franks et al. (1999) respectively. van der Velde et al. (2010) also reported a female-biased ratio 2.2:1. The difference in sex ratios found in these studies is difficult to explain, although it has been suggested to be due to segregation of sexes or differential rates of mortality between the sexes (Franks et al. 1999). Sex ratios can be skewed in some cultured species due to the influence of environmental parameters on the processes of sex determination. Variations in larval rearing temperature have been shown to affect sex ratios in sea bass, *Dicentrachus labrax*, channel catfish, *Paralichthys olivaceus*, and Nile tilapia, *Oreochromis niloticus* (Baroiller et al. 1995, Patiño et al. 1996, Pavlidis et al. 2000). Cobia have been produced in captivity under a variety of temperature regimes throughout the developmental cycle without any reports of skewed sex ratios or reproductive

abnormalities (Faulk and Holt 2005, Faulk et al. 2007, Holt et al. 2007b, Weirich et al. 2007, Benetti et al. 2008). The fish in the current trial were produced in what would be considered normal environmental parameters for cobia, reducing the likelihood that the physical environment has created any imbalance or abnormality (Holt et al. 2007a).

The relatively low number of phenotypic males compared to females in the current study suggests that the intersex fish are possibly male fish symptomatic of a demasculinising and/or feminising influence. It would be beneficial to confirm this through genetic sex markers; however, the sex-determining mechanism and suitable markers for cobia would first need to be established. Given the association of the incidence of intersex and skewed sex ratios, in the current study, it is likely that the source of disruption inducing intersex gonads is also responsible for the female-biased sex ratios. Skewed sex ratios in wild roach, *Rutilus rutilis* and white suckers, *Catostomus commersoni*, have been associated with pollutants derived from wastewater treatment (Alan et al. 2008, Lange et al. 2011). Inbreeding depression has been linked to impaired reproductive performance in salmonid species (Su et al. 1996, Gallardo et al. 2004). It can be excluded as a mechanism producing abnormalities in the current study, as individuals in each cohort were the offspring of wild parents, or wild fish crossed with first generation captive-reared fish. The occurrence of a particular genetic combination that results in the malformation or creates a predisposition to the condition in the presence of potential disrupting mechanisms cannot be ruled out.

Low-level incidence of intersex may occur as a simple malformation in some fish populations. The review by Kinnison et al. (2000) suggests that the intersex condition in farmed salmonids occurs as a natural aberration; due to the dispersed occurrence across a number of genera and geographic regions and the very low incidence of one in several thousand. However, the relatively high incidence of intersex in this study suggests that

some form of external influence has impacted heavily on the gonad development of the cobia in the current trial. Environmental pollutants classified as endocrine disrupting chemicals (EDC) are capable of interfering with the action and/or synthesis of steroid hormones resulting in an imbalance of male and female hormones which may manifest as reproductive abnormalities. The impact of EDCs is heightened at critical developmental stages such as during puberty, and the impacts are often irreversible (Bergman et al. 2013). Purdom et al. (1994) demonstrated that environmental pollutants originating from sewerage treatment resulted in increased circulating vitellogenin levels and the incidence of intersex fish in rainbow trout. The occurrence of intersex fish in wild roach, *Rutilus rutilis,* populations has been linked to feminising agents originating from effluent treatment facilities (Jobling et al. 2002). Further research has shown that the increase in intersex condition has impacted on maturation and fertility of the fish (Jobling et al. 2002). The only reported impact on fish from environmental pollutants in Australian waters was by Kroon et al. (2015) who described increased transcription levels of liver vitellogenin in barramundi, *Lates calcarifer*, and coral trout, *Plectropomus leopardus,* associated with agricultural pesticides. Whilst providing evidence of a feminising effect from EDCs, there was no evidence of a phenotypic change in the fish, or resultant changes to the population dynamics in either species.

Whilst there is compelling evidence in the literature linking EDCs with reproductive abnormalities, originating from anthropogenic pressure in freshwater systems, there is limited evidence of such acute impacts on marine fish populations. The review by Bahamonde et al. (2013) lists 37 species in which the intersex condition was reported in wild fish, of which only one is a marine fish species (Diaz De Cerio et al. 2012). This may be due to the diluting effect of the marine environment. Based on the limited evidence of anthropogenic impact on marine fish and given the relatively low-level urban and

industrial development near the oceanic intake of the Bribie Island Research Centre (BIRC), it seems unlikely that the incidence of intersex observed in this study is the result of locally sourced EDCs (Supplementary file 1). If the EDCs were sourced from the local environment, it would be expected that local wild stocks would be impacted and that several cohorts of cobia produced at BIRC would demonstrate similar incidence of intersex rather than the two isolated incidents reported in this study. Furthermore intersex gonads have not been previously reported from any fish in Australian waters. The source of the EDCs that impacted cobia in this trial could be related to a significant weather event that occurred prior to the spawning of cohort 1 and 2. Flood events have long been associated with increased levels of industrial and agricultural pollutants in local waterways, and may be associated with the intersex anomaly observed in cohorts 1 and 2. The summer of 2012 saw a significant rainfall event in southeast Queensland. Rainfall totals of close to 300 mm occurred within a 24-hr period in the local catchment areas (Bureau of Meteorology 2013). Cohort 1 was spawned and larval rearing began shortly before this weather event occurred, potentially exposing the cobia larvae to flood-transported pollutants. The connection between the flood event and cohort 2, however, is somewhat tenuous, being spawned nine months after the event. The lower incidence of intersex observed in cohort 2 may reflect a similar source of disruption with a lower level impact. There were no significant rainfall events recorded within 12 months prior to or during the production of cohort 3 fish. It remains difficult to explain why a similar impact has not been observed in wild fish. If the macroscopic disruption to the gonad, as seen in the current study, were to occur in wild fish in local or any Australian waters, it would be reasonable to assume that it would be reported at some level. The gonads of sea mullet, *Mugil cephalus*, are a high-value commercial product, sourced from fish harvested from local waters but at this stage no incidence of intersex has been reported, despite

high volume of gonads from the species being processed and examined macroscopically. Intersex fish have been reported from fish from the same family (Bizarro et al. 2014) and *M. cephalus* could serve as a possible indicator of EDCs disrupting gonadal development in local estuarine and marine environment.

As the demand for fishmeal usage in aquafeed increases, so too does the need to identify a suitable economically viable alternative protein sources. Soy bean meal has been demonstrated as a suitable alternative to fish meal for a number of marine species including cobia (Suarez et al. 2013). A potential drawback of the use of such plant-based proteins is the presence of estrogen-mimicking compounds, also known as phytoestrogens. In particular, the isoflavone genistein has been demonstrated to have a feminising effect on a number of fish species. Bennetau-Pelissero et al. (2001) described an increase in vitellogenin levels in both male and female trout, *Oncorhynchus mykiss*, and decreased testosterone and 11-ketotesterone during spawning as well as reduced reproductive performance, when fed diets with genistein at levels similar to those found in a soy-based diet. The presence of genistein has been associated with increased incidence of intersex and reduction of secondary sex characteristics in Japanese medaka, *Oryzias latipes* (Kiparissis et al. 2003). Suarez et al. (2013) examined the effect of substituting fish meal for soy meal on the growth performance of 1.8 kg cobia over a 90-day period. Although not specifically examined in their trial there was no report of reproductive abnormality induced by the treatments. The impact on reproductive development due to phytoestrogens may be more pronounced in fish exposed around the period of sexual differentiation. Green and Kelly (2009) found that channel catfish, *Ictalurus punctatus*, exposed to genistein during sexual differentiation resulted in altered sex ratios and an increase in intersex fish. The level of exposure to genistein in the current

study was not measured and cannot be ruled out as a possible source of disruption to reproductive development.

The clear delineation between the ovarian and testis tissue of the intersex cobia would suggest that the abnormality has occurred before or during differentiation (Kinnison et al. 2000). Manipulation of the plasticity of sexual phenotype is most effective during or directly proceeding sexual differentiation (Nakamura et al. 1998). It is therefore possible that a combination of the aforementioned factors have been involved, with an additive or synergistic effect, to influence the development of the fish during the highly sensitive period of differentiation. Johnstone et al. (1978) described distinct regions of specific germinal material when feminisation was induced with exogenous hormones at the time of sexual differentiation. Had the disruption in the current study occurred after differentiation and there was conversion of existing tissue, a mosaic or mixing of ovarian and testis tissue throughout the gonad would be expected. A similar effect has been observed in carp (*Cyprinus carpio*) and fathead minnows (*Pimephales promelas*) when sex reversal was induced after differentiation by exogenous hormones or exposure to EDCs (Gimeno et al. 1998, Niemuth and Klaper 2015).

The occurrence of intersex cobia is likely to have an impact on commercial production of the species. It has the potential to disrupt broodstock management due to a resultant mismatch in sex ratios. Identification of intersex cobia by gonad biopsy is subjective. There is a tendency to misidentify intersex fish as males due to the testis section of the intersex gonad being proximal to the gonopore. It is unlikely that the intersex fish would be sexually productive and in doing so waste the resources allocated to those individuals. It is also possible that the mechanism that induces the intersex condition could impact on the reproductive potential of the phenotypically male and female fish.

This study demonstrated that female cobia grow significantly faster than male fish and that investigations into monosex culture could lead to significant productivity gains for cobia aquaculture. It was also demonstrated that those cohorts containing intersex fish did not exhibit sexually dimorphic growth. It is likely that the reproductive anomaly is the result of disruption to the endocrine system, which has impacted on, or in this case prevented, the occurrence of sexually dimorphic growth. The exact source of the disruptor or potential mechanism for the disruption remains unclear. It does however warrant further investigation due to the potential impact on both commercial aquaculture and wild fish stocks.

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2.7 References

Alan, M. V., E. M. Lopez, J. D. Woodling, D. O. Norris, L. B. Barber and J. L. Gray (2008). "Reproductive disruption in fish downstream from an estrogenic wastewater effluent." Environmental Science and Technology **42**(9): 3407-3414.

Bahamonde, P. A., K. R. Munkittrick and C. J. Martyniuk (2013). "Intersex in teleost fish: Are we distinguishing endocrine disruption from natural phenomena?" General and Comparative Endocrinology **192**(0): 25-35.

Baroiller, J. F., D. Chourrout, A. Fostier and B. Jalabert (1995). "Temperature and sex chromosomes govern sex ratios of the mouthbrooding Cichlid fish *Oreochromis niloticus*." Journal of Experimental Zoology **273**(3): 216-223.

Beardmore, J. A., G. C. Mair and R. I. Lewis (2001). "Monosex male production in finfish as exemplified by tilapia: applications, problems, and prospects." Aquaculture **197**(1–4): 283-301.

Benetti, D. D., M. R. Orhun, B. Sardenberg, B. O'Hanlon, A. Welch, R. Hoenig, I. Zink, J. A. Rivera, B. Denlinger, D. Bacoat, K. Palmer and F. Cavalin (2008). "Advances in hatchery and grow-out technology of cobia *Rachycentron canadum* (Linnaeus)." Aquaculture Research **39**(7): 701-711.

Bennetau-Pelissero, C., F. Le Menn, B. Davail-Cuisset, C. Helou, B. Breton B, B. Bennetau, G. Corraze and S. J. Kaushik (2001). "Effect of genistein-enriched diets on the endocrine process of gametogenesis and on reproduction efficiency of the rainbow trout *Oncorhynchus mykiss*." General and Comparative Endocrinology **121**(2): 173-187.

Bergman, A., J. Heindel, S. Jobling, K. A. Kidd and R. T. Zoellar (2013). State of the science of endocrine disrupting chemicals - 2012. Switzerland, United Nations Environmental Programme and the World Health Organisation.

Bizarro, C., O. Ros, A. Vallejo, A. Prieto, N. Etxebarria, M. P. Cajaraville and M. Ortiz-Zarragoitia (2014). "Intersex condition and molecular markers of endocrine disruption in relation with burdens of emerging pollutants in thicklip grey mullets (*Chelon labrosus*) from Basque estuaries (South-East Bay of Biscay)." Marine Environmental Research **96**(0): 19-28.

Bonnet, S., P. Haffray, J. M. Blanc, F. Vallée, C. Vauchez, A. Fauré and B. Fauconneau (1999). "Genetic variation in growth parameters until commercial size in diploid and triploid freshwater rainbow trout (*Oncorhynchus mykiss*) and seawater brown trout (*Salmo trutta*)." Aquaculture **173**(1–4): 359-375.

Brown-Peterson, N. J., R. M. Overstreet, J. M. Lotz, J. S. Franks and K. M. Burns (2001). "Reproductive biology of cobia, *Rachycentron canadum*, from coastal waters of the United States." Fishery Bulletin **99**: 15-28.

Bureau of Meteorology, Q. (2013). 2011/2012 Queensland Flood Season. A. G. B. o. Meteorology. http://www.bom.gov.au/hydro/flood/qld/fld_reports/reports.shtml, Queensland Government Department of Natural Resources and Mines.

Diaz De Cerio, O., I. Rojo-Bartolomé, C. Bizarro, M. Ortiz-Zarragoitia and I. Cancio (2012). "5S rRNA and accompanying proteins in gonads: Powerful markers to identify sex and reproductive endocrine disruption in fish." Environmental Science and Technology **46**(14): 7763-7771.

FAO (2013). Fisheries Statistical Database: Aquaculture production 1950-2010. www.fao.org/fishery/statistics/software/fishstat/en.

Faulk, C. K. and G. J. Holt (2005). "Advances in rearing cobia *Rachycentron canadum* larvae in recirculating aquaculture systems: Live prey enrichment and greenwater culture." Aquaculture **249**(1-4): 231-243.

Faulk, C. K., J. B. Kaiser and G. J. Holt (2007). "Growth and survival of larval and juvenile cobia *Rachycentron canadum* in a recirculating raceway system." Aquaculture **270**(1-4): 149-157.

Franks, J. S., J. R. Warren and M. V. Buchanan (1999). "Age and growth of cobia, *Rachycentron canadum*, from the north eastern Gulf of Mexico." Fishery Bulletin **97**: 459-471.

Fry, G. C. and S. P. Griffiths (2010). "Population dynamics and stock status of cobia, *Rachycentron canadum*, caught in Australian recreational and commercial coastal fisheries." Fisheries Management and Ecology **17**(3): 231-239.

Gallardo, J. A., X. García, J. P. Lhorente and R. Neira (2004). "Inbreeding and inbreeding depression of female reproductive traits in two populations of Coho salmon selected using BLUP predictors of breeding values." Aquaculture **234**(1–4): 111-122.

Gimeno, S., H. Komen, S. Jobling, J. Sumpter and T. Bowmer (1998). "Demasculinisation of sexually mature male common carp, *Cyprinus carpio*, exposed to 4-tert-pentylphenol during spermatogenesis." Aquatic Toxicology **43**(2–3): 93-109.

Goudie, C. A., B. A. Simco, K. B. Davis and G. J. Carmichael (1994). "Growth of channel catfish in mixed sex and monosex pond culture." Aquaculture **128**(1–2): 97-104.

Green, C. C. and A. M. Kelly (2009). "Effects of the estrogen mimic genistein as a dietary component on sex differentiation and ethoxyresorufin-O-deethylase (EROD) activity in channel catfish (*Ictalurus punctatus*)." Fish Physiology and Biochemistry **35**(3): 377-384.

Holt, J. G., C. K. Faulk and M. H. Schwarz (2007a). "A review of the larviculture of cobia *Rachycentron canadum*, a warm water marine fish." Aquaculture **268**(1-4): 181-187.

Holt, J. G., J. Kaiser and C. Faulk (2007b). Advances in cobia research in Texas. Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University**:** 45-56.

Ibañez Aguirre, A. L., M. Gallardo-Cabello and X. C. Carrara (1999). "Growth analysis of striped mullet, *Mugil cephalus*, and white mullet, *M. curema* (Pisces: Mugilidae), in the Gulf of Mexico." Fishery Bulletin **97**(4): 861-872.

Imsland, A. K., A. Folkvord, G. L. Grung, S. O. Stefansson and G. L. Taranger (1997). "Sexual dimorphism in growth and maturation of turbot, *Scophthalmus maximus* (Rafinesque, 1810)." Aquaculture Research **28**(2): 101-114.

Jobling, S., N. Beresford, M. Nolan, T. Rodgers-Gray, G. C. Brighty, J. P. Sumpter and C. R. Tyler (2002). "Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents." Biology of Reproduction **66**(2): 272-281.

Jobling, S., S. Coey, J. G. Whitmore, D. E. Kime, K. J. W. Van Look, B. G. McAllister, N. Beresford, A. C. Henshaw, G. Brighty, C. R. Tyler and J. P. Sumpter (2002). "Wild intersex roach (*Rutilus rutilus*) have reduced fertility." Biology of Reproduction **67**(2): 515-524.

Johnstone, R., T. H. Simpson and A. F. Youngson (1978). "Sex reversal in salmonid culture." Aquaculture **13**(2): 115-134.

Kinnison, M. T., M. J. Unwin and F. Jara (2000). "Macroscopic intersexuality in salmonid fishes." New Zealand Journal of Marine and Freshwater Research **34**(1): 125-134.

Kiparissis, Y., G. C. Balch, T. L. Metcalfe and C. D. Metcalfe (2003). Effects of the isoflavones genistein and equol on the gonadal development of Japanese medaka (*Oryzias latipes*), National Institute of Environmental Health Sciences. National Institutes of Health. Department of Health, Education and Welfare**:** 1158.

Kroon, F. J., S. E. Hook, D. Jones, S. Metcalfe, B. Henderson, R. Smith, M. S. J. Warne, R. D. Turner, A. McKeown and D. A. Westcott (2015). "Altered transcription levels of endocrine associated genes in two fisheries species collected from the Great Barrier Reef catchment and lagoon." Marine Environmental Research **104**(0): 51-61.

Lange, A., G. C. Paull, P. B. Hamilton, C. R. Tyler and T. Iguchi (2011). "Implications of persistent exposure to treated wastewater effluent for breeding in wild roach (*Rutilus rutilus*) populations." Environmental Science and Technology **45**(4): 1673-1679.

Liao, I. C., E. M. Leano, C. T. Hsu and C. C. Ku (2007). Marine cage culture of cobia in Taiwan. Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University**:** 131-145.

Lozán, J. (1992). "Sexual differences in food intake, digestive tract size, and growth performance of the dab, *Limanda limanda* L." Netherlands Journal of Sea Research **29**(1–3): 223-227.

Nævdal, G., R. Lerøy and D. Møller (1983). "Sources of variation in weight and length of Atlantic salmon." Fiskeridirektoratets skrifter, Serie Havundersøkelser **17**: 359-366.

Nakamura, M., T. Kobayashi, X.-T. Chang and Y. Nagahama (1998). "Gonadal sex differentiation in teleost fish." Journal of Experimental Zoology **281**(5): 362-372.

Nhu, V. C., H. Q. Nguyen, T. L. Le, M. T. Tran, P. Sorgeloos, K. Dierckens, H. Reinertsen, E. KjÃ¸rsvik and N. Svennevig (2011). "Cobia *Rachycentron canadum* aquaculture in Vietnam: Recent developments and prospects." Aquaculture **315**(1-2): 20-25.

Niemuth, N. J. and R. D. Klaper (2015). "Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish." Chemosphere **135**(0): 38-45.

Panfili, J., M.-C. Ximénès and A. J. Crivelli (1994). "Sources of variation in growth of the European eel (*Anguilla anguilla*) estimated from otoliths." Canadian Journal of Fisheries and Aquatic Sciences **51** 506-515.

Parker, G. A. (1992). "The evolution of sexual size dimorphism in fish." Journal of Fish Biology **41**: 1-20.

Patiño, R., N. C. Parker, J. E. Schoore, C. Uguz, K. B. Davis, B. A. Simco, C. A. Strüssmann and C. A. Goudie (1996). "Sex differentiation of channel catfish gonads: Normal development and effects of temperature." Journal of Experimental Zoology **276**(3): 209-218.

Pavlidis, M., G. Koumoundouros, A. Sterioti, S. Somarakis, P. Divanach and M. Kentouri (2000). "Evidence of temperature-dependent sex determination in the European sea bass (*Dicentrarchus labrax* L.)." Journal of Experimental Zoology **287**(3): 225-232.

Piferrer, F. (2001). "Endocrine sex control strategies for the feminization of teleost fish." Aquaculture **197**(1–4): 229-281.

Purdom, C. E., P. A. Hardiman, V. V. J. Bye, N. C. Eno, C. R. Tyler and J. P. Sumpter (1994). "Estrogenic effects of effluents from sewage treatment works." Chemistry and Ecology **8**(4): 275-285.

Roff, D. A. (1983). "An allocation model of growth and reproduction in fish." Canadian Journal of Fisheries and Aquatic Sciences **40**(9): 1395-1404.

Saillant, E., A. Fostier, B. Menu, P. Haffray and B. a. Chatain (2001). "Sexual growth dimorphism in sea bass *Dicentrarchus labrax*." Aquaculture **202**(3â€“4): 371-387.

Sampaio, L. A., C. B. Moreira, K. C. Miranda-Filho and A. N. Rombenso (2011). "Culture of cobia *Rachycentron canadum* (L) in near-shore cages off the Brazilian coast." Aquaculture Research **42**(6): 832-834.

Shaffer, R. V. and E. V. Nakamura (1989). Synopsis of biological data on the cobia *Rachycentron canadum* (Piscies: Rachycentridae). U. D. o. Commerce. Washington DC USA, FAO Fisheries synopsis **153:** 21.

Shiau, C.-Y. (2007). Biochemical composition and utilization of cultured cobia (*Rachycentron canadum*). Cobia Aquaculture: Research, Development and Commercial

Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University**:** 147-156.

Su, G.-S., L.-E. Liljedahl and G. A. E. Gall (1996). "Effects of inbreeding on growth and reproductive traits in rainbow trout (*Oncorhynchus mykiss*)." Aquaculture **142**(3–4): 139-148.

Suarez, J. A., C. Tudela, D. Davis, Z. Daugherty, M. Taynor, L. Glass, R. Hoenig, A. Buentello and D. D. Benetti (2013). "Replacement of fish meal by a novel non-GM variety of soybean meal in cobia, *Rachycentron canadum*: Ingredient nutrient digestibility and growth performance." Aquaculture **416–417**(0): 328-333.

Thompson, B. A., C. A. Wilson, J. H. Render and M. Beasley (1992). Age, growth and reproductive biology of greater amberjack and cobia from Louisiana waters. W. US Department of Commerce, DC. **(US Dept Commerce NOAA Agreement NA90AAH-MF089):** 55.

Thorpe, J. E. (1994). "Reproductive strategies in Atlantic salmon, *Salmo salar* L." Aquaculture Research **25**(1): 77-87.

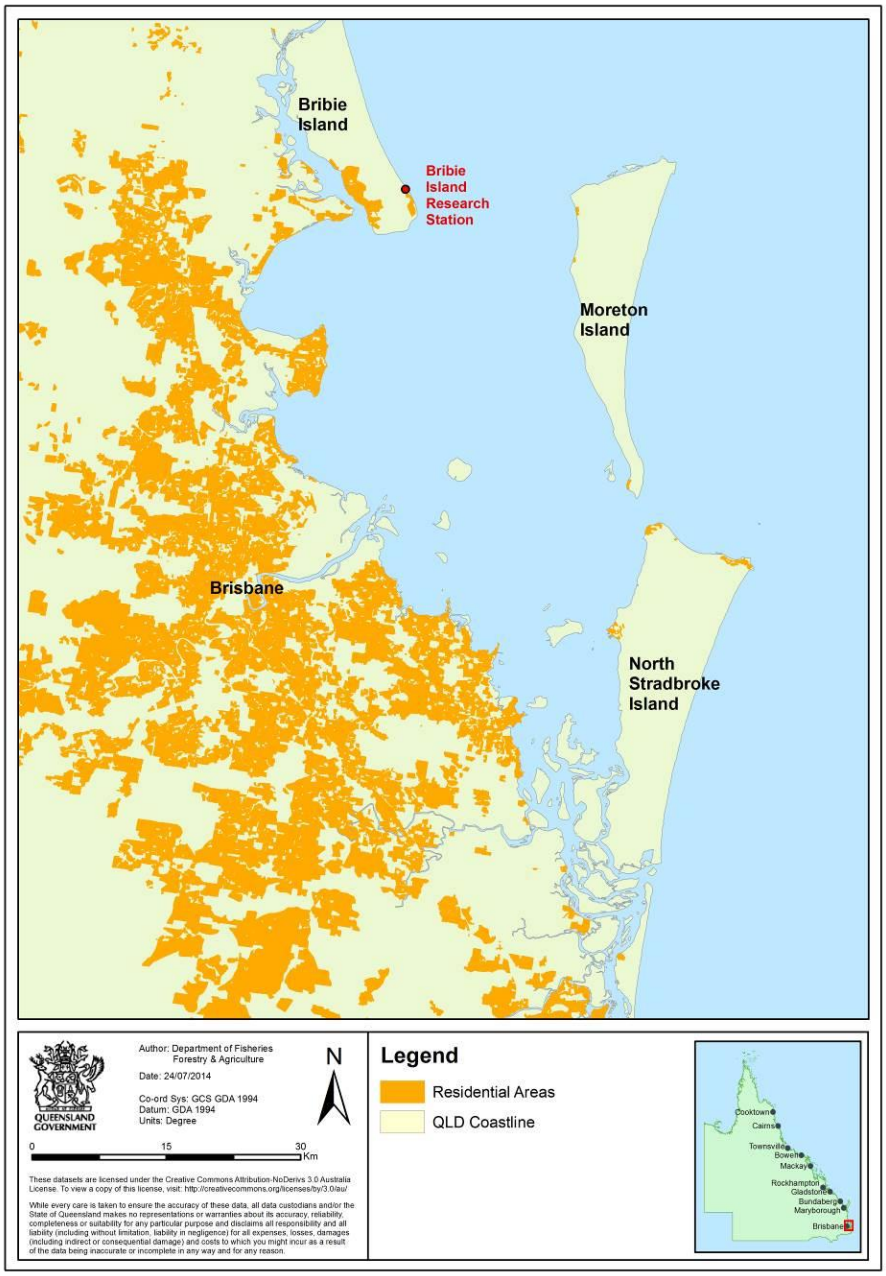
Toguyeni, A., B. Fauconneau, T. Boujard, A. Fostier, E. R. Kuhn, K. A. Mol and J.-F. Baroiller (1997). "Feeding behaviour and food utilisation in tilapia, *Oreochromis niloticus*: Effect of sex ratio and relationship with the endocrine status." Physiology & Behavior **62**(2): 273-279.

van der Velde, T., S. Griffiths and G. Fry (2010). "Reproductive biology of the commercially and recreationally important cobia (*Rachycentron canadum*) in northeastern Australia." Fisheries Science **76**(1): 33-43.

Weirich, C. R., A. D. Stokes, T. I. J. Smith, W. E. Jenkins, M. R. Denson, J. R. Tomasso, J. Chappel and D. Burnside (2007). Cobia aquaculture research in South Carolina, USA: Captive reproduction, pond nursery production, and selected environmental requirements of juveniles. Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University**:** 19-44.

2.7.1 Supplementary File 1

The location of the Bribie Island Research Centre (BIRC) showing the relatively low level urban and industrial development near the research centre.



Chapter 3: Hormonal manipulation strategies to enhance reproductive development in cobia (*Rachycentron canadum*).

This chapter is presented as a manuscript to be submitted for publication in Aquaculture Research.

Hormonal manipulation strategies to enhance reproductive development in cobia (*Rachycentron canadum*).

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3.1 Abstract

Cobia broodstock held at the Bribie Island Research Centre have demonstrated reproductive dysfunction, ranging from the absence of vitellogenic development, to a failure to undergo final oocyte maturation and spawning. Two trials were conducted to examine the effect of repeated injection of luteinising hormone releasing hormone analogue (LHRHa) and human chorionic gonadotropin (hCG) on stimulating vitellogenesis and supporting ovarian maturation in cobia broodstock. In the first trial, two injections of 15 µg/kg body weight LHRHa were given two weeks apart. In the second trial four injections of 500 IU/kg body weight hCG were given on a weekly basis. Each trial was conducted as temperature and photoperiod approached or was at the peak summer conditions. Ovarian development was assessed by regular biopsies and a methodology using the proportional distribution of different oocyte stages present in the sample was devised to describe the developmental state of the ovary. Plasma 17β-estradiol (E2) levels were also determined at regular intervals during the trials. The ovarian development of individual fish was highly variable within treatments and across trials. Although based on relatively low numbers of individuals, there was no evidence to suggest that either hCG or LHRHa was effective in stimulating and supporting ovarian development in cobia at the doses used in the current study. Plasma E2 concentrations were highly variable between individual fish in each treatment, with no change as a result of LHRHa administration. There was an increase in relative E2 concentration subsequent to the third hCG administration, followed by a rapid decease two weeks after the final injection. Those fish that initiated vitellogenesis continued to develop regardless of treatment with exogenous hormones. While hormonal therapy is effective for inducing

spawning in cobia, these results suggest that this hormone therapy is not an effective approach to initiating or supporting early stage ovarian development in cobia.

Key words

Cobia, *Rachycentron canadum*, LHRHa, hCG, hormonal manipulation, oocyte assessment.

Highlights

- A method was developed to provide a more comprehensive assessment of ovarian development in cobia.
- Hormonal manipulation was ineffective in initiating vitellogenesis in cobia.

3.2 Introduction

Cobia, *Rachycentron canadum*, is a large benthopelagic fish species that is endemic to all tropical and subtropical waters across the globe with the exception of the eastern Pacific (Shaffer and Nakamura 1989). Growth rates that exceed 5kg per year, adaptability to commercially available aquafeeds, excellent palatability, and temperature and salinity tolerance are some of the biological attributes that make cobia an exceptional candidate for aquaculture (Holt et al. 2007, Shiau 2007, Weirich et al. 2007).

Commercial cobia aquaculture began in Taiwan in the late nineties and has since been adopted by several nations through the Asian-Pacific, the USA and South America (Liao et al. 2007, Benetti et al. 2008, Nhu et al. 2011, Sampaio et al. 2011). Cobia research and development began in Australia in 2007, focusing largely on introducing cobia as an alternative and off season crop for prawn farms in Queensland (Dutney and Palmer 2008). Viable cobia larvae have been produced at Queensland's Department of Agriculture and Fisheries, Bribie Island Research Centre (BIRC); however, hormonal induction of spawning has resulted in irregular and generally poor outputs. Highly variable gonad development, observed across a number of mature sized female fish, has been a key underlying factor associated with poor spawning success. The reproductive dysfunctions observed in cobia at BIRC are similar to those described for other species, reviewed by Zohar and Mylonas (2001). These include an absence of, or only partial, vitellogenic development and post vitellogenic oocytes that fail to undergo final oocyte maturation (FOM) and ovulation.

Whilst captivity and the associated human presence itself can be a stressor that may inhibit gonadal maturation, captive reared fish often miss critical environmental and/or social cues responsible for initiating gonad maturation and spawning (Mylonas et al.

2010). Many salmonid species for example, partake in mass spawning migrations in which they are exposed to vast changes in hydrology, salinity, temperature, water depth and substrate. Certain tropical species are reliant on rainfall and floods to stimulate and synchronise spawning (Lam 1983). The environmental parameters required for some species to spawn cannot always be replicated in captivity; however, in many cases the reproductive dysfunctions have been overcome through manipulation of the environment or the use of exogenous hormones.

The most common reproductive dysfunction in captive reared fish occurs when the animals undergo vitellogenesis as normal, but fail to undergo FOM and ovulation, and therefore spawning (Mylonas et al. 2010). As such, a majority of research on hormonal manipulation has focused on this aspect of reproductive development, as reviewed by Zohar (1989), Zohar and Mylonas (2001), Mylonas et al. (2010). A variety of hormones that act on different sites in the hypothalamus-pituitary-gonadal axis (HPG) have been used to induce ovulation and spawning. Human chorionic gonadotropin (hCG) is a gonadotropin used in spawning induction of a multitude of fish species (Zohar and Mylonas 2001), which acts at the level of the gonads to stimulate the synthesis and release of sex steroids (Mylonas and Zohar 2007). A variety of synthetic gonadotropin releasing hormone analogues (GnRHa), including luteinising releasing hormone analogue (LHRHa), that were developed commercially for application in human medicine, are also implemented in the hormonal manipulation of a range of captive finfish broodstock (Mylonas et al. 2010). GnRHa acts directly on the pituitary to stimulate the release of endogenous gonadotropins. GnRHa therapies are therefore generally the most utilised method for stimulating final oocyte maturation and spawning; see reviews by Mylonas and Zohar (2000) and Mylonas et al. (2010).

There are limited examples of the use of hormonal manipulations to stimulate vitellogenesis, as most fish species successfully initiate vitellogenesis in captivity. In species where vitellogenesis must be initiated in captivity, such as Mekong catfish, *Pangasius bocourti* (Cacot et al. 2002) and European eels, *Anguilla anguilla* (Palstra et al. 2010), repeated injections of gonadotropins have been shown to induce vitellogenesis. Matsuyama et al. (1995) successfully induced vitellogenesis in red sea bream (*Pagrus major*) using repeated administration of LHRHa. An understanding of the effectiveness of exogenous hormonal treatment is beneficial, even in those species that will spawn spontaneously in captivity. Hormonal manipulation of broodstock fish affords a level of control to provide regular seed production, as well as facilitating more controlled and regulated spawning that is essential for selective breeding. Both LHRHa and hCG are readily available for hormonal manipulation of fish and have been shown to successfully induce maturation and spawning in cobia broodstock (Franks et al. 2001, Nguyen et al. 2010). However, there is no information available concerning the use of hormonal therapies to initiate and support vitellogenesis in cobia.

An accurate assessment of ovarian development is fundamental to successful hormone induction of spawning. Cobia do not develop obvious external signs of advancing sexual maturity, such as pronounced external changes in body shape, alterations in jaw shape or other secondary sex characteristics, that occur in some other fish species (Crim and Glebe 1990). Wallace and Selman (1981) described the three main types of ovarian development that are generally in observed in fish; synchronous, when all oocytes develop in unison and are spawned in a single event, group synchronous, when two or more distinct groups of oocytes at different developmental stages are present concurrently, from which fish are capable of multiple distinct spawning events associated with seasonal or lunar cycles and asynchronous, when oocytes at all stages of

development are observed, allowing ovulation to occur on a daily on continuous basis over a protracted period. Asynchronous and group synchronous oocyte development have been observed in cobia at BIRC over several spawning seasons (Dutney, unpubl. data) and has been reported in wild cobia, consistent with multiple spawning throughout the reproductive season (Biesiot et al. 1994, Lotz et al. 1996). While several studies have examined spawning activity in captive cobia (Arnold et al. 2002, Liao et al. 2004, Holt et al. 2007, Benetti et al. 2008) oocyte development has not been reported in detail. There is a need to improve existing methods used for assessing oocyte samples in cobia. Incorporating this inherent asynchrony into the measurement of ovarian development will provide a more accurate assessment of the impact of exogenous influences, such as hormones or environmental conditions, on reproductive development.

The most common method of assessing ovarian development of marine fish in commercial or research aquaculture facilities is via gonadal biopsy, referred to as canulation. This involves gently inserting hollowing plastic tubing, of approximately 1 mm inner diameter, into the oviduct to a depth of 5-10 cm. Gentle suction is applied to the tube as it is withdrawn from the ovary and the contents are then expelled onto a microscope slide for examination (Partridge et al. 2002). Ovarian development is typically then quantified by calculating the average diameter of the ten largest oocytes or by examining the most advanced group of oocytes present in the sample (Davis 1982, Partridge et al. 2002). Studies on wild cobia populations conducted by van der Velde et al. (2010) used a similar approach, combining histological examination of the gonads and staging ovarian development according to the most advanced group of oocytes in a section. However, an evaluation based on only the largest oocytes in the sample may not provide true indication of ovarian development, as it would also depend on what proportion of the oocytes reached advanced stages. If there has been only partial

maturation or asynchronous development of the ovary, assessing only the largest oocytes in the sample will provide a false or exaggerated measure of ovarian development. In such cases, there is a need incorporate all oocyte stages present in a sample in order to accurately assess the development of the ovary.

The primary aim of this study was to conduct a preliminary investigation to gain an understanding of the effect of repeated doses of exogenous hormones, LHRHa and hCG on circulating sex steroids and on stimulating early reproductive development in female cobia. As part of this study, a simple, commercially applicable assessment method was developed to provide a more accurate measure of overall development of the ovary in cobia. This approach was based on a measure of the relative abundance of all oocyte stages within an ovarian biopsy sample. This new measurement tool was then used to assess the effect of exogenous hormones on ovarian development in cobia.

3.3 Methods

3.3.1 General husbandry

The broodstock maturation system at BIRC consists of four 30,000 L fibreglass tanks each with a separate enclosed recirculating system consisting of 500 µm pre-filter screens, dual 1 kw transfer pumps, zeolite media filter, 150 W UV steriliser, 13 kw heat/chill unit, foam fractionator and moving bed bioreactor. All tanks were fitted with internal bottom drains, and overflow outlets that facilitate the collection of eggs post-spawn. Each tank was fitted with a vinyl cover to exclude natural light and prevent escape. Lighting was provided by two twin 37 W fluorescent lights.

Fish were fed to satiety five days per week on a mixture of squid, prawns and pilchards (*Sardinops* spp.). Pilchards were supplemented with a vitamin mixture as per Appendix 1.

Water quality measurements including temperature, dissolved oxygen, salinity, and pH were recorded daily, along with general observations of fish condition, behaviour and feeding response for each broodstock tank. The pH of the system was maintained above 7.5 by adding sodium carbonate as required. Total ammonia nitrogen (TAN) was measured weekly. All water quality parameters remained stable and within normal operating levels for broodstock systems at BIRC for both trials (Table 3.1).

Table 3.1 The range of water quality measurements taken daily in the hCG and LHRHa trial tanks.

Trial	Temperature (°C)	Dissolved oxygen (ppm)	Salinity (ppt)	pH	Total ammonia nitrogen (ppm)
HCG	26.9-27.7	>5.0	34.5-35.7	7.5-8.0	<0.6
LHRHa	25.9-27.1	>5.0	35.6-36.8	7.9-8.3	<0.5

Prophylactic disease treatments were conducted immediately before the trial and again after four weeks. The water level in the recirculating systems was lowered to approximately 4000 L and 200 ppm of formalin was added. After 60 minutes, clean, temperature adjusted water, was flushed through the system at a rate of approximately 150 L/min for 20 minutes. The tank was then refilled at the same rate to the operating level with temperature adjusted water.

Fish were sedated prior to any sampling or examination. Water in the broodstock tanks was lowered to a working depth of approximately 400 mm, with a volume of 4000-5000 L. Light sedation of fish was obtained by adding 10 ppm of AQUI-S® (Aqui-S New Zealand Ltd.) to the entire tank. For heavy sedation, the fish were transferred to a 600 L tank

containing 25 ppm AQUI-S®, allowing for various examinations including ovarian sampling (canulation), weight checks and blood sampling.

3.3.2 Broodstock

Cobia broodstock used in the LHRHa trial were wild-caught fish sourced from the outer reaches of Moreton Bay in October 2011. The fish were held in quarantine at BIRC for approximately 5 weeks, during which time they were allowed to recover from any injuries resulting from capture and then given three, one hour, 200 ppm formalin treatments for at three day intervals. During the quarantine period PIT tags were inserted subcutaneously into the dorsal musculature close to the base of the first dorsal spine, allowing fish to be individually identified. At the completion of the quarantine period, in mid-November, a total of 8 females and 3 males ranging from 3 to 6.5 kg were transferred from the quarantine system to a single broodstock maturation system and allowed to settle for one week before baseline sampling was conducted. An additional three wild caught male fish of similar size captured the previous year were also added to the system at the commencement of the trial. The prescribed photoperiod was representative of the maximum ambient daylight for the region at 14 hr light:10 hr dark, at the point of stocking. Temperature was slowly increased at approximately 0.5 °C per week from 25.5 °C to a maximum of 27 °C. Fish weight ranged from 4.8 to 9.2 kg at the completion of the trial.

The cobia broodstock used in the hCG trial were first generation captive reared fish produced at BIRC. The fish selected for the trial were those that showed minimal or an absence of ovarian development in response to maximised seasonal photoperiod and temperature. Fish were two years old at the commencement of the trial and the weight ranged from 6.5 to 16 kg. Fish were selectively allocated to treatment and control groups

in an attempt to provide similar weights and ovarian development in each group. Prescribed photoperiod was 14 hr light:10 hr dark at the point of stocking. Temperature ranged from 26.9 – 27.7 °C during the trial period. Fish weight ranged from 8 to 19 kg at the completion of the trial.

3.3.3 Analysis of ovarian development

Ovarian samples were taken by inserting 1mm plastic tubing (canula) through the gonopore and approximately 5-10 cm into the ovary, and applying gentle suction. The extracted ovarian tissue was placed into a petri dish and held on ice until analysis. For analysis, a sample of the extracted tissue was placed on a microscope slide with a small amount of seawater and a cover slip. Gentle pressure was applied to the cover slip to flatten the sample to provide close to a single layer of oocytes.

Oocytes were classified into five stages based on using similar criteria to that described by Morehead *et al*. (1998). Staging was based on the developmental stage of cobia oocytes and the coinciding oocyte diameter. Table 3.2 describes the physical parameters of each oocyte stage. There was some overlap in stage classification between oocyte diameter and the development stage of the oocyte. To maintain simplicity and to ensure the methods remained commercially applicable, oocyte diameter was used as the primary defining parameter.

Oocyte samples were viewed with a Nikon Eclipse Ti inverted compound microscope and digital images were captured by a Nikon digital camera mounted on the microscope. Measurements and calculations were conducted using NIS Elements software. Ovarian development was assessed by first selecting the total sample area of approximately 30 mm^2 from the field of view of the captured image. Any void area, not containing oocytes, was measured and subtracted from the total sample area to provide a total oocyte area.

All oocytes at stage 2 and above were outlined and individual diameters and area were calculated using the imaging software. The total area of stage 2 to 5 oocytes was subtracted from the total oocyte area to calculate the area of stage 1 oocytes. The oocyte stage percentage was then calculated by dividing the area of each stage by the total oocyte area, as follows:

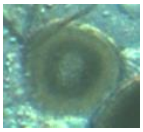
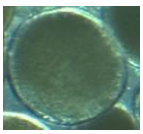
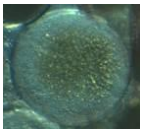
Oocyte area = Total sample area – Void

Stage 1 area = Oocyte area – Stage (2+3+4+5)

Oocyte stage percentage = Stage area / Oocyte area x 100

The use of percentage area of each oocyte stage, rather than a percent distribution, was used due to the difficulty in counting individual stage 1 or previtellogenic oocytes. Previtellogenic oocytes were difficult to separate and to manipulate into a single layer in order to count individual oocytes. The assessment of proportional area ensured that a measure of the previtellogenic oocytes could be incorporated into the assessment yet remained technically feasible for use in commercial hatchery operations.

Table 3.2 Cobia oocyte stages, based on size range and description[a]

Stage	Oocyte Stage	Description	Size Range (µm)	
1	Primordial	Clear oocyte	< 170	
2	Cortical Alveoli	Endogenous yolk vesicles form a distinct granular ring	170 – 430	
3	Vitellogenic	Exogenous yolk accumulation (oocyte opaque)	430 – 685	
4	Maturing	Yolk fuses, oil drop coalesces and oocyte clears	685 – 1025	
5	Hydrated	Oil drop coalesced, oocyte clear	> 1025	

[a] Adapted from Morehead et al. (1998)

3.3.4 Experimental design and sampling

The LHRHa study examined a total of eight fish, held in a single tank, over an eight week period, with four receiving LHRHa treatment and four control fish. The hCG study was conducted over six weeks, in which a total of seven fish were used in a single tank with four fish treated with hCG and three fish remained as control animals. Fish were selectively allocated to either the treatment or control group, ensuring that the compositions of groups were matched based on similar weight and ovarian developmental status. Hormonal treatments were applied to fish following sedation as described above. LHRHa was administered intramuscularly via a cholesterol based pellet (Lee et al. 1986) at a dose of 15 µg/kg. HCG was dissolved according to the manufacturer's instructions and administered via intraperitoneal injection at a rate of 500 IU/kg. Fish in the control group underwent the same procedures without receiving the injection.

At the commencement of both the LHRHa and hCG studies, fish were weighed, oocyte samples collected as described above, blood samples collected and the first hormone injection was administered. Subsequent sampling and hormone treatment for each trial is described in Table 3.3.

Table 3.3 Sample schedule for the LHRHa and hCG trials, showing the weeks in which each sample type was taken

Trial	Weight	Canulation	Blood sample	Hormone injection
LHRHa	0,2,4,8	0,2,4,8	0,4,8	0,2
hCG	0,4,6	0,2,4,6	0,2,4,6	0,1,2,3

Blood sampling was conducted by placing the sedated fish ventral side uppermost in a supportive cradle and collecting 0.5 ml samples from the caudal vein using a heparinised 21 G x 38 mm needle and 1 ml syringe. Blood was then transferred to a 1.5 ml heparinised polypropylene tube and embedded in shaved ice. Heparin was prepared at a

concentration of 9 units/µl. Needles and syringes were prepared by drawing and expelling heparin solution through them; 5 µl of heparin was pipetted and added to polypropylene tubes. Blood plasma was separated by centrifugation at 4000 rpm for 20 minutes at 4 °C within two hours of collection. Four 100 µL aliquots of plasma from each sample were then held at -80 °C until required.

The concentration of plasma 17β-estradiol was quantified using an EIA competitive assay kit (Cayman Chemical ACE ™) according to the manufacturer's instructions. Plasma samples were used undiluted and without extraction.

3.3.5 Statistical analysis

The time-series nature of the data was taken into account by an analysis of variance of repeated measures (Rowell and Walters 1976), via the AREPMEASURES procedure of GenStat (2013). This forms an approximate split-plot analysis of variance (split for time). The degree of temporal autocorrelation was estimated using Greenhouse-Geisser epsilon, and probability levels adjusted for this. The residual plots were examined for each analysis, and as these showed approximate normality, no transformations were considered. Statistical analysis of ovarian development was conducted separately on the sum percentage of stage 2 to 5, stage 3 to 5, and stage 4 to 5. The start weight of the fish was included as a covariate in the analysis.

3.4 Results

3.4.1 Analysis of ovarian development

The high variability in oocyte size and stage observed ovarian biopsies of cobia (Figure 3.1) cannot be accounted for using a single figure descriptor. The difference between the traditional method of assessment and that developed in the current study is

demonstrated in Figure 3.2. The devised percentage area method measured and accounted for the consistency of oocyte size within a sample and in doing so provides a better indication of overall development of the ovary and the readiness of fish to spawn.

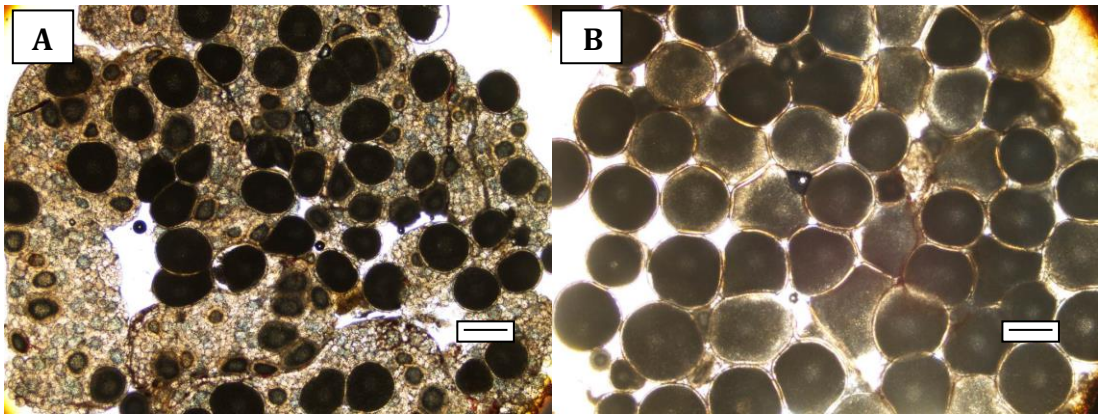


Figure 3.1 Cobia oocyte images.
A - Highly variable oocyte stages as a function of asynchronous development and **B** - Well developed and consistent oocytes stages. Scale bar = 500µm.

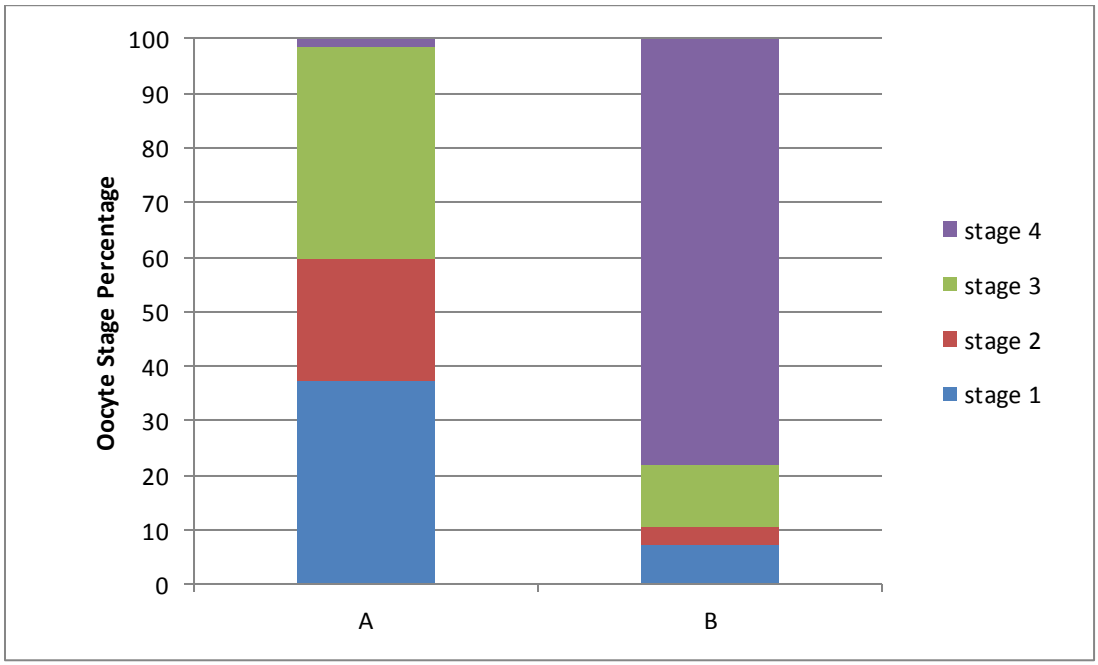


Figure 3.2 Percent area of oocyte stages of sample A and B shown in Figure 3.1.

3.4.2 LHRHa Trial

Ovarian development was highly variable in both the treatment and control animals. Two fish in each group failed to show any change in ovarian development through the course of the trial, with oocytes remaining at stage 1. The other two fish in each group demonstrated substantial ovarian development, evidenced by an increased percentage of oocytes at stage 3 or higher (Figure 3.3). Those fish that did develop showed a steady increase in development over the eight-week trial period. There was no significant difference between the hormone treated and untreated fish at any level of sum percentage ovarian development ($p>0.05$). There was no statistical interaction between treatment and time ($p>0.05$) to suggest differing patterns of development (Figure 3.4). In general, those fish that initiated development continued through to maturity; however, those fish that did not initiate any form of development failed to develop at any level through the trial period. There was no evidence that LHRHa injection had any effect on initiating or supporting continued ovarian development in cobia.

E2 concentrations were highly variable within control and treatment animals at each sample point. There was no relationship between E2 levels and ovarian development (Figure 3.3) and no evidence of an increase in E2 concentration in response to hormonal injection (Figure 3.4). There was no significant difference between the E2 concentrations of treated and untreated fish ($p>0.05$) (Figure 3.5).

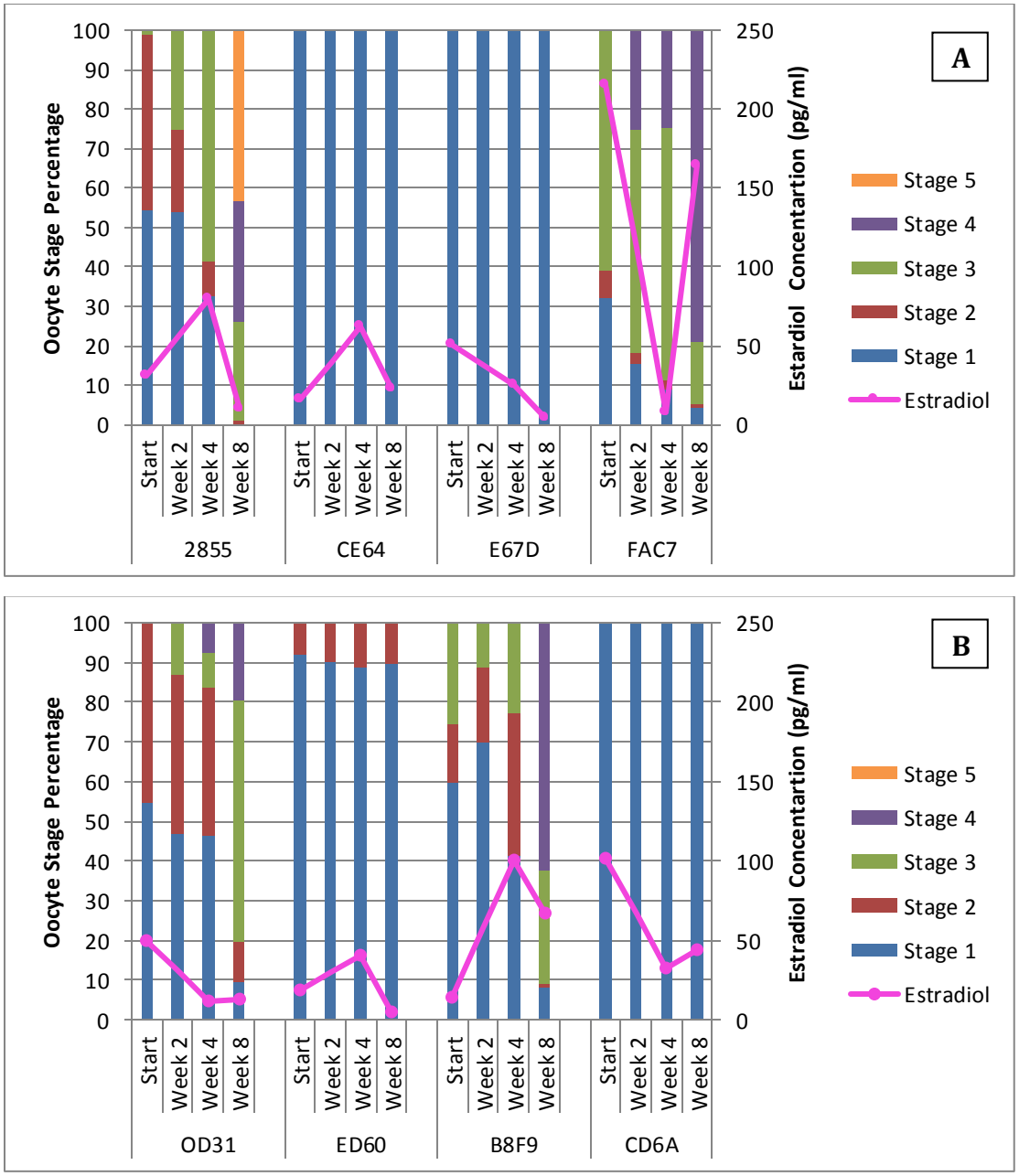


Figure 3.3 Percent area of oocyte stages and 17β-estradiol concentrations of cobia in the LHRHa trial.
A - Untreated fish and **B** - Treated fish.

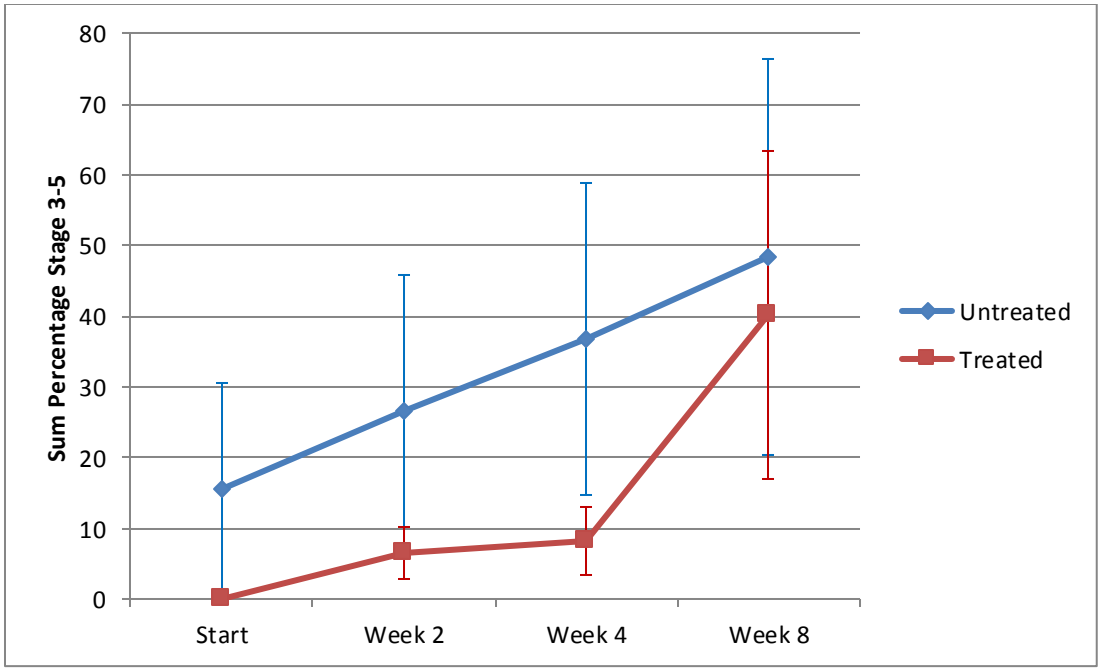


Figure 3.4 Mean sum percentage of oocytes stage 3 to stage 5 of LHRHa treated and untreated cobia (±SE).

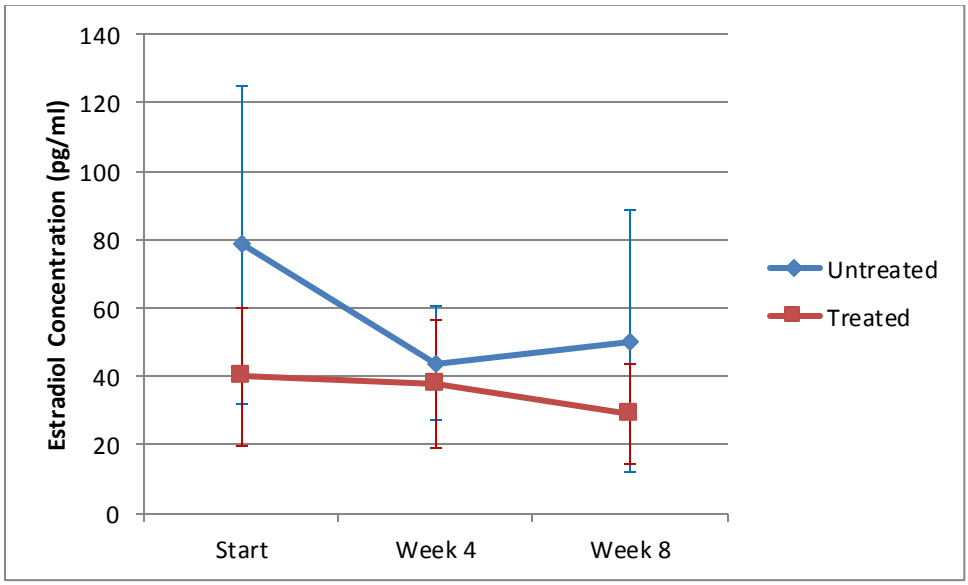


Figure 3.5 Plasma 17β-estradiol concentrations of LHRHa treated and untreated cobia (±SE).

3.4.3 HCG trial

Ovarian development was highly variable in both the treatment and control animals. Two fish in each group showed little or no change in ovarian development during the trial. One

fish in the control group demonstrated moderate ovarian development, while two fish in the treatment group showed substantial development. One fish in the treatment group developed rapidly at T2, demonstrated by the presence of a moderate level of stage 5 oocytes, showed moderate regression at T4 and then substantial redevelopment at T5 (Figure 3.6).

There was no significant difference between hormone treated and untreated fish at any level of sum percentage ovarian development ($p>0.05$). There was no statistical interaction between treatment and time ($p>0.05$) to suggest differing patterns of development (Figure 3.7). Those fish that initiated development continued to develop through to maturity regardless of treatment effect. There was no evidence that repeated hCG injection was effective in initiating or supporting continued ovarian development in cobia.

There was no relationship between plasma E2 concentration and ovarian development. There was no significant difference between the E2 concentrations of treated and untreated fish ($p>0.05$) (Figure 3.8). Plasma E2 was highly variable in the untreated fish, primarily due to one fish with very high concentration at each sample point.

Examining plasma E2 concentrations as a percentage change showed very little relative change in untreated fish. Treated fish showed a moderate increase in E2 concentration at T4, following the second hCG injection, before a substantial drop by T6 (Figure 3.9).

When ovarian development was initiated it developed rapidly, especially in the fish that were hormone treated. Within a two week period two fish progressed from approximately 80% previtellogenic oocytes to a sum percentage stage 3-5 of approximately 80%.

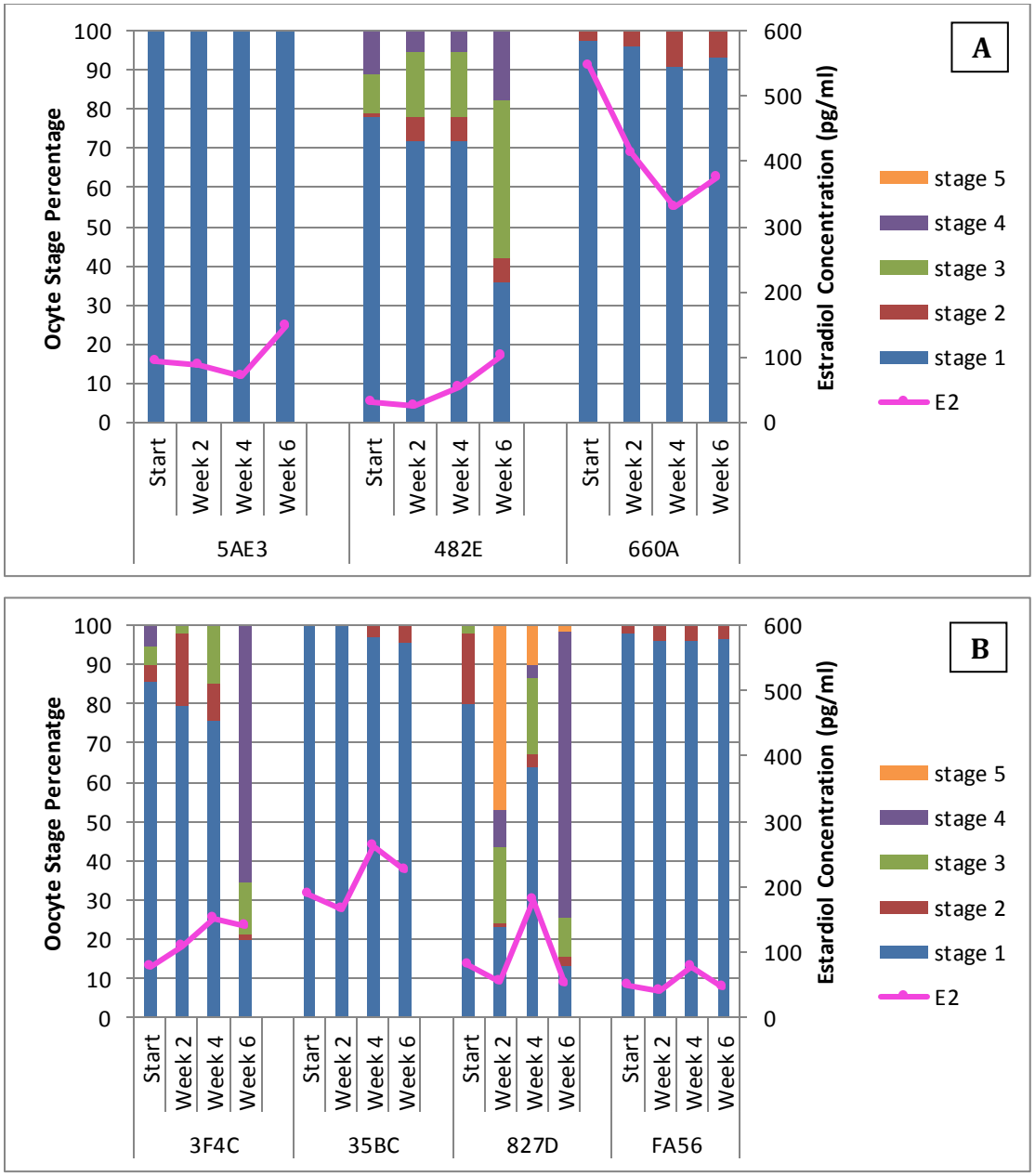


Figure 3.6 Percent area of oocyte stages and 17β-estradiol concentrations of cobia in the hCG trial. **A** - Untreated and **B** - Treated fish.

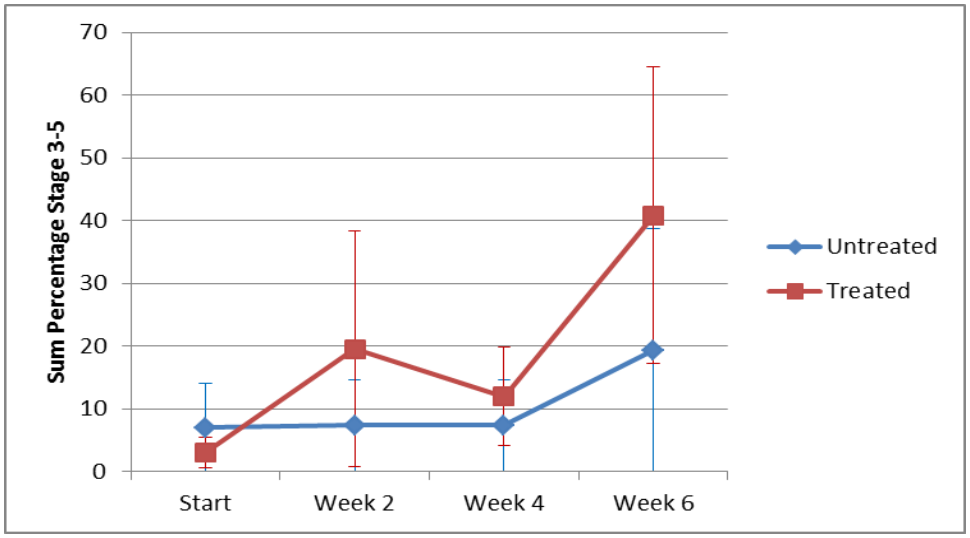


Figure 3.7 Mean sum percentage of oocytes stage 3 to stage 5 in hCG treated cobia and untreated cobia (±SE).

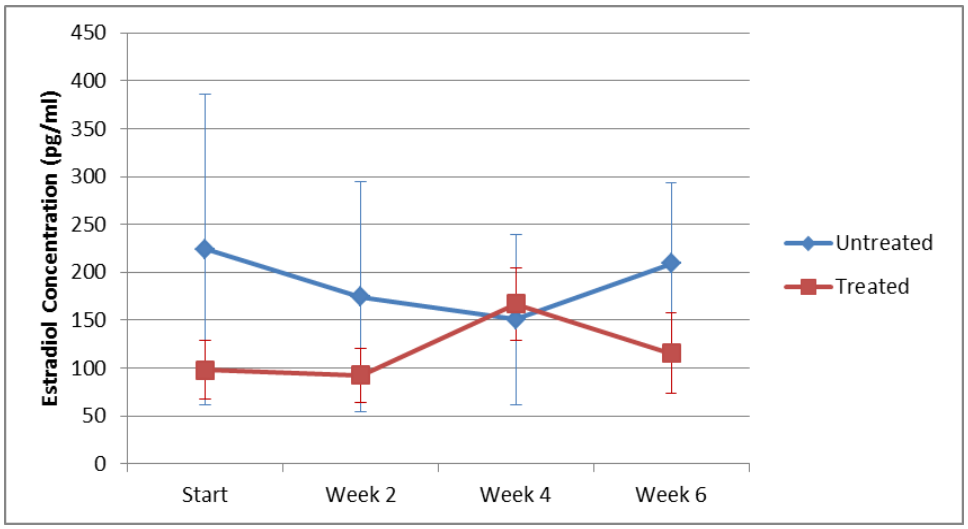


Figure 3.8 Plasma 17β-estradiol concentrations in hCG treated and untreated cobia (±SE).

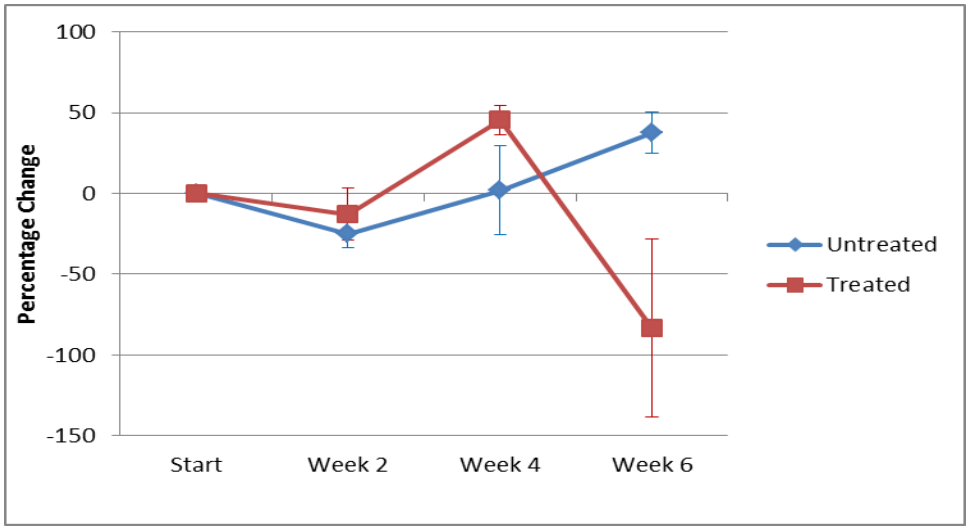


Figure 3.9 The percentage change in plasma 17β-estradiol concentration in hCG treated and untreated cobia (±SE).

3.5 Discussion

The current study demonstrates that ovarian development in cobia can be highly variable within individual fish and between individuals within the same population. Marine broodstock, including cobia, are considered suitable for hormonal induction when the ten largest oocytes are 500 µm (Partridge et al. 2002, Benetti et al. 2007, Weirich et al. 2007). Based on this assumption, both of the fish sampled in Figure 3.1 would be suitable for hormone induction. However, previous unpublished hormone induction trials at BIRC suggest it would be highly unlikely that sample A would spawn, whereas sample B could be successfully hormone induced. Asynchronous and group synchronous ovarian development observed in the current trial has also been described in wild cobia populations (Biesiot et al. 1994, Lotz et al. 1996). A factor contributing to the limited success for hormone induction of cobia at BIRC in the past may have been due to the methodologies used to assess ovarian development of broodfish, which assumed a unimodal distribution. A single-figure descriptor derived from the average of the largest

oocytes in a sample did not account for the asynchronous development observed in cobia. The use of such a measure to assess ovarian development could falsely indicate the readiness of an individual for hormone induced spawning, resulting in variable outcomes from hormonal induction of spawning.

The approach described in the current study effectively incorporates the various oocyte stages present in ovarian biopsy samples from cobia. The overlap in oocyte size and developmental state observed when defining oocyte stages is common when attempting to categorise the continuum of development that occurs in ovarian maturation (West 1990). The decision to use oocyte diameter as the defining parameter provided a distinction between the stages to aid assessment and analysis yet ensure the method was commercially appropriate. Measuring ovarian development by assessing proportions is more complicated than measuring selected oocytes; however, it provides a more accurate quantification of ovarian maturation and the suitability of individual fish for hormone induction. The use of parameters such as the proportion of oocytes at stage 3 or higher provides a straightforward measure of development status and a potential threshold for decision-making for spawning induction. These parameters can then be readily applied in both research and commercial settings.

This study set out to examine the effect of LHRHa and hCG treatment on circulating sex steroids and vitellogenic development in cobia. Limited sample sizes were used in both trials within the current study. This is often a drawback of experimental work conducted on large fish, in which the availability of suitable fish and infrastructure are often limiting (Mushiake et al. 1998, Mylonas et al. 2013a, Yazawa et al. 2015). Furthermore, the current study required animals that were at a mature size or age, but had shown limited ovarian development during what is considered the reproductive season of the species (Holt et al. 2007). Due to the limited sample sizes it is difficult to draw definitive conclusions from

the study; however, it does provide some insights into the reproductive physiology of cobia.

The application of LHRHa has been previously shown to induce vitellogenesis in red sea bream, *Pagrus major* (Matsuyama et al. 1995). However, more commonly, chronic administration of LHRHa has been shown to enhance, rather than initiate vitellogenesis, as demonstrated in milk fish, *Chanos chanos* (Lee et al. 1986). Studies conducted on the Pacific herring, *Clupea harengus pallasi,* and Atlantic salmon, *Salmo salar* demonstrated a response to GnRHa from vitellogenic oocytes; however, there was no evidence of an effect on previtellogenic oocytes (Crim et al. 1983, Carolsfeld et al. 1988). There was no evidence from the results of the current study to suggest that LHRHa was capable of stimulating vitellogenesis in cobia. Furthermore, there was no change in plasma E2 concentrations to suggest that LHRHa had an effect on circulating sex steroids. These results are broadly consistent with the proposition by Mylonas and Zohar (2000) that gonadotropin releasing hormone analogue (GnRHa) is generally not effective in stimulating vitellogenesis. Whilst the current study applied LHRHa only via cholesterol pellets at a consistent dose rate, Matsuyama et al. (1995) found LHRHa to be equipotent at a variety of dose rates and irrespective of administration via cholesterol pellet or osmotic pump. The release of GnRH by the hypothalamus regulates the pituitary to release the gonadotropins (GtH) luteinising hormone (LH) and follicle stimulating hormone (FSH). The GtHs then stimulate the synthesis of sex steroids from the gonads, primarily E2 in females, to elicit and regulate gonadal development. Follicle stimulating hormone is associated with vitellogenesis and LH with the regulation of final oocyte maturation in fish with synchronous ovarian development. However, both FSH and LH may contribute to the control of vitellogenic development in fish with asynchronous ovarian development (see review by Mylonas et al. 2010). The administration of LHRHa

would be expected to stimulate endogenous GtH to facilitate ovarian development; however, the current study shows that cobia, as with the majority of fish species, did not respond accordingly. There remains limited explanation for the relative inability of GnRHa to initiate vitellogenesis, largely due to the limited knowledge of the functions of the brain and pituitary during this stage of development (Mylonas and Zohar 2007).

The use of GtH, such as hCG, has been generally more effective for the induction of vitellogenesis compared with GnRH. Repeated doses of salmon and carp pituitary extract, injected on a weekly basis over several months, have been shown to effectively induce vitellogenesis in the Japanese and European eel (Suetake et al. 2002, Palstra et al. 2010). Successful induction of vitellogenesis in Mekong catfish has been realised by administering multiple daily injections of human chorionic gonadotropin (hCG) at low doses. Maturation and ovulation is then induced using two stage injections with higher doses of hCG (Cacot et al. 2002). There was however, no evidence in the current study to suggest that hCG administration is able to initiate or support vitellogenesis in cobia. As with the LHRHa treated fish those fish that initiated vitellogenesis continued to develop regardless of their exposure to treatment or control conditions.

Plasma E2 was highly variable between individual cobia. Examining the percent change across each time point in the hCG trial compensated for the variability in absolute levels and enabled the assessment of change in E2 concentrations in response to hormone injection. There was an increase in E2 concentrations in cobia shortly after the third hCG injection, followed by a rapid decline in the proceeding weeks. It therefore appears that there might have been a physiological response to the administration of hCG; however, it was insufficient to facilitate a change in ovarian development. Variations in dose rate, frequency and application strategies such as sustained release methods, have been shown to influence the response to exogenous hormones in a number of fish species

(Mylonas and Zohar 2000). Such variations may yield an improved response in cobia to hCG, to facilitate progression through to ovarian development.

The measurement of E2 as an indicator of ovarian development in cobia appears to have limited application. The current study showed no relationship between plasma E2 and ovarian development of cobia in. Estradiol is produced in the ovaries of fish in response to increased levels of GtH and stimulates vitellogenin synthesis in the liver to facilitate ovarian development (Nagahama and Yamashita 2008). As such, it would be expected that E2 concentration would increase in accordance with ovarian development. This relationship; however, was not evident in cobia, irrespective of the hormone treatment applied. Ovarian development in cobia appears to occur independently of plasma E2 concentrations. In contrast, E2 concentration in most marine fish increases linearly or exponentially, depending on the species, to peak concomitant with ovarian maturation (Scott et al. 1980, Morehead et al. 2000, Dahle et al. 2003, King and Pankhurst 2003). Using diethyl ether extraction and RIA analysis, Scott et al. (2013) reported levels of plasma E2 in roach, *Rutilis rutilis*, prior to gonadal recrudescence that were similar to those reported for cobia in the current study. However, once ovarian development commenced, the levels were up to 15 times higher than seen in the current study (Scott et al. 2013).

In a study using a method of E2 analysis similar to that in the current study, Palstra et al. (2010) describes E2 concentrations that were lower than those in cobia. They report concentrations of approximately 50 pg/ml in eels prior to hormone manipulation; however they found E2 levels increased almost 10 fold in response to hormonal manipulation. Such an acute response to hormonal manipulation and association with ovarian development was not observed in cobia in the current study. Given that the methods used to analyse E2 were able to detect low levels, similar to those reported in

some fish species, it is expected that a significant increase would be detected. Mylonas et al. (2013b) described low levels of plasma E2 in meagre, *Argyrosomus regius*, similar to those of the current study, demonstrating that oogenesis can still proceed in the presence of relatively low level sex steroids. The relatively low concentration and variability in plasma E2 observed in this trial suggests that E2 analysis may have limited application for predicting ovarian development.

One further factor that may have influenced the observed irregularity in plasma E2 and lack of response in the hCG trial may be related to prior disruption to gonad development. Although the fish used in the hCG trial were confirmed as phenotypic females, they originated from a cohort of fish that had a significant level of gonad malformation (Chapter 2). A significant number of fish in this cohort demonstrated abnormal gonad structure and were shown to have altered growth dynamics, possibly due to endocrine disrupting chemicals (EDC). Environmental pollutants classified as EDCs are capable of interfering with the action and/or synthesis of steroid hormones resulting in an imbalance of male and female hormones which may manifest as reproductive abnormalities (Jobling et al. 2002). The occurrence of gonad malformation, and the potential for disruption within the cohort, does not explain the variable and irregular patterns in E2 concentration observed in the LHRHa trial. The impact of exposure to endocrine disruption on the reproductive function of phenotypic female cobia warrants further investigation.

The current study was able to demonstrate and incorporate a methodology that improves the accuracy of quantifying ovarian development in cobia that is applicable to both commercial operations and research facilities. The limited samples used in the study makes it difficult to draw definitive conclusions; however, the study found that as with most fish species, GnRHa had no effect on stimulating or supporting oogenesis in cobia.

While there was limited evidence to suggest a physiological change in response to hCG administration there was no evidence to suggest an effect on ovarian development.

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3.7 References

Arnold, C. R., J. B. Kaiser and G. J. Holt (2002). "Spawning of cobia *Rachycentron canadum* in captivity." Journal of the World Aquaculture Society **33**(2): 205-208.

Benetti, D. D., M. R. Orhun, B. Sardenberg, B. O'Hanlon, A. Welch, R. Hoenig, I. Zink, J. A. Rivera, B. Denlinger, D. Bacoat, K. Palmer and F. Cavalin (2008). "Advances in hatchery and grow-out technology of cobia *Rachycentron canadum* (Linnaeus)." Aquaculture Research **39**(7): 701-711.

Benetti, D. D., M. R. Orhun, I. Zink, F. Cavalin, B. Sardenberg, K. Palmer, B. Denlinger, D. Bacoat and B. O'Hanlon (2007). Aquaculture of cobia (*Rachycentron canadum*) in the Americas and the Caribbean. Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan**:** 57-78.

Biesiot, P. M., R. E. Caylor and J. S. Franks (1994). "Biochemical and histological changes during ovarian development of cobia, *Rachycentron canadum*, from the northern Gulf of Mexico." Fishery Bulletin **92**: 686-696.

Cacot, P., M. Legendre, T. Q. Dan, L. T. Tung, P. T. Liem, C. Mariojouls and J. Lazard (2002). "Induced ovulation of *Pangasius bocourti* (Sauvage, 1880) with a progressive hCG treatment." Aquaculture **213**(1–4): 199-206.

Carolsfeld, J., N. M. Sherwood, H. Kreiberg and S. A. Sower (1988). "Induced sexual maturation of herring using GnRH 'quick-release' cholesterol pellets." Aquaculture **70**(1–2): 169-181.

Crim, L. W., D. M. Evans and B. H. Vickery (1983). "Manipulation of the Seasonal Reproductive Cycle of the Landlocked Atlantic Salmon (*Salmo salar*) by LHRH Analogues Administered at Various Stages of Gonadal Development." Canadian Journal of Fisheries and Aquatic Sciences **40**(1): 61-67.

Crim, L. W. and B. D. Glebe (1990). Reproduction. Methods for Fish Biology. C. B. Schreck and P. B. Moyle. Bethesda, American Fisheries Society**:** 529-553.

Dahle, R., G. L. Taranger, Ø. Karlsen, O. S. Kjesbu and B. Norberg (2003). "Gonadal development and associated changes in liver size and sexual steroids during the reproductive cycle of captive male and female Atlantic cod (*Gadus morhua* L.)."

Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **136**(3): 641-653.

Davis, T. (1982). "Maturity and sexuality in barramundi, (*Lates calcarifer* (Bloch)), in the Northern Territory and south-eastern Gulf of Carpentaria." Marine and Freshwater Research **33**(3): 529-545.

Dutney, L. and P. Palmer (2008). Farmed cobia making progress at BIARC. Queensland Aquaculture News, Queensland Government Department of Primary Industries and Fisheries**:** 6-7.

FAO (2013). Fisheries Statistical Database: Aquaculture production 1950-2010. www.fao.org/fishery/statistics/software/fishstat/en.

Franks, J. S., J. T. Ogle, J. M. Lotz, L. C. Nicholson, D. N. Barnes and K. M. Larsen (2001). "Spontaneous spawning of Cobia, *Rachycentron canadum*, induced by Human Chorionic Gonadotropin (HCG), with comments on fertilization, hatching and larval development." Proceedings of the Gulf and Caribbean Fisheries Institute **52**: 598-609.

Holt, J. G., C. K. Faulk and M. H. Schwarz (2007). "A review of the larviculture of cobia *Rachycentron canadum*, a warm water marine fish." Aquaculture **268**(1-4): 181-187.

Holt, J. G., J. Kaiser and C. Faulk (2007). Advances in cobia research in Texas. Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University**:** 45-56.

Jobling, S., N. Beresford, M. Nolan, T. Rodgers-Gray, G. C. Brighty, J. P. Sumpter and C. R. Tyler (2002). "Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents." Biology of Reproduction **66**(2): 272-281.

King, H. R. and N. W. Pankhurst (2003). "Ovarian growth and plasma sex steroid and vitellogenin profiles during vitellogenesis in Tasmanian female Atlantic salmon (*Salmo salar*)." Aquaculture **219**(1–4): 797-813.

Lam, T. J. (1983). Environmental influences on gonadal activity in fish. Fish Physiology. D. J. R. W.S. Hoar and E. M. Donaldson, Academic Press. **Volume 9, Part B:** 65-116.

Lee, C. S., C. S. Tamaru, J. E. Banno and C. D. Kelley (1986). "Influence of chronic administration of LHRH-analogue and/or 17α-methyltestosterone on maturation in milkfish, *Chanos chanos*." Aquaculture **59**(2): 147-159.

Lee, C. S., C. S. Tamaru and C. D. Kelley (1986). "Technique for making chronic-release LHRH-a and 17α-methyltestosterone pellets for intramuscular implantation in fishes." Aquaculture **59**(2): 161-168.

Liao, I. C., T.-S. Huang, W.-S. Tsai, C.-M. Hsueh, S.-L. Chang and E. M. LeaÃ±o (2004). "Cobia culture in Taiwan: current status and problems." Aquaculture **237**(1-4): 155-165.

Liao, I. C., E. M. Leano, C. T. Hsu and C. C. Ku (2007). Marine cage culture of cobia in Taiwan. Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University**:** 131-145.

Lotz, J. M., R. M. Overstreet and J. S. Franks (1996). "Gonadal maturation in the cobia, *Rachycentron canadum*, from the northcentral Gulf of Mexico." Gulf Research Reports **9**(3): 147-159.

Matsuyama, M., H. Takeuchi, M. Kashiwagi, K. Hirose and H. Kagawa (1995). "Induced gonadal development and spawning of immature red sea bream (*Pagrus major*) with LHRH-a administration in different ways during winter season." Fisheries Science **61**(3): 472-477.

Morehead, D. T., N. W. Pankhurst and A. J. Ritar (1998). "Effect of treatment with LHRH analogue on oocyte maturation, plasma sex steroid levels and egg production in female striped trumpeter *Latris lineata* (Latrididae)." Aquaculture **169**(3-4): 315-331.

Morehead, D. T., A. J. Ritar and N. W. Pankhurst (2000). "Effect of consecutive 9- or 12-month photothermal cycles and handling on sex steroid levels, oocyte development, and reproductive performance in female striped trumpeter *Latris lineata* (Latrididae)." Aquaculture **189**(3-4): 293-305.

Mushiake, K., K. Kawano, T. Kobayashi and T. Yamasaki (1998). "Advanced spawning in yellowtail, (*Seriola quinqueradiata*), by manipulations of the photoperiod and water temperature." Fisheries science **64**(5): 727-731.

Mylonas, C. and Y. Zohar (2000). "Use of GnRHa-delivery systems for the control of reproduction in fish." Reviews in Fish Biology and Fisheries **10**(4): 463-491.

Mylonas, C. C., A. Fostier and S. Zanuy (2010). "Broodstock management and hormonal manipulations of fish reproduction." General and Comparative Endocrinology **165**(3): 516-534.

Mylonas, C. C., N. Mitrizakis, C. A. Castaldo, C. P. Cerviño, M. Papadaki and I. Sigelaki (2013a). "Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity II. Hormonal induction of spawning and monitoring of spawning kinetics, egg production and egg quality." Aquaculture **414–415**(0): 318-327.

Mylonas, C. C., N. Mitrizakis, M. Papadaki and I. Sigelaki (2013b). "Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity I. Description of the annual reproductive cycle." Aquaculture **414–415**(0): 309-317.

Mylonas, C. C. and Y. Zohar (2007). Promoting oocyte maturation, ovulation and spawning in farmed fish. The fish oocyte: from basic studies to biotechnical applications. P. J. Babin, J. CerdÃ and E. Lubzens. Dordrecht, Springer**:** 437-474.

Nagahama, Y. and M. Yamashita (2008). "Regulation of oocyte maturation in fish." Development, Growth & Differentiation **50**: S195-S219.

Nguyen, H. Q., T. M. Tran, H. Reinertsen and E. Kjørsvik (2010). "Effects of dietary essential fatty acid levels on broodstock spawning performance and egg fatty acid composition of cobia, *Rachycentron canadum*." Journal of the World Aquaculture Society **41**(5): 687-699.

Nhu, V. C., H. Q. Nguyen, T. L. Le, M. T. Tran, P. Sorgeloos, K. Dierckens, H. Reinertsen, E. KjÃ¸rsvik and N. Svennevig (2011). "Cobia *Rachycentron canadum* aquaculture in Vietnam: Recent developments and prospects." Aquaculture **315**(1-2): 20-25.

Palstra, A. P., D. Schnabel, M. C. Nieveen, H. P. Spaink and G. E. E. J. M. van den Thillart (2010). "Temporal expression of hepatic estrogen receptor 1, vitellogenin1 and vitellogenin2 in European silver eels." General and Comparative Endocrinology **166**(1): 1-11.

Partridge, G. J., G. I. Jenkins and K. R. Franklin (2002). Manual for the hatchery production of snapper (*Pagrus auratus*) and black bream (*Acanthopagrus butcheri*). Fremantle,

Fisheries Research and Development Corporation and the Aquaculture Development Unit, WA Maritime Training Centre, Challenger TAFE.

Sampaio, L. A., C. B. Moreira, K. C. Miranda-Filho and A. N. Rombenso (2011). "Culture of cobia *Rachycentron canadum* (L) in near-shore cages off the Brazilian coast." Aquaculture Research **42**(6): 832-834.

Scott, A. P., V. J. Bye and S. M. Baynes (1980). "Seasonal variations in sex steroids of female rainbow trout (*Salmo gairdneri* Richardson)." Journal of Fish Biology **17**(5): 587-592.

Scott, A. P., T. Hill, N. Beresford, T. J. Runnalls, J. P. Sumpter and T. Ellis (2013). "Out-of-season production of 17,20β-dihydroxypregn-4-en-3-one in the roach *Rutilus rutilus*." Journal of Fish Biology **83**(2): 233-249.

Shaffer, R. V. and E. V. Nakamura (1989). Synopsis of biological data on the cobia *Rachycentron canadum* (Piscies: Rachycentridae). U. D. o. Commerce. Washington DC USA, FAO Fisheries synopsis **153:** 21.

Shiau, C.-Y. (2007). Biochemical composition and utilization of cultured cobia (*Rachycentron canadum*). Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University**:** 147-156.

Suetake, H., K. Okubo, N. Sato, Y. Yoshiura, Y. Suzuki and K. Aida (2002). "Differential expression of two gonadotropin (GTH) β subunit genes during ovarian maturation induced by repeated injection of salmon GTH in the Japanese eel *Anguilla japonica*." Fisheries Science **68**(2): 290-298.

van der Velde, T., S. Griffiths and G. Fry (2010). "Reproductive biology of the commercially and recreationally important cobia (*Rachycentron canadum*) in northeastern Australia." Fisheries Science **76**(1): 33-43.

Wallace, R. A. and K. Selman (1981). "Cellular and dynamic aspects of oocyte growth in teleosts." American Zoologist **21**: 325-343.

Weirich, C. R., A. D. Stokes, T. I. J. Smith, W. E. Jenkins, M. R. Denson, J. R. Tomasso, J. Chappel and D. Burnside (2007). Cobia aquaculture research in South Carolina, USA: Captive reproduction, pond nursery production, and selected environmental

requirements of juveniles. Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University: 19-44.

West, G. (1990). "Methods of assessing ovarian development in fishes: a review." Marine and Freshwater Research **41**(2): 199-222.

Yazawa, R., Y. Takeuchi, K. Amezawa, K. Sato, G. Iwata, N. Kabeya and G. Yoshizaki (2015). "GnRHa-induced spawning of the Eastern little tuna (Euthynnus affinis) in a 70-m3 land-based tank." Aquaculture **442**: 58-68.

Zohar, Y. (1989). Fish reproduction: its physiology and artificial manipulation. Fish culture in warm water systems: problems and trends. M. Shilo and S. Sarig. Boca Raton, Florida, CRC Press: 65-119.

Zohar, Y. and C. C. Mylonas (2001). "Endocrine manipulations of spawning in cultured fish: from hormones to genes." Aquaculture **197**(1-4): 99-136.

3.8 Appendices

Appendix 1

Broodstock Vitamin Supplement

Supplement was offered to compensate for suspected vitamin loss in stored frozen baitfish food. The formulation was developed by Dr K Williams, (Queensland Department of Primary Industries Nutritionist) based on known requirements of fish generally.

The supplement is obtained as two separate components: (1) dry premix (Northern Fisheries Vitamin Premix Code D 062/0 Rhone-Poulenc Animal Nutrition Pty. Ltd) and choline chloride viscous liquid. These are kept separate until required so as to maximize the shelf life of the vitamins. Dry premix is freezer stored and has about 3-6 months shelf life while the liquid choline chloride is refrigerated and very stable.

Mixing Instructions: To 980 ml of distilled water add 100 grams of dry premix and 20 ails of choline chloride. Mix vigorously and store in a dark coloured contained under refrigeration. In use agitate frequently to keep mixture reasonably well suspended. Make up about one week's supply at a time since shelf life of the refrigerated made up mixture is only about a week.

Administration: A 5 ml graduated (Lyppards brand) plastic disposable or preferably stainless steel (Model 74 Vaccinator) auto syringe is used to inject vitamin supplement into the broodstock food. The agitated mixture is injected at the rate of one ml per 50 grams pilchard when broodstock are being fed at 2% body weight per day: If feeding rate is decreased to 1%, then 2ml per 50 grams of bait fish is injected.

Formulation

Compound	Unit	Amount /kg premix	Allowance per kg brood fish per day
Vitamin A	I.U.	2×10^6	80
Vitamin D_3	I.U.	0.8×10^6	32
Vitamin E (DL-α-tocopherol)	g	40	1.6
Vitamin K3	g	2	0.08
Ascorbic acid	g	40	1.6
Thiamin	g	4	0.16
Riboflavin	g	4	0.16
Pyroxidine	g	4	0.16
Pantothenic acid	g	10	0.4
Biotin	mg	100	4
Nicotinic acid	g	30	1.2
Folic acid	g	1	0.04
Vitamin B12	mg	4	0.16
Choline chloride	g	200	8
Inositol	g	50	2
PABA	g	20	0.8
Ethoxyquin	g	30	1.2
Dextrose	g	to 1.0 kg	--

Chapter 4: The influence of photothermal manipulation on reproductive development of captive cobia (*Rachycentron canadum*).

This chapter is presented as a manuscript to be submitted for publication in Aquaculture Journal.

The influence of photothermal manipulation on reproductive development of captive cobia (*Rachycentron canadum*).

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4.1 Abstract

Two cohorts of cobia (*Rachycentron canadum*) broodstock, sourced from separate annual spawnings, were assessed on a monthly basis to examine the ovarian development and plasma 17β-estradiol (E2) of individual fish in response to photothermal manipulation. In 2013, eight female and four male broodstock fish were maintained in each of four 30,000L tanks. Two tanks were placed on a compressed phototherm regime and two tanks were maintained on an ambient phototherm. In 2014, two tanks each containing eleven females and six males were placed on a compressed phototherm and one tank with nine females and five males was held in ambient conditions. Ovarian development was limited, in terms of the number of individuals and the level of development, in the 2013 trial, irrespective of the phototherm regime. There was no significant difference in development between the compressed and ambient phototherm regime. Ovarian development was significantly higher in the 2014 trial compared to 2013, with a majority of fish developing to maturity. There was no significant difference in overall ovarian development between the compressed and ambient phototherm in 2014 at the completion of the trial. There was however a significant interaction between time and treatment demonstrating that ovarian development advanced earlier under a compressed phototherm compared to the ambient phototherm. Ovarian development was most rapid when temperature was 24-27 °C and photoperiod was 12.5 - 14 hrs. Plasma E2 concentrations were relatively low in comparison to other marine species throughout each trial period. There was no relationship between E2 concentration and ovarian development of cobia in either trial. Fish in the 2013 trial showed sporadic development in which ovarian samples contained low numbers of late stage oocytes amongst a large percentage of previtellogenic oocytes. Ovarian samples from the 2014

fish showed more regular progression in development. The possibility of reproductive dysfunction due to exposure to endocrine disruption in the early life history stages of the 2013 fish is discussed.

Key words
Cobia, *Rachycentron canadum*, ovarian development, photoperiod, temperature

Highlights

- Ovarian development can be advanced with the use of photothermal manipulation.
- Different response to photothermal manipulation was observed between two cohorts of cobia in relation to levels of gonad malformation.
- No relationship between levels of circulating 17β-estradiol and ovarian development was evident.

4.2 Introduction

Most fish, in their natural environment, display some degree of seasonality in reproductive activity. Coordinating the reproductive cycle with seasonal changes in climate and photoperiod ensures that spawning coincides with the environmental conditions most suited for the growth and survival of juveniles (Bromage et al. 2001). Photothermal manipulation has been used to improve broodstock management by extending the production seasons and facilitating out of seasoning spawning of a large range of fish species, see reviews by (Zohar 1989, Bromage et al. 2001, Pankhurst and Porter 2003, Pankhurst and King 2010). This allows for the regular seed supply for on growing and allows producers to meet the market requirement for annual supply of product. For example, the spawning season of Atlantic salmon (*Salmo salar*) is extended by implementing an accelerated photoperiod (Taranger et al. 1998), while exposure to low water temperature prior to ovulation will further advance and synchronise spawning (Taranger et al. 1999). Out of season larval supply of striped trumpeter, *Latris lineata*, and gilthead sea bream, *Sparus aurata*, is facilitated by exposing broodstock to a compressed phototherm regime (Zohar et al. 1995, Morehead et al. 2000). Precise control of gonadal recrudescence and year-round spawning in southern flounder (*Paralichthys lethostigma)* is achieved by manipulation of photoperiod and temperature (Watanabe et al. 2006).

Cobia, *Rachycentron canadum*, is a large benthopelagic species that is endemic to all tropical and subtropical waters across the globe with the exception of the eastern Pacific (Shaffer and Nakamura 1989). Commercial cobia aquaculture began in Taiwan in the late nineties and has since been adopted by several nations through the Asian-Pacific, the USA and South America (Liao et al. 2007, Benetti et al. 2008, Nhu et al. 2011, Sampaio et al.

2011). Global production is dominated by China, which produces approximately 40,000 of the global annual production of 43,000 metric tonnes (T) (FAO 2013).

Cobia broodstock are brought to maturity using a wide variety of aquaculture systems and management techniques. Methods used for maturation of broodstock include capture of wild fish in spawning condition, selecting suitable fish from ocean grow out cages, holding fish in spawning ponds and maintaining fish in environmentally controlled tank systems (Liao et al. 2004, Holt et al. 2007). Studies that consider specific aspects of environmental or hormonal induction of cobia spawning have also been conducted (Arnold et al. 2002, Holt et al. 2007, Benetti et al. 2008, Stieglitz et al. 2012); however, they are generally production focussed and examine only the outputs from the entire population without recognising the relative contribution of individuals. Benetti et al. (2008) reported successful repeated volitional spawning from cobia held in environmental controlled recirculating systems. Stieglitz et al. (2012) proposed that exposure to sustained high temperatures was sufficient to provide out of season volitional spawning in cobia. Each of these studies examined spawning activity of the broodstock population, and whilst was able to demonstrate effective system for spawning cobia, they did not analyse the development or contribution of individuals. The failure of some individuals to spawn spontaneously during the reproductive season has been reported, with limited information available on the developmental status of those individuals (Benetti et al. 2008). There remains a paucity of information available concerning the reproductive development of individuals and the acute stimuli to initiate and support ovarian development of cobia.

The current study differs from previous research conducted on cobia broodstock, in that it assesses the progression of ovarian development of individual fish, rather than the spawning activity of the broodstock population. The active examination and assessment

of individuals within a population provides improved production efficiencies, ensuring that all fish that are maintained in a population are capable of contributing to spawning events. Furthermore, it facilitates controlled spawning events in order to maintain the genetic integrity of stock and to implement selective breeding programmes. Through regular assessment of ovarian development and sex steroids, this study aims to gain a better understanding of ovarian development of cobia across the reproductive season and to monitor the response of individuals to photothermal manipulation.

The analysis of plasma sex steroids is widely used to provide information on the reproductive development of fish. The androgen 11-Ketotestosterone plays a major role in spermatogenesis (Devlin and Nagahama 2002), and the estrogen 17β-estradiol (E2) in ovarian development by inducing vitellogenin synthesis in the liver (Nagahama and Yamashita 2008). Elevated plasma E2 levels are correlated with ovarian maturation in Atlantic salmon, *Salmo salar*, (King and Pankhurst 2003) and Atlantic cod, *Gadus morhua*, (Karlsen et al. 2014) and have been used to examine the influence of environmental parameters on gonad development (Norberg et al. 2004). There is currently no information available on sex steroid profiles in cobia. The current study set out to gain an improved understanding of the reproductive physiology of cobia through the analysis of E2 and its relationship with ovarian development in cobia.

The primary aim of this study was to gain a better understanding of the effect of photothermal manipulation on ovarian development in cobia through regular assessment of morphological and physiological development of individual fish within broodstock populations.

4.3 Methods

4.3.1 General Husbandry

Cobia were maintained at the broodstock maturation system at BIRC, Queensland, Australia, which consists of four 35,000 L fibreglass tanks each with a separate enclosed recirculating system consisting of 500 µm prefilter screens, dual 1 kW transfer pumps, zeolite media filter, 150 W ultraviolet steriliser, 13 kW heat/chill unit, foam fractionator and moving bed bioreactor. All tanks were fitted with internal bottom drains, and overflow outlets that facilitate the collection of eggs post-spawn. Each tank was fitted with a vinyl cover to exclude natural light and prevent escape. Lighting was provided by two twin 37 W fluorescent lights.

Fish were fed to satiety five days per week on a mixture of squid, prawns and pilchards (*Sardinops* spp.). Pilchards were supplemented with a vitamin premix (as per Appendix 1).

Water quality measurements including temperature, dissolved oxygen, salinity, and pH were recorded daily, along with general observations of fish condition, behaviour and feeding response for each broodstock tank. The pH of the system was maintained above 7.5 by adding sodium carbonate as required. Total ammonia nitrogen (TAN) was measured weekly. Prophylactic disease treatments were conducted immediately before the trial and again four weeks into the trial. The water level in the recirculating systems was lowered to approximately 4000 L and 200 ppm of formalin was added. After 60 minutes, clean temperature adjusted water was flushed through the system at a rate of approximately 150 L/min for 20 minutes. The tank was then refilled at approximately 150 L/min to the operating level. All water quality parameters remained stable and within normal operating levels for broodstock systems at BIRC for both trials (Table 4.1).

Table 4.1 Water quality measurement range for the 2013 and 2014 trial period.

Trial	DO (ppm)	Salinity (ppt)	pH	TAN (ppm)
2013	>5.0	34.3-36.3	7.6-8.1	<0.5
2014	>5.0	35.4-36.6	7.4-8.3	<0.5

Fish were sedated prior to any sampling or examination. Water in the broodstock tanks was lowered to a working depth of approximately 400 mm, with a volume of 4000-5000 L. Light sedation of fish was obtained by adding 10 ppm of AQUI-S® (Aqui-S New Zealand Ltd.) to the entire tank. For heavy sedation, the fish were transferred to a 600 L tank containing 25 ppm AQUI-S®, allowing for various examinations including ovarian sampling (canulation), weight checks and blood sampling.

4.3.2 Experimental design and sampling

Trials were carried out in 2013 and 2014. The broodstock cobia used in both trials were first generation captive reared fish produced and raised at BIRC. Fish were selectively allocated to treatment and control groups in an attempt to provide similar weights and levels of ovarian development in each group. Fish were sampled at the commencement of the trials and every four weeks thereafter.

Four broodstock tanks were used in the 2013 trial. Two were placed on an ambient phototherm and two were on a compressed phototherm. Eight female and four males were held in each tank. At each of the four sample points, fish weight, oocyte and blood samples were taken from all female fish.

In the 2014 trial two tanks were placed on a compressed phototherm regime. Eleven females and six males were placed in each tank. One tank, containing nine females and five males was placed on an ambient phototherm regime. Fish in the 2014 trial were sampled five times in total. At each sample point fish weight was measured and oocyte samples taken from all females, and blood samples were taken from the six females from

each tank. Blood samples were taken from the same individual fish across the sample points.

The phototherm used was similar in each trial. The compressed phototherm provided maximum photoperiod of 14 hours in September and maximum temperature of 28 °C in October, approximately three months in advance of ambient conditions. Photoperiod and temperature had not reached the seasonal maximum under ambient conditions by the conclusion of the trials. Temperature readings and photoperiod settings for each trial are presented in Figure 4.1.

Ovarian samples were taken by inserting 1mm plastic tubing (canula) through the gonopore and approximately 5-10 cm into the ovary. The extracted ovarian tissue was placed on a microscope slide with a small amount of seawater and a cover slip. Gentle pressure was applied to the cover slip to flatten the sample to provide close to a single layer of oocytes.

Oocyte samples were viewed with a Nikon Eclipse Ti inverted compound microscope and digital images were captured by a Nikon digital camera mounted on the microscope. Measurements and calculations were conducted using NIS Elements software. Ovarian development was assessed according to Chapter 2. Fish were considered to have matured sufficiently for hormonal induction of spawning when more than 75% of the oocyte samples were measured as stage 3 or above. Stage 3 oocytes were defined by exogenous yolk accumulation, in which the oocyte becomes opaque and oocyte diameter range from 430 – 685 µm. Stage 4 and 5 were defined as yolk fuses, oil drop coalesces, oocyte clears, diameter 685 – 1025 µm and oil drop coalesced, oocyte clear, diameter > 1025 µm respectively. The oocyte stage percentage was then calculated by dividing the area of each stage by the total oocyte area, as follows:

Oocyte area = Total sample area – Void

Stage 1 area = Oocyte area – Stage (2+3+4+5)

Oocyte stage percentage = Stage area / Oocyte area x 100

Individuals were placed ventral side up in a supportive cradle and 0.5 ml blood samples were collected from the caudal vein using a heparinised 21 G x 38 mm needle and 1 ml syringe. Blood was then transferred to two 1.5 ml heparinised polypropylene tubes and embedded in shaved ice. Plasma was separated by centrifugation at 4000 rpm for 20 minutes at 4 °C. Two 200 µL aliquots of plasma from each sample were then held in separate 1.5 ml polypropylene tubes at -80 °C until required.

The concentration of plasma 17β-estradiol was quantified using an EIA competitive assay kit (Cayman Chemical ACE ™) according to the manufacturer's instructions. Plasma samples were used undiluted and without extraction.

4.3.3 Statistical analysis

The time-series nature of the data was taken into account by an analysis of variance of repeated measures (Rowell and Walters 1976) via the AREPMEASURES procedure of GenStat (2013). This forms an approximate split-plot analysis of variance (split for time). The degree of temporal autocorrelation was estimated using Greenhouse-Geisser epsilon, and probability levels adjusted for this. The residual plots were examined for each analysis, and as these showed approximate normality, no transformations were considered. Statistical analysis of ovarian development was conducted on the sum percentage of stage two and above, stage three and above, and stage four and above, at each sample point. Fish weight was found to be not significant for treatments and was therefore removed from further analysis.

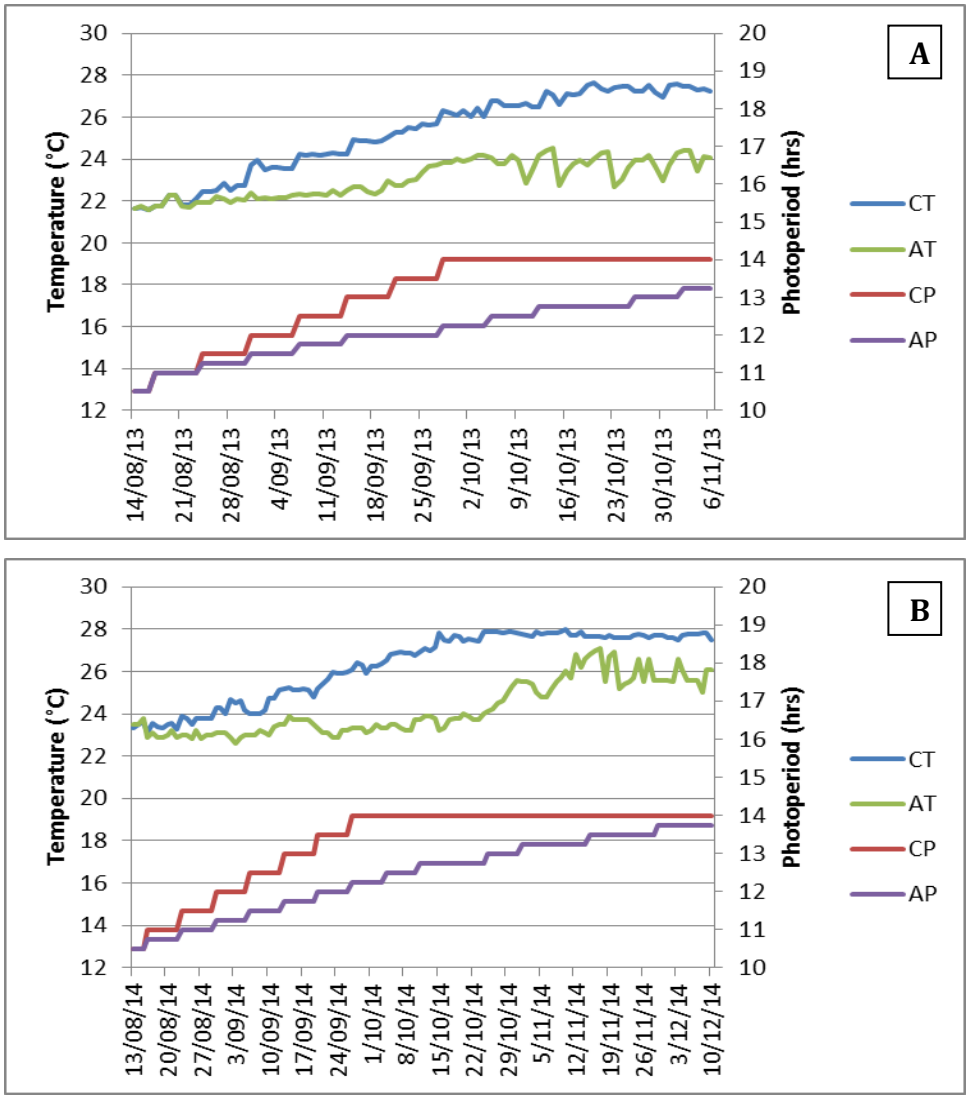

Figure 4.1 Phototherm regime used to assess the impact of photothermal manupulation on cobia in 2013 (**A**) and 2014 (**B**).
Temperature readings for the compressed (CT) and ambient (AT) phototherm and photoperiod settings for the compressed (CP) and ambient (AP) phototherm.

4.4 Results

4.4.1 Growth rates

Growth rates of cobia in each of the trials were considered normal for broodstock conditioning at BIRC (Figure 4.2). During the 2014 trial one fish from the compressed phototherm and two fish from the ambient tanks were considered to be of poor health,

based on a loss of weight. The compromised health of the fish was considered more likely to impact development than the treatment regime; as such they were removed from the analysis.

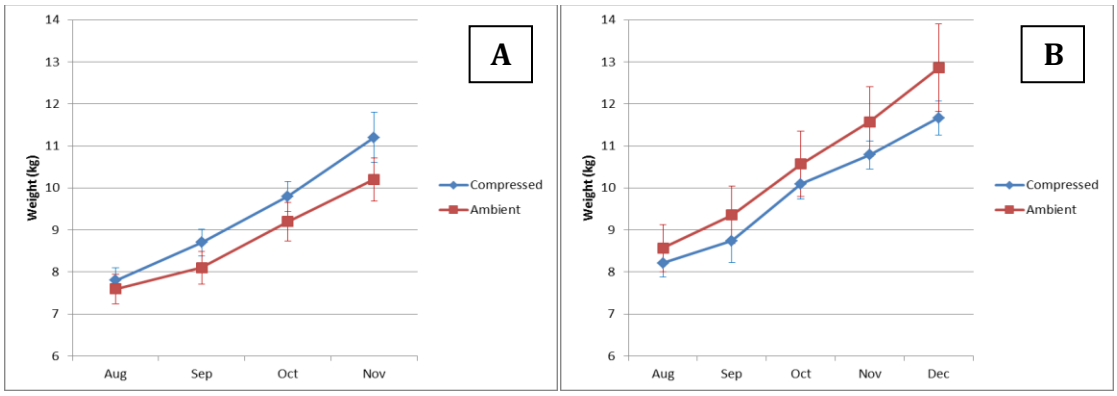

Figure 4.2 Average weight of cobia used in the 2013 (**A**) and 2014 (**B**) phototherm trials.

4.4.2 Ovarian development

2013 Trial

Ovarian development was generally low in fish from both the ambient and compressed phototherm regimes. Only eight of the sixteen fish in the ambient and five of the sixteen fish in the compressed phototherm had stage 3 oocytes during the trial period. The remaining fish demonstrated little or no ovarian development. Only two fish in the ambient and one fish in the compressed phototherm regime developed sufficiently to be considered for hormonal induction of spawning by the completion of the trial (Figure 4.3, Table 4.2). No spontaneous spawning occurred.

There was no significant difference between the sum percentage level ovarian development of fish from the ambient and compressed phototherm regimes ($p>0.05$) (Figure 4.4).

Blood samples were not analysed from the 2013 trial due to the limited development exhibited in each treatment and possibility of compromised results as a function of factors outside the treatment effects.

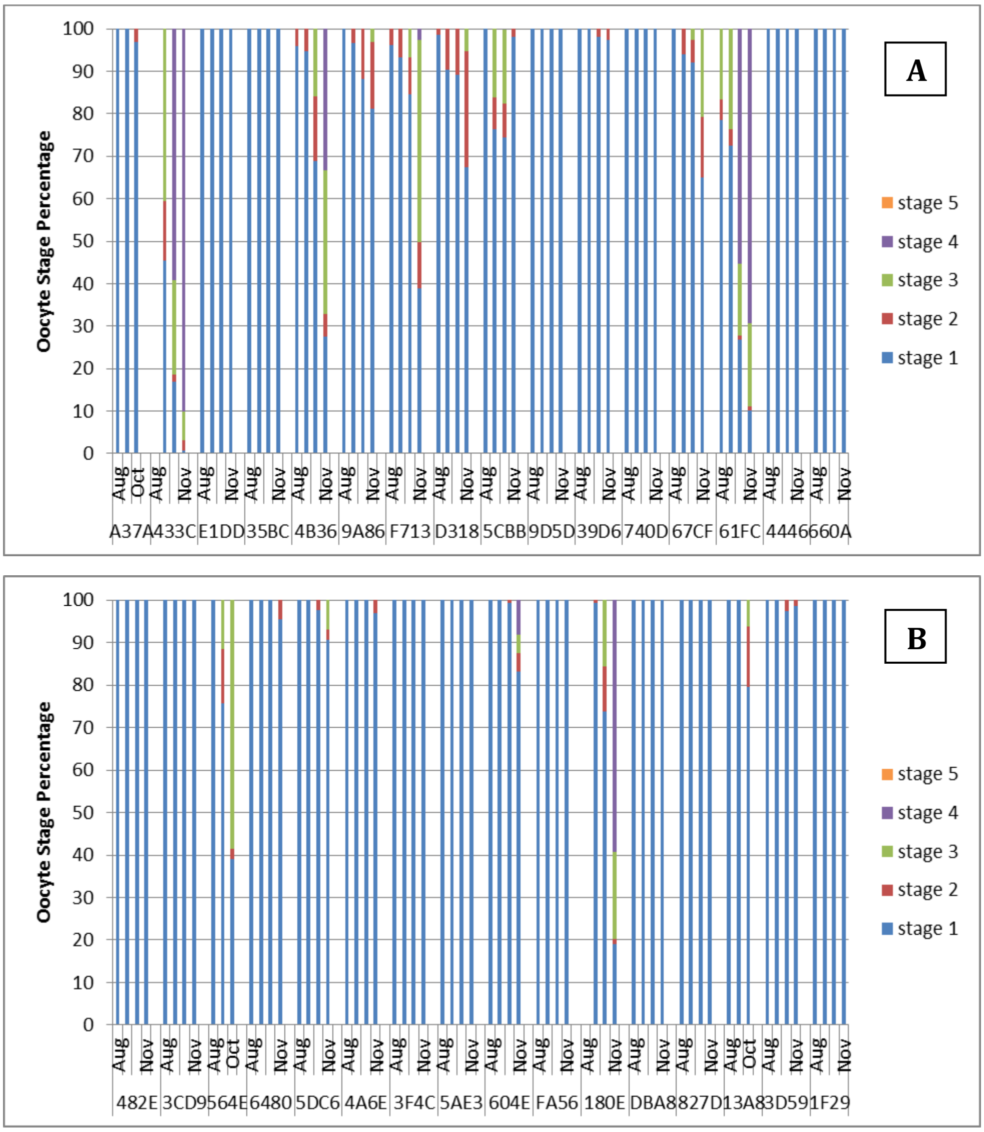

Figure 4.3 Oocyte stages of individual cobia in 2013 trial.
Percent area calculated as per section 2.2, held in 2013 ambient (**A**) and compressed (**B**) phototherm regimes (PIT tag numbers displayed below).

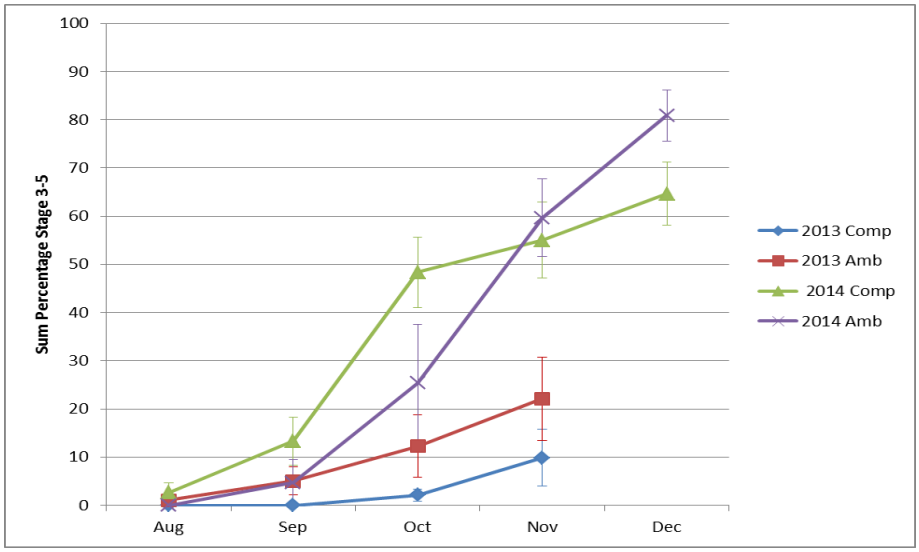

Figure 4.4 Cobia oocytes from compressed and ambient phototherm regimes in 2013 and 2014. The sum percentage of stages three to five cobia oocytes from compressed and ambient phototherm regimes in 2013 and 2014 (±SE).

2014 Trial

All fish in 2014, except two in the compressed phototherm, developed a minimum of stage 3 oocytes. Six of the seven fish in the ambient and thirteen of the twenty-one fish in the compressed phototherm regime developed sufficiently to be considered for hormonal induction of spawning at the completion of the trial (Figure 4.5, Table 4.2). No spontaneous spawning was recorded. Stage 3+ oocytes were maintained in a majority of individuals for 2 to 3 months with limited atresia present (Figure 4.5).

There was no significant difference, at any sum percentage level of ovarian development, between fish in the ambient and compressed phototherm when analysed across the entire trial period ($p>0.05$). There was however a significant interaction between time and treatment in the sum percentage of stage 2-5, 3-5 and 4-5 ($p=0.008$, $p=0.014$, $p=0.041$, respectively) suggesting a change in the pattern of development in response to the treatment, where ovarian development in the compressed phototherm advanced earlier than the ambient phototherm (Figure 4.4). The fish in the compressed phototherm

were more advanced at the October sample point. There was no difference between the two regimes at subsequent sample points.

There was a noticeable spike in development in the compressed phototherm between September and October, associated with temperature of 24-27 °C and photoperiod of 12.5-14 hrs. A similar spike in development occurred under ambient conditions between October and November associated with temperatures of 23-26 °C and a photoperiod of 12.5-13.5 hrs (Figure 4.4).

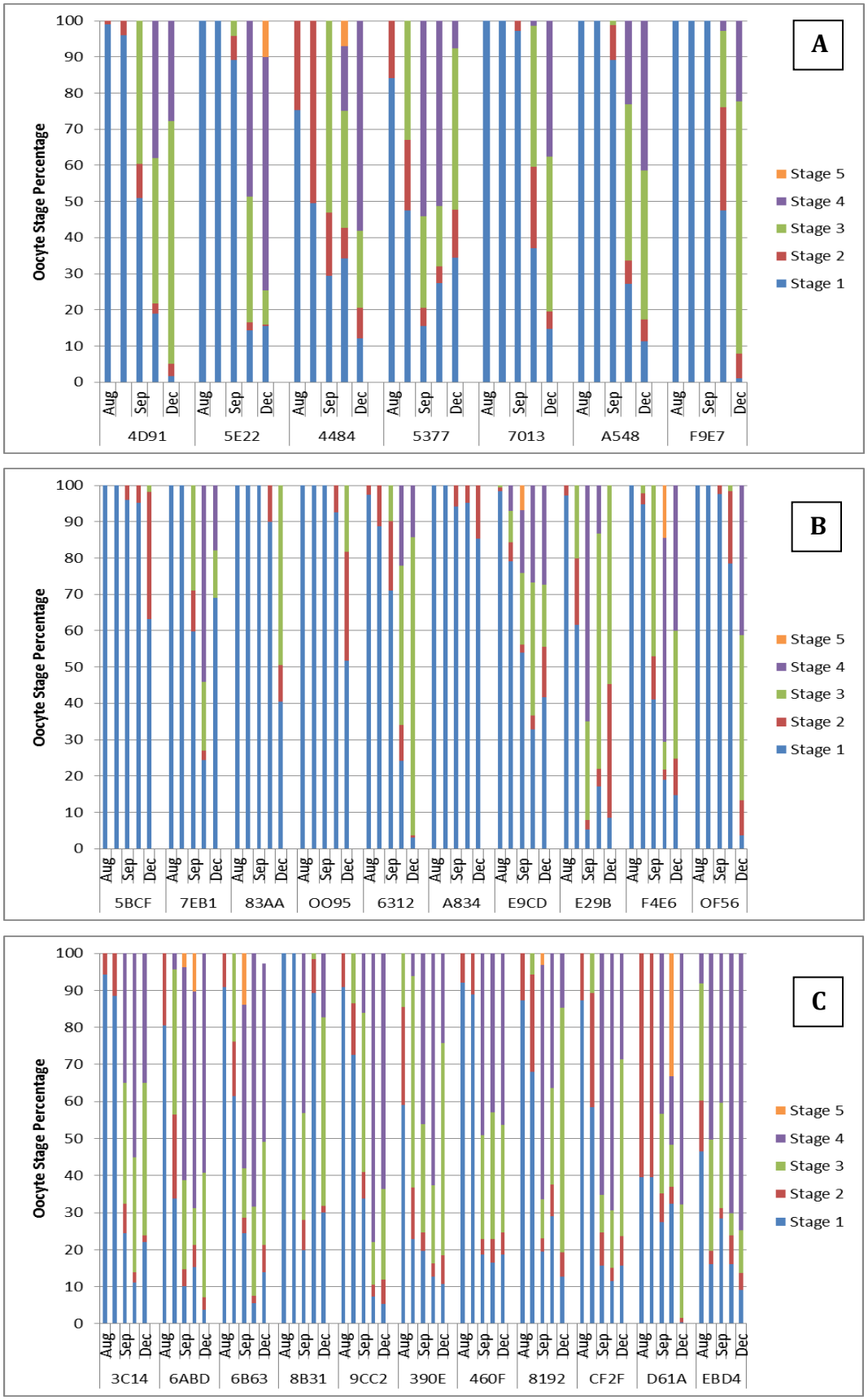

Figure 4.5 Oocyte stages of individual cobia in 2014 trial.

Percent area of oocyte stages of individual cobia, calculated as per section 2.2, held in the 2014 ambient (**A**) and compressed (**B**,**C**) phototherm regimes (PIT tag numbers displayed below).

Estradiol concentrations

Estradiol concentrations were highly variable in fish from both the ambient and compressed phototherm regimes (Figure 4.6). There was no significant difference between the E2 concentrations in the two regimes and no interaction between treatment and time to suggest a change in pattern ($p>0.05$). There was no relationship between E2 levels and ovarian development and no evidence of an increase in circulating E2 in response to photothermal manipulation (Figure 4.7).

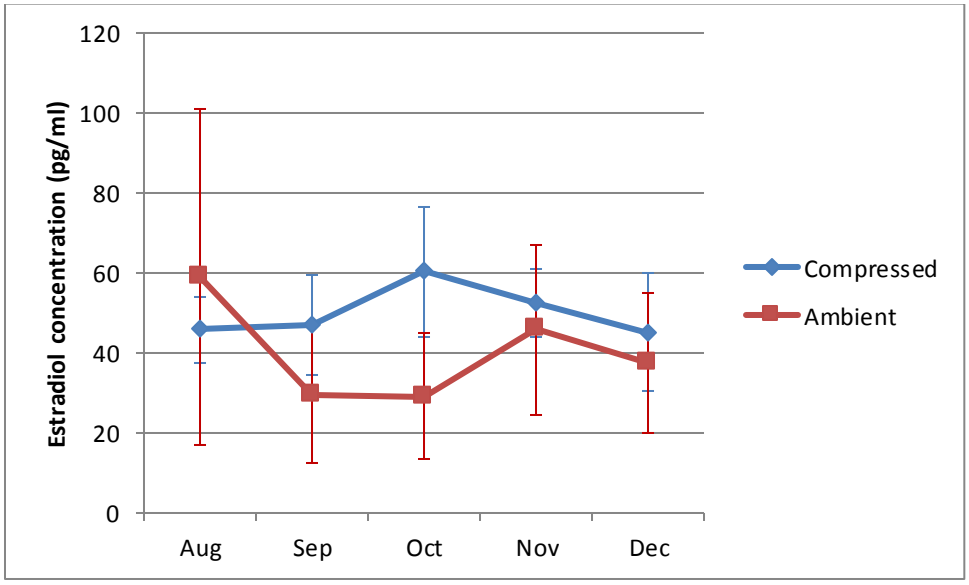

Figure 4.6 Mean 17β-estradiol concentrations of cobia females from the 2014 compressed and ambient phototherm regimes.

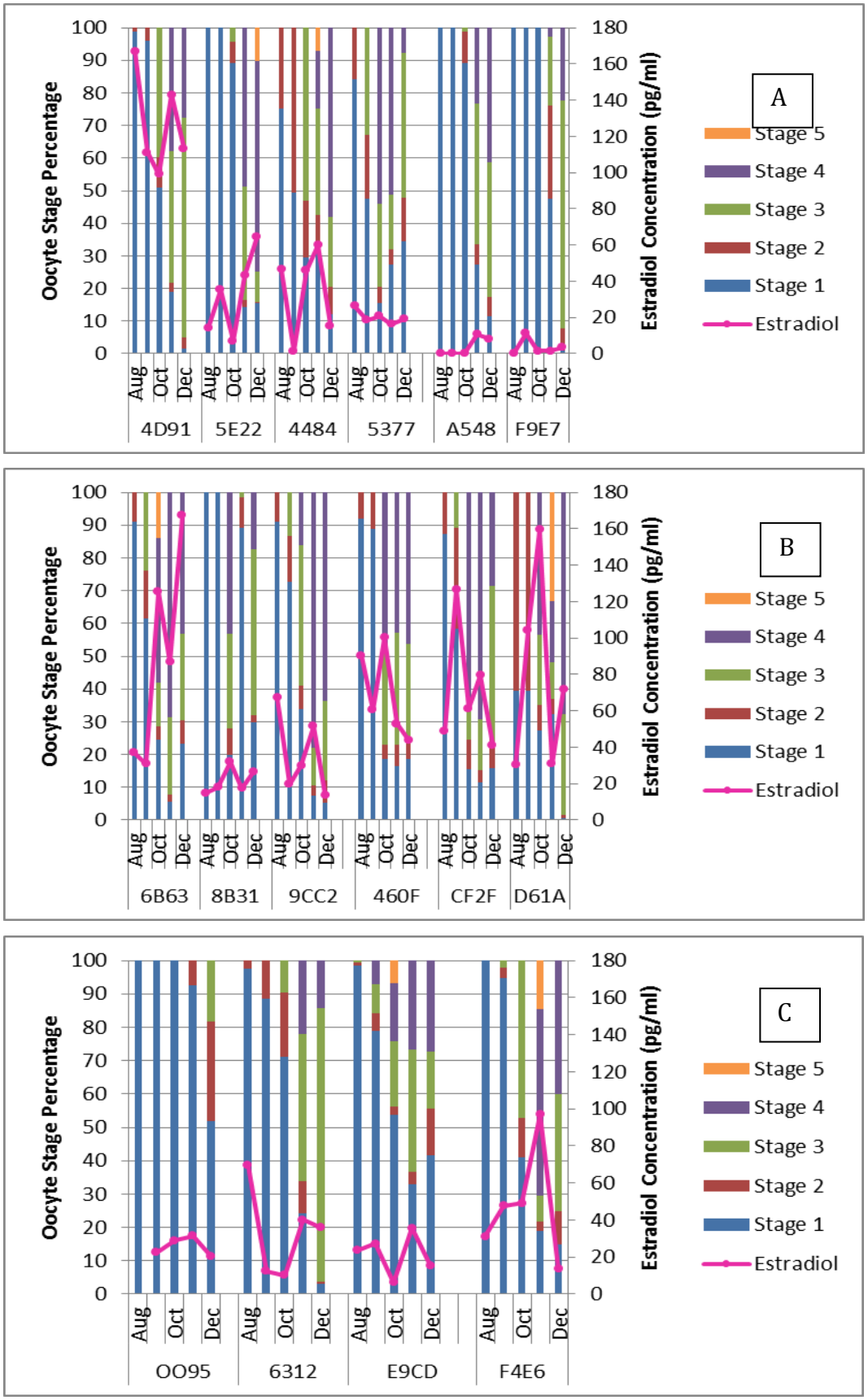

Figure 4.7 Plasma 17β-estradiol concentration and oocyte stages of individual cobia, 2014.

Plasma 17β-estradiol concentration and coinciding percent area of oocyte stages of individual cobia, calculated as per section 2.2, held in the 2014 ambient (A) and compressed (B,C) phototherm regimes(PIT tag numbers displayed below).

4.4.3 Comparison of year classes

Gonadal development progressed differently in the two cohorts, with ovarian development in 2014 significantly higher than that of 2013 at the respective sample points in October and November ($p<0.001$) (Figure 4.4).

A developmental anomaly was observed in fish from both treatments of the 2013 trial. Ovarian development appeared to be sporadic and highly asynchronous during the September and October sample points. This was evidenced by low numbers of mid to late stage oocytes mixed with a large percentage of previtellogenic and early stage oocytes (Figure 4.8).

The oocyte samples observed in 2014 were considered more regular for cobia broodstock. Although different stages of oocytes were identified within samples, there was an obvious progression of development within samples, and more synchrony compared with 2013 samples (Figure 4.9).

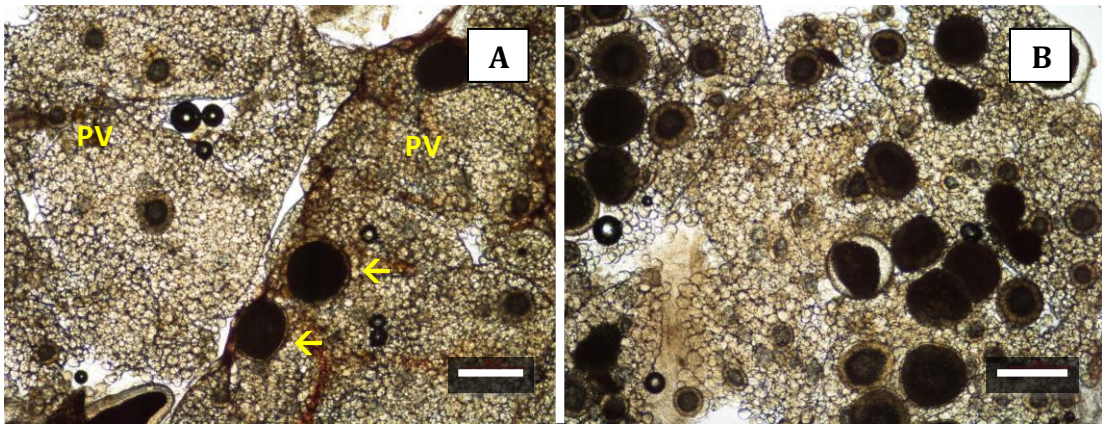

Figure 4.8 Oocyte development typical of cobia in the 2013 photothermal manipulation trial. Oocyte samples taken from a cobia broodstock in October (**A**) and November (**B**) 2013 demonstrating the highly asynchronous and sporadic development typical of cobia in the 2013 photothermal manipulation trial. Low numbers of mid to late stage

oocytes (arrows) are seen mixed with a large percentage of previtellogenic (PV) and early stage oocytes. Scale bar = 500µm.

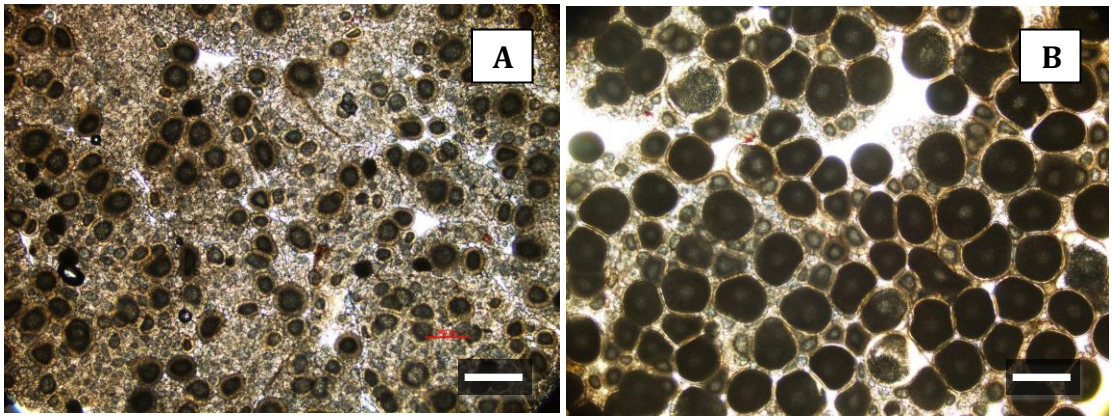

Figure 4.9 Oocyte development typical of cobia in the 2014 photothermal manipulation trial. Oocyte samples taken from a cobia broodstock in September (**A**) and October (**B**) 2014; considered typical for cobia broodstock demonstrating regular progression in the development of oocytes within samples and more synchronous development in comparison to the 2013 samples (Figure 4.8). Scale bar = 500µm.

Hormone induction of broodstock at the conclusion of the 2013 trial produced very low numbers of viable larvae. The numbers of fish which spawned, the fecundity and fertilisation were all considered well below that expected for the production of cobia (Table 4.3). Hormone induction of spawning in 2014 was highly successful with good numbers of fish spawning, and fecundity and fertilisation at levels expected from cobia broodstock (Table 4.3). As a result, fewer spawning inductions were undertaken.

Table 4.2 Numbers of female cobia broodstock suitable for induction[a] in each phototherm treatment in 2013 and 2014

	2013		2014	
Sample Point	Ambient (n=16)	Compressed (n=16)	Ambient (n=7)	Compressed (n=21)
August	0	0	0	0
September	0	0	0	1
October	1	0	1	6
November	2	1	2	11
December	Not measured	Not measured	6	13

[a] fish were considered suitable for induction when more than 75% of sample area contained oocytes at Stage 3 or above.

Table 4.3 Hormone induction results for 2013 and 2014

Trial	Number of inductions	Successful inductions	Total fish induced	Spawning fish	Average fecundity (000)	Average fertilisation rate
2013	11	64%	17	53%	623.1	14.6%
2014	3	100%	6	67%	2016.7	72.2%

Those fish identified as females in the 2013 trial were confirmed as female during post mortem examination. There was no evidence of testicular tissue in any female fish to infer any level of intersex. However approximately 50% of the fish had some level of malformation in the form a misshapen ovaries. Post mortem examination of a subsample of twelve females used in the 2014 trial found no evidence of testicular tissue with 25% of the ovaries considered misshapen.

4.5 Discussion

The aim of the current study was to gain an improved understanding of the influence photothermal manipulation on ovarian development of cobia. The results demonstrated a contrasting response to photothermal manipulation across two cobia cohorts. Ovarian development was limited to relatively few individuals in the 2013 cohort irrespective of the phototherm regime. Conversely, a majority of the fish in 2014 developed well with a measurable response to a compressed phototherm regime, highlighting how variations among cohorts can have a significant effect on seed production and commercial operations. The 2014 trial demonstrated that ovarian development could be advanced with exposure to a compressed phototherm regime. Photothermal manipulation is a well demonstrated method to advance gonadal development in aquaculture in order to extend the reproductive season of broodstock and also to provide out of season spawning of fish

to provide a regular supply of juvenile fish for on-growing, (see reviews by Zohar (1989), Bromage et al. (2001) and Pankhurst and King (2010)). Extending the reproductive season of cobia by maintaining elevated water temperature has been shown by Stieglitz et al. (2012). Their study was focussed on the frequency of volitional spawning from the broodstock population as the measurable outcome. As such, the progress of ovarian development was not considered. In contrast, the current study was structured to examine both ovarian development and spawning frequency of individuals, in an attempt to better understand the physiological response to external stimuli.

The 2014 trial was able to demonstrate that ovarian development is maximised when temperatures reach 24-26°C with a photoperiod of 12.5-13.5 hours. Although spontaneous spawning did not occur in the current study, the association with temperature and development aligns well with the findings of Stieglitz et al. (2012), in which spawning begins when the water temperature reaches 26 °C. It seems reasonable to assume that in the study by Stieglitz et al. (2012), spawning was preceded by a period of maximal ovarian development. Stieglitz et al. (2012) suggest that temperature is more influential on cobia spawning than photoperiod; demonstrated by continued spawning when water temperature remained elevated and photoperiod was decreased to that of the off-season. Whilst temperature is influential in the spawning activity of cobia (Stieglitz et al. 2012), and is likely to be the most determinant environmental factor; the influence of photoperiod on initiating ovarian development in cobia cannot be excluded, especially considering that cobia commonly occur in sub-tropical and warm temperate waters (Shaffer and Nakamura 1989). It is possible that both temperature and photoperiod influence gonadal recrudescence in cobia; however examination of temperature and photoperiod independently was outside the scope of the current project. Photoperiod is capable of providing an unambiguous date signal and is

considered to be the principal environmental determinant to stimulate reproductive development in temperate and cold water species; while temperature has a role in cueing the precise timing of maturation and spawning (Bromage et al. 2001). Due to the limited variation in the annual photoperiod and temperature in the tropics, the environmental cues associated with reproductive development are more subtle. Whilst both photoperiod and temperature remain as likely proximate cues for gonad development, it is likely that temperature has a greater influence than photoperiod (Zohar 1989, Pankhurst and Porter 2003, Stieglitz et al. 2012). Spawning activity in the tropics may also coincide with the lunar cycle, periods of increased productivity following high rainfall or shifts in oceanic currents (Bromage et al. 2001, Pankhurst and Porter 2003, van der Velde et al. 2010). Such cues may not be as predictable as photoperiod but signal an acute environmental change indicating appropriate conditions for reproduction (Pankhurst and Porter 2003).

Oocyte integrity was maintained in post vitellogenic oocytes for up to three months in a large percentage of fish in the compressed phototherm treatments of the 2014 trial. A number of studies describe the protracted spawning season of cobia for up to six months, extending through to 15 months when water temperatures remain elevated (Holt et al. 2007, Benetti et al. 2008, Stieglitz et al. 2012). However, limited information is available on the regularity or frequency of repeat spawns by individuals and the relative contribution by individuals to spawning events. The current trial was able to demonstrate that individual cobia can be maintained in a state suitable for hormone induction for at least three months during the reproductive season. Mylonas et al. (2013) describes a similar scenario in meagre, *Agryrosomus regius*, highlighting the advantage to hatchery production of having fish available for hormonal induction for extended periods over the reproductive season. Maintaining control of spawning events through hormonal

manipulation allows for the spawning of selected individuals to maintain genetic integrity and to implement selective breeding strategies.

As described in Chapter 3, the plasma E2 concentration in cobia in the current study was low in comparison to other fish species. Estradiol is produced in the ovaries of fish in response to increased levels of GtH and stimulates the production of hepatic vitellogenin and as such typically increases during vitellogenesis (Nagahama 1983). In most marine fish E2 concentration increases linearly or exponentially, depending on the species, to peak concomitant with ovarian maturation (Scott et al. 1980, Morehead et al. 2000, Dahle et al. 2003, King and Pankhurst 2003). The level of plasma E2 in the current trial was comparable to that of turbot, *Scophthalmus maximus,* at approximately 50 pg/ml, however such low levels in turbot were assumed to be a function of examining fish prior to ovarian development (Imsland et al. 2013). Scott et al. (2013) described E2 concentrations under 100 pg/ml in roach, *Rutilus rutilus,* prior to vitellogenesis. However, once ovarian development commenced, plasma E2 increased to 15 times that of the current study. Although their study used RIA as opposed to EIA in the current study, a similar pronounced increase in cobia would be detectable given that the concentration measured were towards the lower limit of detection for the EIA test.

In a number of fish species E2 concentrations can act as a predictor of maturation and ovarian development (Crim and Glebe 1990); however, the current study was unable to demonstrate a relationship between E2 concentration and ovarian development in cobia. The average E2 concentrations were consistently low throughout both the ambient and compressed phototherm treatments, despite a significant increase in ovarian development over time. Likewise, at an individual level, there was no consistent pattern of plasma E2 concentration and increasing oocyte sizes. Mylonas et al. (2013) showed an increase in the average oocyte diameter of meagre, *Argyrosomus regius,* from

approximately 150 µm to 550 µm across a three-month period when coinciding plasma E2 was less than 200 pg/ml. There was no significant increase in plasma E2 during this period when vitellogenesis occurred. The results of the current study show that, similar to meagre, oogenesis in cobia can still proceed in the presence of relatively low level sex steroids.

The fish used in the current trials were of the same cohorts described in Chapter 2, in which the occurrence of intersex cobia was first reported. All of the fish used in the 2013 trial and the subsample taken from the 2014 trial were confirmed post mortem as phenotypic females. However, it is possible that the mechanism associated with the intersex condition is also associated with the difference in ovarian development observed between the 2013 and 2014 trials. The results of Chapter 2 demonstrated that cobia did not exhibit sexually dimorphic growth when intersex fish were present in a cohort; whereas in the relative absence of intersex fish (0.5%), female cobia were 30% larger than males at harvest size. Given that the growth dynamics of the cohorts used in the present study had been altered, an impact on reproductive development would be possible. Although both cohorts used in the current contained intersex fish, the reported occurrence was higher in the 2013 cohort, at 16.9% compared with 6.9% in the 2014 cohort. The relatively low level numbers of individuals to develop to maturity coupled with the observed sporadic development within the ovary suggests that the female fish from the 2013 cohort were impacted by this anomaly. The impact on phenotypic females was further evidenced by the relatively poor results of hormone induced spawning and the misshapen gonads observed in the post mortem examination of the fish. The difference in reproductive output between the two cohorts in the present study coincides with the higher incidence of intersex fish within the 2013 cohort. In the absence of a direct comparison with a cobia cohort without intersex present, it cannot be suggested

that the 2014 cohort have not been also compromised in some way; however, the 2014 cohort showed the group synchronous development expected in cobia (Biesiot et al. 1994) as well as spawning outputs that were consistent with previous broodstock manipulation at BIRC (Dutney unpublished data).

The negative impact of endocrine disrupting compounds (EDC) and the intersex condition on the reproductive capacity of both male and female fish has been well demonstrated in other fish species. A majority of studies have found an impact on male fish as a result of estrogen mimicking compounds (Scholz and Kluver 2009). Exposure of male fish to EDCs can result in altered secondary sex characteristics, increased vitellogenin levels, reduced fertility, and partial or complete feminisation (Kiparissis et al. 2003, Harris et al. 2011, Lange et al. 2011, Kroon et al. 2015, Niemuth and Klaper 2015). The development and reproductive potential of male cobia was not examined in this study, however it is likely that the male reproductive capacity has also been impacted and contributed to the poor results from the hormone induction of spawning trials in 2013. Due to the feminising effect of many EDCs, the impact on female fish are often less obvious. Lange et al. (2011) found that female roach (*Rutilus rutilis*) exposed to high level EDCs, sufficient to feminise 98% of the population, were capable of reproducing normally, however there was evidence of degeneration of oocytes with either retarded or enhanced maturation of the ovary. Increased level of oocyte atresia has also been reported in roach exposed to EDCs (Jobling et al. 2002). Oocyte atresia was also evident in Japanese medaka (*Oryzias latipes*) along with delayed maturation as result of EDC exposure (Kiparissis et al. 2003). Chapter 2 discusses the possible causes of the intersex in cobia including exposure to phytoestrogens, such as genistein, by-products of effluent treatment facilities and agricultural and industrial run off, following exposure to floodwaters. Identifying the source of the proposed endocrine disruption was outside the

scope of this study. It does however warrant further investigation, due to the potential impact on aquaculture production and wild fisheries stocks.

4.6 Acknowledgements

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4.7 References

Arnold, C. R., J. B. Kaiser and G. J. Holt (2002). "Spawning of cobia *Rachycentron canadum* in captivity." Journal of the World Aquaculture Society **33**(2): 205-208.

Benetti, D. D., M. R. Orhun, B. Sardenberg, B. O'Hanlon, A. Welch, R. Hoenig, I. Zink, J. A. Rivera, B. Denlinger, D. Bacoat, K. Palmer and F. Cavalin (2008). "Advances in hatchery and grow-out technology of cobia *Rachycentron canadum* (Linnaeus)." Aquaculture Research **39**(7): 701-711.

Biesiot, P. M., R. E. Caylor and J. S. Franks (1994). "Biochemical and histological changes during ovarian development of cobia, *Rachycentron canadum*, from the northern Gulf of Mexico." Fishery Bulletin **92**: 686-696.

Bromage, N., M. Porter and C. Randall (2001). "The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin." Aquaculture **197**(1-4): 63-98.

Crim, L. W. and B. D. Glebe (1990). Reproduction. Methods for Fish Biology. C. B. Schreck and P. B. Moyle. Bethesda, American Fisheries Society**:** 529-553.

Dahle, R., G. L. Taranger, Ø. Karlsen, O. S. Kjesbu and B. Norberg (2003). "Gonadal development and associated changes in liver size and sexual steroids during the reproductive cycle of captive male and female Atlantic cod (*Gadus morhua* L.)." Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **136**(3): 641-653.

Devlin, R. H. and Y. Nagahama (2002). "Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences." Aquaculture **208**(3–4): 191-364.

FAO (2013). Fisheries Statistical Database: Aquaculture production 1950-2010. www.fao.org/fishery/statistics/software/fishstat/en.

Harris, C. A., P. B. Hamilton, T. J. Runnalls, V. Vinciotti, A. Henshaw, D. Hodgson, T. S. Coe, S. Jobling, C. R. Tyler and J. P. Sumpter (2011). "The consequences of feminization in breeding groups of wild fish." Environ Health Perspect **119**(3): 306-311.

Holt, J. G., C. K. Faulk and M. H. Schwarz (2007). "A review of the larviculture of cobia *Rachycentron canadum*, a warm water marine fish." Aquaculture **268**(1-4): 181-187.

Imsland, A. K., S. Gunnarsson, B. Roth, A. Foss, S. Le Deuff, B. Norberg, H. Thorarensen and T. Helming (2013). "Long-term effect of photoperiod manipulation on growth, maturation and flesh quality in turbot." Aquaculture **416–417**(0): 152-160.

Jobling, S., N. Beresford, M. Nolan, T. Rodgers-Gray, G. C. Brighty, J. P. Sumpter and C. R. Tyler (2002). "Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents." Biology of Reproduction **66**(2): 272-281.

Karlsen, Ø., C. Mittelholzer, E. Andersson, B. Norberg and G. L. Taranger (2014). "Continuous light affects onset of puberty and associated changes in pituitary gonadotropin subunit transcript levels, and plasma estradiol-17β and testosterone levels in Atlantic cod (*Gadus morhua* L.) females." Aquaculture **424–425**: 95-103.

King, H. R. and N. W. Pankhurst (2003). "Ovarian growth and plasma sex steroid and vitellogenin profiles during vitellogenesis in Tasmanian female Atlantic salmon (*Salmo salar*)." Aquaculture **219**(1–4): 797-813.

Kiparissis, Y., G. C. Balch, T. L. Metcalfe and C. D. Metcalfe (2003). Effects of the isoflavones genistein and equol on the gonadal development of Japanese medaka (*Oryzias latipes*), National Institute of Environmental Health Sciences. National Institutes of Health. Department of Health, Education and Welfare**:** 1158.

Kroon, F. J., S. E. Hook, D. Jones, S. Metcalfe, B. Henderson, R. Smith, M. S. J. Warne, R. D. Turner, A. McKeown and D. A. Westcott (2015). "Altered transcription levels of endocrine associated genes in two fisheries species collected from the Great Barrier Reef catchment and lagoon." Marine Environmental Research **104**(0): 51-61.

Lange, A., G. C. Paull, P. B. Hamilton, C. R. Tyler and T. Iguchi (2011). "Implications of persistent exposure to treated wastewater effluent for breeding in wild roach (*Rutilus rutilus*) populations." Environmental Science and Technology **45**(4): 1673-1679.

Liao, I. C., T.-S. Huang, W.-S. Tsai, C.-M. Hsueh, S.-L. Chang and E. M. Leano (2004). "Cobia culture in Taiwan: current status and problems." Aquaculture **237**(1-4): 155-165.

Liao, I. C., E. M. Leano, C. T. Hsu and C. C. Ku (2007). Marine cage culture of cobia in Taiwan. Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University**:** 131-145.

Morehead, D. T., A. J. Ritar and N. W. Pankhurst (2000). "Effect of consecutive 9- or 12-month photothermal cycles and handling on sex steroid levels, oocyte development, and reproductive performance in female striped trumpeter *Latris lineata* (Latrididae)." Aquaculture **189**(3-4): 293-305.

Mylonas, C. C., N. Mitrizakis, M. Papadaki and I. Sigelaki (2013). "Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity I. Description of the annual reproductive cycle." Aquaculture **414–415**(0): 309-317.

Nagahama, Y. (1983). The functional morphology of teleost gonads. Fish Physiology. W. S. Hoar, D. J. Randall and E. M. Donaldson. New York, Academic Press. **IXA:** 223-275.

Nagahama, Y. and M. Yamashita (2008). "Regulation of oocyte maturation in fish." Development, Growth & Differentiation **50**: S195-S219.

Nhu, V. C., H. Q. Nguyen, T. L. Le, M. T. Tran, P. Sorgeloos, K. Dierckens, H. Reinertsen, E. KjÃ¸rsvik and N. Svennevig (2011). "Cobia *Rachycentron canadum* aquaculture in Vietnam: Recent developments and prospects." Aquaculture **315**(1-2): 20-25.

Niemuth, N. J. and R. D. Klaper (2015). "Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish." Chemosphere **135**(0): 38-45.

Norberg, B., C. L. Brown, O. Halldorsson, K. Stensland and B. T. Björnsson (2004). "Photoperiod regulates the timing of sexual maturation, spawning, sex steroid and thyroid hormone profiles in the Atlantic cod (*Gadus morhua*)." Aquaculture **229**(1–4): 451-467.

Pankhurst, N. W. and H. R. King (2010). "Temperature and salmonid reproduction: implications for aquaculture." Journal of Fish Biology **76**(1): 69-85.

Pankhurst, N. W. and M. Porter (2003). "Cold and dark or warm and light: variations on the theme of environmental control of reproduction." Fish Physiology and Biochemistry **28**: 385-389.

Rowell, J. G. and R. E. Walters (1976). "Analysing data with repeated observations on each experimental unit." Journal of Agricultural Science **87**: 423-432.

Sampaio, L. A., C. B. Moreira, K. C. Miranda-Filho and A. N. Rombenso (2011). "Culture of cobia *Rachycentron canadum* (L) in near-shore cages off the Brazilian coast." Aquaculture Research **42**(6): 832-834.

Scholz, S. and N. Kluver (2009). "Effects of endocrine disrupters on sexual, gonadal development in fish." Sex Dev **3**(2-3): 136-151.

Scott, A. P., V. J. Bye and S. M. Baynes (1980). "Seasonal variations in sex steroids of female rainbow trout (*Salmo gairdneri* Richardson)." Journal of Fish Biology **17**(5): 587-592.

Scott, A. P., T. Hill, N. Beresford, T. J. Runnalls, J. P. Sumpter and T. Ellis (2013). "Out-of-season production of 17,20β-dihydroxypregn-4-en-3-one in the roach *Rutilus rutilus*." Journal of Fish Biology **83**(2): 233-249.

Shaffer, R. V. and E. V. Nakamura (1989). Synopsis of biological data on the cobia *Rachycentron canadum* (Piscies: Rachycentridae). U. D. o. Commerce. Washington DC USA, FAO Fisheries synopsis **153:** 21.

Stieglitz, J. D., D. D. Benetti, R. H. Hoenig, B. Sardenberg, A. W. Welch and S. Miralao (2012). "Environmentally conditioned, year-round volitional spawning of cobia (*Rachycentron canadum*) in broodstock maturation systems." Aquaculture Research **43**(10): 1557-1566.

Taranger, G. L., C. Haux, S. O. Stefansson, B. Björn Thrandur, B. T. Walther and T. Hansen (1998). "Abrupt changes in photoperiod affect age at maturity, timing of ovulation and plasma testosterone and oestradiol-17β profiles in Atlantic salmon, *Salmo salar*." Aquaculture **162**(1–2): 85-98.

Taranger, G. L., S. O. Stefansson, F. Oppedal, E. Andersson, T. Hansen and B. Norberg (1999). Photoperiod and temperature affects gonadal development and spawning time in Atlantic salmon (*Salmo salar* L.). Proceedings of VIth International Symposium on Reproductive Physiology of Fish. G. L. Taranger, B. Norberg, S. O. Stefansson et al. Bergen, University of Bergen.

van der Velde, T., S. Griffiths and G. Fry (2010). "Reproductive biology of the commercially and recreationally important cobia (*Rachycentron canadum*) in northeastern Australia." Fisheries Science **76**(1): 33-43.

Watanabe, W. O., C. A. Woolridge and H. V. Daniels (2006). "Progress toward year-round spawning of southern flounder broodstock by manipulation of photoperiod and temperature." Journal of the World Aquaculture Society **37**(3): 256-272.

Zohar, Y. (1989). Fish reproduction: its physiology and artificial manipulation. Fish culture in warm water systems: problems and trends. M. Shilo and S. Sarig. Boca Raton, Florida, CRC Press: 65-119.

Zohar, Y., M. Harel, S. Hassin and A. Tandler (1995). Gilthead sea bream (*Saprus aurata*). Broodstock management and egg and larval quality. N. Bromage and R. Roberts. Oxford, Blackwell Science: 94-117.

Chapter 5: Identification of gender in captive reared cobia (*Rachycentron canadum*) using 11-Ketotestosterone analysis.

This chapter is presented as a manuscript to be submitted for publication in Aquaculture Research.

Identification of gender in captive reared cobia (*Rachycentron canadum*) using 11-Ketotestosterone analysis

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5.1 Abstract

The early identification of gender is a useful tool in broodstock management. It can provide cost savings by the reducing the number of fish retained as broodstock, whilst ensuring that the desired sex ratio is maintained. This becomes increasingly important when a species demonstrates sexually dimorphic growth and broodstock selection is based on growth. The aim of this study was to test the suitability using the levels of the androgen 11-ketotestosterone (11KT) as a tool to identify the gender of individual cobia (*Rachycentron canadum*). Two cohorts of captive reared cobia were progressively examined as pre- and post-pubescent fish at different periods of the reproductive season. Blood samples were taken at regular intervals to determine the influence of sex, size and season on the concentration of plasma 11KT. The concentration of 11KT was also measured in tissue samples, in the form of fin clips, to investigate the potential of developing a less invasive method to identify gender. Although 11KT was significantly higher in males than females, there was insufficient separation of its concentration in each sex to identify the gender of individuals when the mean population weight was less than 2 kg or the fish were sampled during the winter months. The gender of individual cobia could be identified by analysing plasma 11KT between the months of October and March, provided the mean population weight was 2 kg or above. The measurement of 11KT concentrations in fin clip samples did not provide an accurate indication of plasma 11KT and as such was not suitable for predicting gender of cultured juvenile cobia.

Key words
Cobia, *Rachycentron canadum*, 11-ketotestosterone, 11KT, gender identification.

Highlights

- The concentration of plasma 11-Ketotestosterone (11KT) was significantly higher in male than female fish; irrespective of size or season.
- There was sufficient separation of plasma 11KT in male and female fish to identify gender between the months of October and March, provided the mean population weight was 2 kg or above.
- The gender of mature fish could not be distinguished during the winter months.
- Fin clip samples did not provide an accurate indication of plasma 11KT.

5.2 Introduction

The cost effective and regular supply of high quality seed stock is essential for the successful commercial production of species. It is a fundamental requirement of a broodstock facility to ensure that high quality oocytes and larvae are available as required. This is achieved by maintaining the genetic integrity of stock and optimising nutrition and environmental conditions (Partridge et al. 2002). The maintenance of broodstock represents a significant capital outlay and operating cost to commercial aquaculture ventures. As such, there is a need to maintain cost efficiency whilst continuing to supply high quality seed stock. It is a requirement of some hatchery facilities to retain and on-grow juvenile fish for use as future broodstock. This occurs when there are logistical and biosecurity issues associated with broodstock size and the transfer of fish between sites. Under these conditions, a large number of fish are retained and on grown until the gender of fish can be positively identified. Broodstock are then selected based on fish quality and are allocated in a prescribed sex ratio (Benetti et al. 2007). The early identification of the gender of individuals allows this selection process to take place sooner, while maintaining the desired sex ratio. Reducing the number of fish retained increases broodstock management efficiency as it offers considerable cost saving to the operation.

Cobia, *Rachycentron canadum*, is a large benthopelagic species that is endemic to all tropical and subtropical waters across the globe with the exception of the eastern Pacific (Shaffer and Nakamura 1989). Commercial cobia aquaculture began in Taiwan in the late 1990s and has since been adopted by several nations through the Asian-Pacific and the Americas (Liao et al. 2007, Benetti et al. 2008, Nhu et al. 2011, Sampaio et al. 2011). Cobia research and development began in Australia in 2007, focusing largely on introducing

cobia as an alternative and off season crop for prawn farms in Queensland (Dutney and Palmer 2008).

Cobia broodstock have been brought to maturity using a wide variety of aquaculture systems and management techniques. Cobia exhibit year-round spawning in tropical conditions, with peaks in spring and autumn (Liao et al. 2004). In subtropical conditions, reproductive development in cobia is associated with increasing water temperatures (Chapter 4) and spawning coincides with the peak of summer (Benetti et al. 2008, Stieglitz et al. 2012). Methods used for the provision of mature cobia broodstock include the capture of wild fish in spawning condition, selection of suitable fish from ocean-based grow-out cages, husbandry of fish in dedicated spawning ponds and maintenance of fish in environmentally controlled tank systems (Liao et al. 2004, Holt et al. 2007). Cobia typically begin to mature sexually at approximately 2 kg in males and 3 kg in females which is approximately 10-12 months of age depending on growing conditions (Benetti et al. 2008).

Cobia broodstock at the Bribie Island Research Centre (BIRC) in Queensland, Australia are maintained at the hatchery from juveniles through to maturity, to ensure genetic integrity of captive reared broodstock and to maintain biosecurity. At BIRC, broodstock cobia are selected based on a variety of factors including size, health and vigour. However, as demonstrated in Chapter 2, female cobia are significantly larger than males when the mean weight of the population is above 2 kg. Selecting broodstock based on size beyond this point will result in skewed sex ratios. Cobia do not develop sexually dimorphic phenotypic features that are indicative of advancing sexual maturity, such as pronounced external changes in body shape, alterations in jaw shape or other secondary sex characteristics, that occur in some other fish species (Crim and Glebe 1990). The early

identification of the gender of cobia would allow the largest fish of each sex to be selected sooner in the production cycle while maintaining the desired sex ratio.

The sex of cobia is readily identified by gonad biopsy, referred to as canulation (Partridge et al. 2002, Benetti et al. 2007), whereby a sample of gonadal tissue is collected and examined. This technique is commonly used in marine finfish broodstock; however, it is more accurate and efficient when the fish are close to maturity. It can be particularly difficult to obtain a definitive sample from male cobia when they are immature or outside of the reproductive season. Alternative methods that analyse the sex steroids of both male and female fish have been successfully used to identify gender in a number of species (Feist et al. 2004, Chu-Koo et al. 2009). Similarly, vitellogenin levels have been used to identify females, or steroid induced and environmentally-feminised fish (Purdom et al. 1994, Takemura and Oka 1998, Moncaut et al. 2003). Sex steroids increase in fish coincides with the onset of pubescence, and fluctuate in both sexes according the reproductive season of the species (Devlin and Nagahama 2002). The androgen 11-ketotestosterone (11KT) plays a major role in spermatogenesis in male teleosts and is considered the most influential steroid in male reproductive development (Nagahama 1994, Devlin and Nagahama 2002). Both testosterone (T) and 11KT tend to increase steadily during the quiescent period, with a rapid increase associated with the peak of the reproductive cycle (Mylonas et al. 2013). However; 11KT often occurs at significantly higher levels than T during the peak of the cycle, most likely as a function of the role of T in the synthesis of 11KT (Scott et al. 1980).

The standard procedure for measuring steroid concentrations in fish is through the analysis of blood plasma (Barkowski and Haukenes 2014), typically quantified through the use of radioimmunoassay (RIA) or enzyme immunoassay (EIA). This process requires the collection of blood from fish, an invasive process that requires significant handling

and heavy sedation of the fish, thereby posing an inherent risk to fish health (Ellis et al. 2013). Sex steroids such as 11KT and lipoprotiens such as vitellogenin, are also known to be present at quantifiable levels in a range of other body tissues (Moncaut et al. 2003, Van Veld et al. 2005, Barkowski and Haukenes 2014). The analysis of sex steroids in mucous, muscle and fin samples has been used to identify the gender of fish and provides a less invasive method for sexing (Schultz et al. 2005). This is particularly useful in those species that are difficult to anaesthetise, such as some tuna species (Hutchinson et al. 2012). The concentration of 11KT from mucous or muscle samples has been shown to be an effective tool to identify the gender in species such as koi, *Cyprinus carpio*, white bass *Morone chrysops* and yellowfin tuna, *Thunnus albacares* (Schultz et al. 2005, Hutchinson et al. 2012, Barkowski and Haukenes 2014).

Sex steroid synthesis is strongly cyclic in nature occurring in response to pituitary hormone release, which is, in turn a function of factors such as environmental conditions, social conditions, and physiological status (Redding and Patiño 1993). Therefore developing a protocol to accurately to predict cobia gender using 11KT levels will need to take into account factors such as fish age, size, reproductive status and season. The aim of this study was to assess the feasibility of using 11KT as a means of identifying gender in cobia, and to describe the seasonal profile of 11KT in captive male cobia. In order to develop a protocol with strong commercial applicability, the study also focussed on early identification of gender, together with a comparative assessment of tissue levels of 11KT, as a less invasive sampling method.

5.3 Methods

5.3.1 General husbandry

The cobia used in the study were first generation offspring produced primarily from wild broodstock held and maintained at the Bribie Island Research Centre, Queensland, Australia. Larvae were grown in semi-intensive green-water tank systems for two weeks post hatch and then transferred to extensive pond production for weaning and on-growing to approximately 10 g. The juvenile fish were then maintained in a single 5000 L tank with flow-through seawater before being allocated to the respective trial. Fish were fed to satiation on commercially available marine fish diets: twice daily for five days per week, and once daily for two days per week. The fish used in the trial were subsampled from two populations of 100 fish from two different cohorts. Individual fish in each cohort were identified using T-bar tags. Cohort 1 originated from a mass spawning on 19/01/2012 involving up to three wild caught females, one captive reared female and four wild caught males. Cohort 2 originated from a mass spawning of two wild caught females and three wild caught males on 21/10/2012; of which one male and one female were also used in the spawning population of cohort 1.

The fish in cohort 1 were initially grown in a single 30,000 L tank fitted with an independent recirculating aquaculture system (RAS). On 20/03/13 the population was split evenly between two 30,000 L tanks fitted with identical RAS, where they were maintained until the completion of the trial. The fish in cohort 2 were maintained in a single 10,000 L circular tank fitted with flow through seawater. In order to maintain suitable water temperature through the winter period, on 27/5/13 and at an average weight of 627 g, the fish were transferred a single 10,000 L tank that was part of a 50,000

L RAS. In early spring, on 18/09/2013, the population was split and returned to two 10,000 L flow through systems where they remained until the completion of the trial.

The growing conditions remained similar for each tank of fish within each cohort after the population split. The maximum stocking densities were 2.6 kg/m^3 and 17.2 kg/m^3 in cohorts 1 and 2. Temperature, salinity, dissolved oxygen and pH were measured and recorded daily using YSI professional plus multiparameter meter. Total ammonia nitrogen (TAN) was measured weekly when stocking densities exceeded 5 kg/m^3 in recirculating systems.

5.3.2 Sampling methods and 11KT analysis

Fish were sedated prior to any sampling or examination. Light sedation of fish was obtained by adding 10 ppm of AQUI-S® (Aqui-S New Zealand Ltd.) to the entire tank. For heavy sedation, the fish were transferred to a 600 L tank containing 25 ppm AQUI-S®, allowing for various examinations including weight checks and blood and tissue sampling.

Blood samples were collected from the caudal vein of individuals that were placed ventral side up in a supportive cradle. Samples of 0.5 ml were collected using a heparinised 21 G x 38 mm needle and 1 ml syringe. Blood was then transferred to two 1.5 ml heparinised polypropylene tubes and embedded in shaved ice for a maximum of one hour before further processing. Plasma was separated by centrifugation at 4000 rpm for 20 minutes at 4ºC. Two 200 µL aliquots of plasma from each sample were then held at -80°C until required. Fin clips were taken from the tip of the ventral lobe of the caudal fin and the tip of the pectoral fin using single hole punch, to provide a tissue sample disc of approximately 5 mm diameter. Following the comparison of weights from caudal and pectoral fin clips described below, further analysis was conducted on the caudal fin clips

only. Fin clips were placed into 1.5 ml polypropylene tubes, embedded in dry ice and transferred to -80°C storage within one hour of sampling. The supportive cradle was hosed down thoroughly with freshwater between samples. The hole punch and the associated instruments used for tissue samples were rinsed with 100% ethanol between samples. The age, size of fish and the month when each sample was taken in each cohort are detailed in Table 5.1.

The concentration of 11-ketotestosterone (11KT) in the plasma and tissue samples was quantified using an EIA competitive assay kit (Cayman Chemical ACE ™) according to the manufacturer's instructions. Plasma samples were used undiluted and without extraction. Steroids were extracted from tissue samples by adding 500 µL of diethyl ether to the polypropylene tube containing the fin clip. Each sample then underwent pulse vortex three times for 30 seconds each time. The tissue sample was then removed from the tube and the diethyl ether evaporated off overnight. Steroids were brought into suspension by the addition of 110 µL of EIA buffer and briefly pulse vortexed 3 times. The extracted fin clips were placed on paper towel and allowed to dry overnight in a fume cupboard and weighed the following day.

The gender of the individuals was confirmed by post mortem examination at the completion of each trial. Intersex fish, as described in Chapter 2 were present in both cohorts and removed from the analysis.

Table 5.1 Sampling strategy used to measure 11KT in cobia.

Cohort[a]	Sample point	Sample month	Age (months)	Mean weight (kg)	No. of females	No. of males
1	1	Jan	11	1.8	7	3
1	2	Mar	13	2.8	10	7
1	3	Aug	18	5.8	10	5
1	4	Nov	21	7.1	8	4
2	1	Aug	9	1.1	10	4
2	2	Oct	11	2.0	8	4
2	3	Feb	14	4.1	8	4

[a] Two cohorts at different weights were examined at various stages of the reproductive season.

5.3.3 Data analysis

The comparison of 11KT concentration between male and female fish at each sample point was analysed by ANOVA, using Genstat 16th Edition. The comparison between the concentration of 11KT extracted from fin clip with plasma concentrations was conducted on samples from cohort 1 sample points 2 and 3 and from cohort 2 sample point 1. Fin clip data obtained from the assay were converted to pg/fin clip and then divided by the fin clip weight to provide pg/g of extracted tissue. Any values below the level of detection for the assay were excluded from the analysis. Plasma 11KT of individual fish was entered into a scatter plot to visualise the separation between the 11KT levels in male and female fish at each sample point.

5.4 Results

5.4.1 Plasma 11KT

The plasma 11KT concentrations ranged from 3.8 to 170 pg/ml in individual male cobia and 1.2 to 30 pg/ml in females. At any one sampling point, the mean 11KT concentration of male cobia was significantly higher than in females in both cohort 1 and 2 ($p<0.05$) (Figure 5.1 and 5.2). 11KT concentrations were highest when the fish were 2 kg or above and from spring through until the mid-summer. There was a rapid spike in plasma 11KT in the spring when the fish were an average of 7.1 kg.

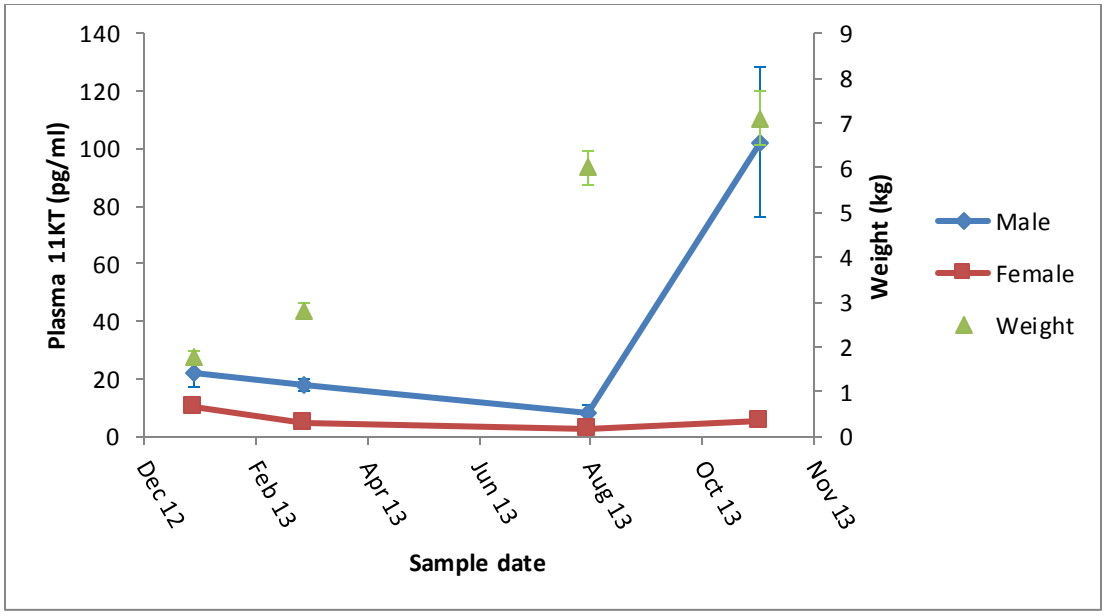

Figure 5.1 The average concentration of plasma 11-ketotestosterone (11KT) and weight (±SE) of cobia from cohort 1.

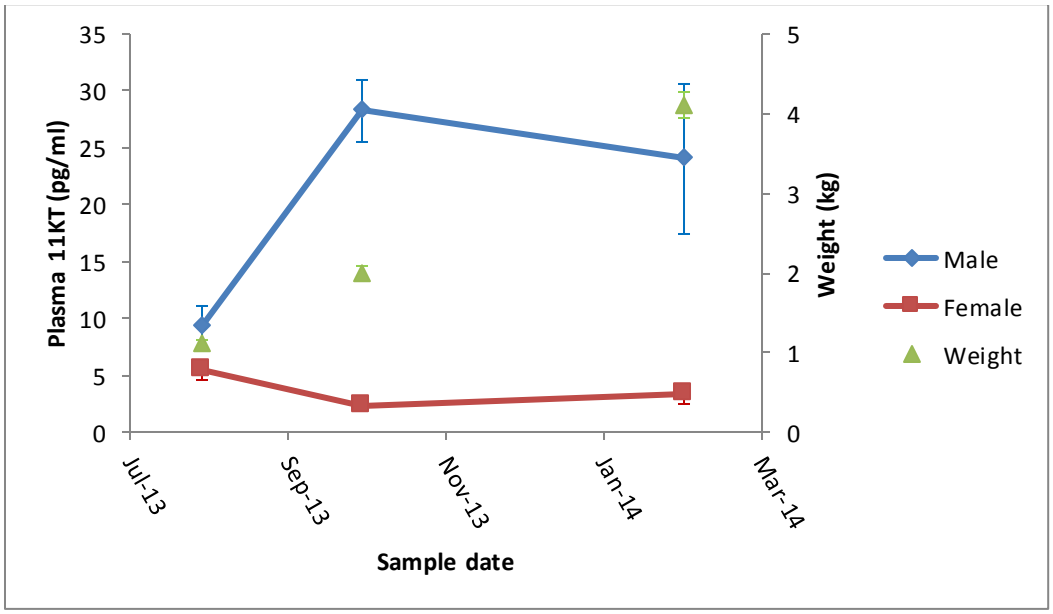

Figure 5.2 The average concentration of plasma 11-ketotestosterone (11KT) and weight (±SE) of cobia from cohort 2.

5.4.2 Identification of gender

Cohort 1

The gender of individual cobia could be readily distinguished using plasma concentrations of 11KT in late spring (November), when the population mean was 7.1 kg (Figure 5.3). Male and female fish could not be distinguished in mid-summer (January), when the mean weight of the population was 1.8 kg. The gender of individuals could be identified by the end of the summer (March) when the population mean was 2.8 kg and the fish were 13 months old. However, during the winter (August), when the fish were an average weight of 5.8 kg, there was insufficient distinction between male and females to distinguish gender, despite of them being large enough to be considered mature.

Cohort 2

The gender of individual cobia could not be distinguished in August, when the mean population weight was 1.1 kg. There was sufficient separation between the plasma concentration of male and female cobia to identify the gender of individuals when the population mean was 2.0 kg, in October and 4.1 kg in February (Figure 5.4).

The threshold for determining female fish was below 10 pg/ml and above 15 pg/ml for male fish at each sample point in which gender could be identified. There was no distinction between males and females when the mean population weight was less than 2 kg or when the fish were sampled during the winter period.

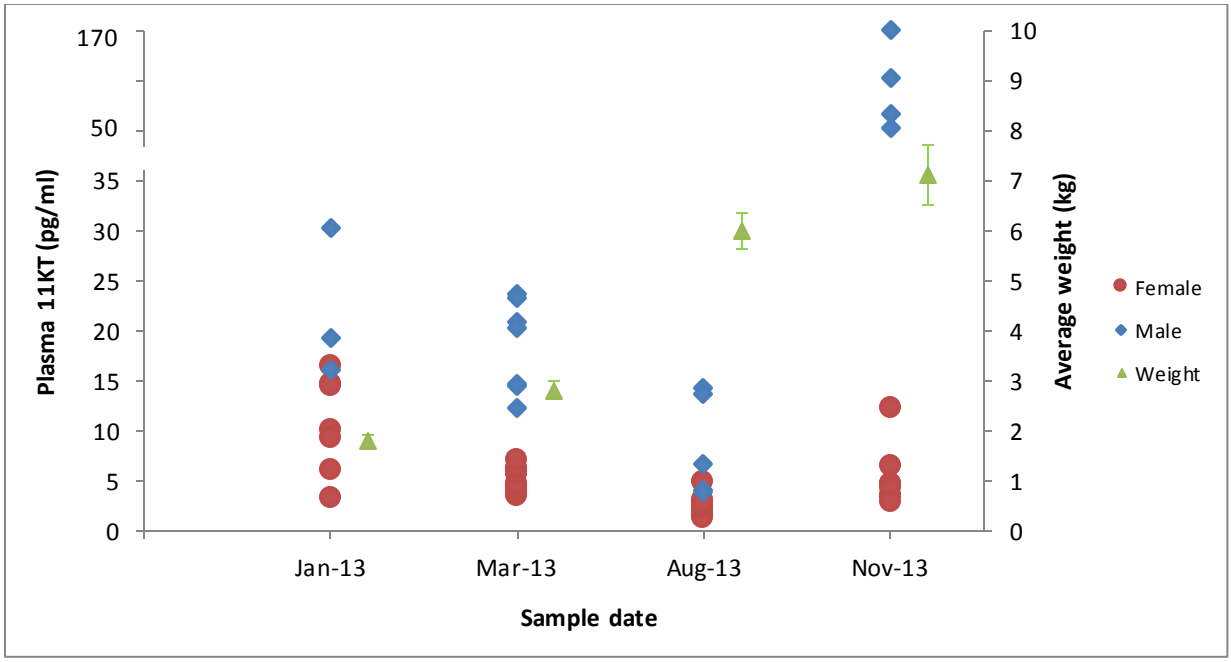

Figure 5.3 Scatter plot of plasma 11-ketotestosterone concentration of individuals and average weight (±SE) of cobia from cohort 1.
Samples were taken in January, (11 months post hatch), March (13 months post hatch), August (18 months post hatch) and November (21 months post hatch).

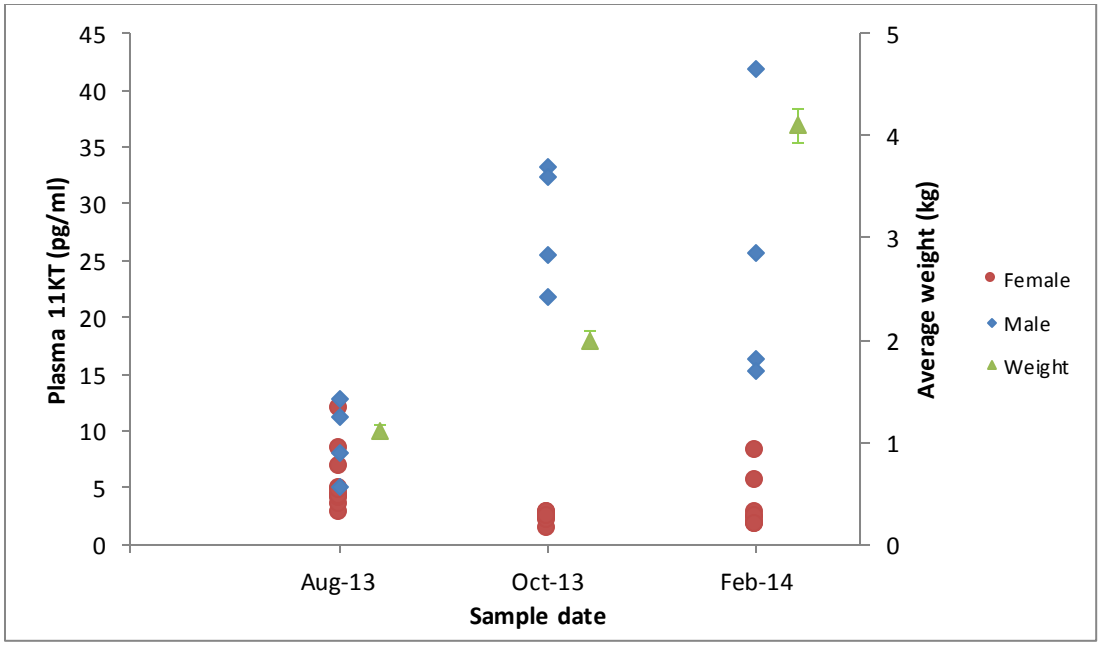

Figure 5.4 Scatter plot of plasma 11-ketotestosterone concentration of individuals and average weight (±SE) of cobia from cohort 2.
Samples were taken in August (9 months post hatch), October (11 months post hatch) and February (14 months post hatch).

5.4.3 Comparison of caudal and pectoral fin clips

The weight range of fin clips from the pectoral fin was greater than those taken from the caudal fin. The variability relative to the mean was similar in each data set with the coefficient of variation calculated at 19.7% and 21.9% in caudal and pectoral fin clips respectively (Table 5.2).

Table 5.2 Weights of fin clips taken from the caudal and pectoral fin of cobia[a].

Fin	Range (mg)	Mean (mg)	Standard Deviation	Coefficient of Variation (%)
Caudal	18.0 - 34.9	26.3	5.19	19.7
Pectoral	9.1 - 28.4	18.0	3.92	21.9

[a] Sampled cobia population mean weight of 2.8 kg.

5.4.4 Comparison of 11KT in fin clips and plasma

Determination of 11KT concentration in fin clips did not provide an accurate prediction of the 11KT concentration in the plasma. When the data from the three sample points analysing fin clip 11KT was pooled, there was a poor correlation between the 11KT concentration in fin clips and plasma when examined per fin clip (Figure 5.5, R^2=0.32). To account for the variation in fin clip weight described in Table 5.2 a second analysis was conducted with 11KT levels corrected for fin clip weight. There was again poor correlation between fin clip and plasma 11KT concentration (Figure 5.6, R^2=0.06).

The March sample point had the highest absolute values of plasma 11KT of the three sample points used for fin clip analysis. It was also the only point analysed that had sufficient separation of plasma 11KT between males and females to identify gender (Figure 5.3). Due to the large range of values and level of separation, the degree of correlation in the March data was analysed separately. There was a significant correlation between fin clips and plasma ($r=0.79$, d.f.=12, $p<0.01$) to suggest that fin clips can provide an indication of circulating 11KT (Figure 5.7). However, the separation of values

observed in the plasma was not apparent in the range of values from fin clips, with overlap in values between males and females preventing the ability to discriminate the sexes (Figure 5.7).

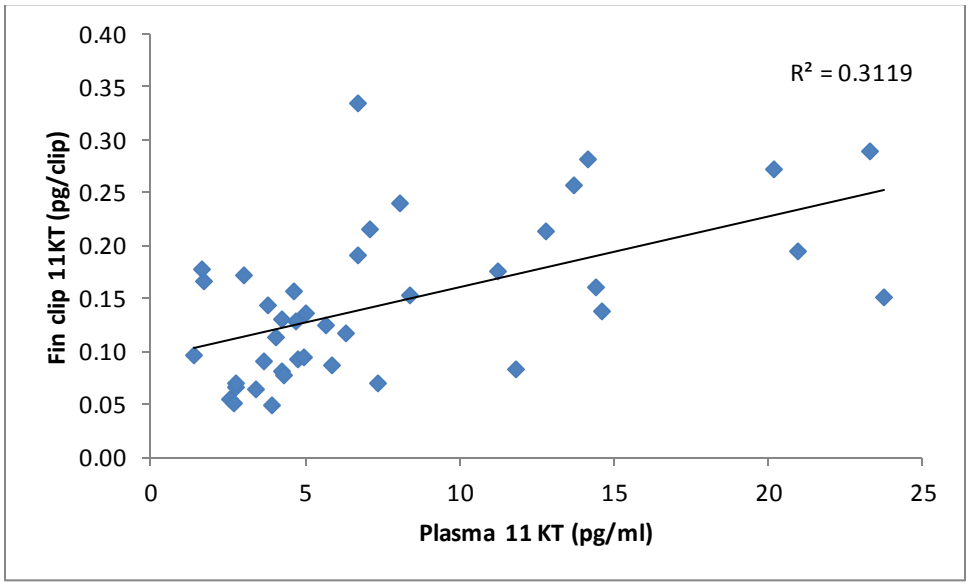

Figure 5.5 Comparison of 11-ketotestosterone concentrations of cobia measured in the plasma and per fin clip sample.

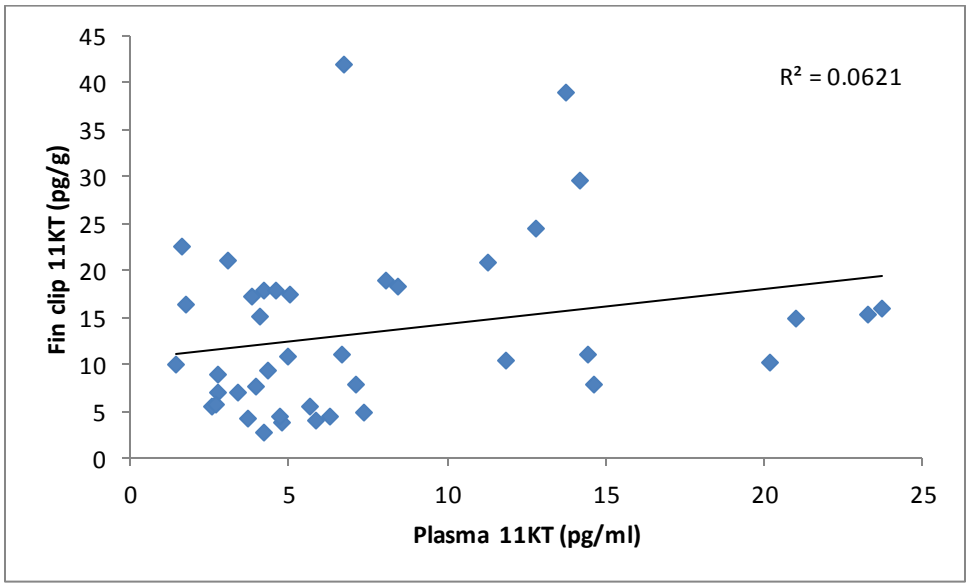

Figure 5.6 Comparison of 11-ketotestosterone concentrations of cobia measured in the plasma and per gram of fin clip.

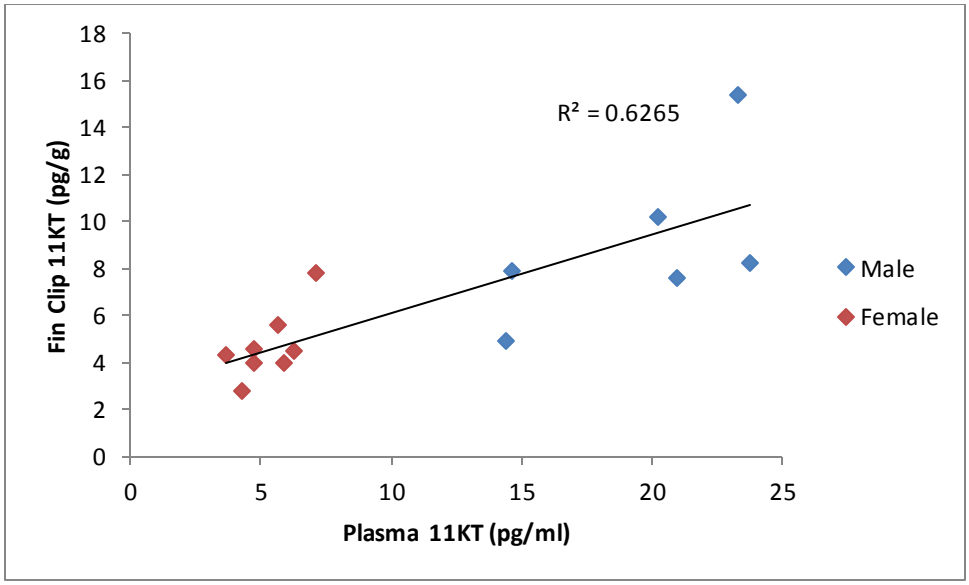

Figure 5.7 Comparison of 11-ketotestosterone concentrations of cobia measured in the plasma and per gram of fin clip from cohort 1 in March.

5.5 Discussion

The current study demonstrated that the concentration of plasma 11KT in cobia was influenced by season and the size and sex of fish. As such, the capacity of plasma 11KT concentration to accurately predict the gender of an individual was also influenced by both fish size and season. Plasma 11KT was consistently low in female fish, whereas the levels observed in males varied significantly. The highest levels observed were in males that were approaching mature size of 2 kg or above and during the peak of the summer reproductive season. The seasonal cycle of sex steroids, and specifically 11KT, has been observed in a variety of fish species and is consistent with the findings of the present study (Scott et al. 1980, Mylonas et al. 2013).

The limited sample points in the current study did not enable an extensive seasonal profile of 11KT in cobia; however it did show 11KT concentration in cobia to be lower than that observed in other species. The plasma 11KT concentration in rainbow trout, *Oncorhyncus mykiss*, peaked at 250 ng/ml (Scott et al. 1980) and male hapuka, *Polyprion oxygeneios*, peaked at 6 ng/ml (Kohn et al. 2013). The levels observed in the current study

were at least an order of magnitude lower with the highest reading of 170 pg/ml. Similar low levels were reported in male meagre, *Argyrosomus regius*, outside the reproductive season (Mylonas et al. 2013). However, the seasonal peak of 800 pg/ml observed in meagre was considerably higher than that observed in cobia. Mylonas et al. (2013) used diethyl ether extraction and EIA to analyse plasma 11KT, whereas the current study used plasma directly, without extraction. Whilst this may create some variability when comparing absolute levels, it is unlikely that it would be the reason that no significant increase in 11KT was observed in cobia, especially considering that the levels recorded were toward the lower detection limit of the assay. Similar low level sex steroids were reported in Chapter 3 and 4, when examining 17β-estradiol (E2) in female cobia during the peak of the reproductive cycle suggesting a generally low level of circulating steroid hormones occurs overall in cobia. The low 11KT levels recorded in the current trial may be the result of examining fish that were approaching or only recently maturing. A rapid spike in 11KT was observed when the fish were larger than 7 kg, early in the reproductive season. Further examination of these larger and possibly more mature fish, through to the reproductive peak in January, may have shown 11KT levels similar to those recorded in other fish species. The low concentrations of sex steroids observed in Chapter 3 and 4 may also be a function of examining fish during their first reproductive season. Although demonstrating gonadal development in the presence of low circulating sex steroids, they may increase in subsequent reproductive season to levels more typical of marine fish. Further investigation is warranted to profile the sex steroids of cobia across multiple reproductive seasons.

The current study demonstrated that cobia gender can be identified using the analysis of plasma 11KT, provided that the fish were 2 kg or greater and sampled during the months of October through until March, coinciding with increased reproductive development in

the species (Chapter 4). The distinction between sexes was not clear when the fish were under 2 kg or when larger fish were sampled during the winter months. A similar size and season dependent impact on the ability of sex steroid analysis to predict gender has been reported for a number of fish species, where the accuracy of the technique is reliant upon measurements being taken from adult fish during the reproductive season (Feist et al. 2004, Chu-Koo et al. 2009, Kohn et al. 2013). There is limited evidence in the literature of successful gender identification of immature fish using steroid analysis. Similarly the analysis of vitellogenin to identify gender relies on the fish being at a mature age (Barkowski and Haukenes 2014). Kohn et al. (2013) suggested that steroid analysis could be used to identify underdeveloped fish; however the ability to predict gender under these conditions would likely be limited. The concurrent analysis of E2 and 11KT, to provide an 11KT:E2 ratio, improves the identification of gender in a number of species. E2 is considered a key steroid in female gonad development and the analysis of both steroids provides improved differentiation between the sexes in some species (Chu-Koo et al. 2009, Kohn et al. 2013). Estradiol concentrations in female cobia, are however low in comparison with other species, even in post pubescent fish in the peak of the reproductive cycle (Chapter 4). As such, the efficacy of concurrent measurement of E2 to improve the prediction of cobia gender at an earlier stage is questionable.

Male cobia were able to be identified by plasma 11KT analysis in this study before they were actively spermiating. At this point positive identification via canulation remains technically difficult due to the fish being immature. However, as a function of the rapid growth rate of cobia, there is a relatively short period of time, perhaps only a few months, from when 11KT is effective until a positive identification could be obtained more readily by canulation or the fish would be expected to be running ripe. When cobia are approaching maturity, canulation is relatively cheap and simple compared with steroid

and vitellogenin analysis. Analysis of plasma steroids carries the inherent risk to fish health associated with blood sampling and as with vitellogenin analysis requires suitable laboratory facilities and equipment and suitably trained personnel. This brings the commercial application and viability of this method into question.

The secondary aim of the project was to examine the use of tissue samples to provide a less invasive method to predict the level of circulating 11KT. The analysis of 11KT concentrations in fin clip samples did not provide an accurate indication of plasma levels, and in doing so failed to provide a less invasive method to identify the gender of cobia. A similarly poor correlation was described in the study by Schultz et al. (2005) when comparing 11KT concentrations in muscle tissue to plasma in Koi, *Cyprinus carpio*. Their study was however, able to demonstrate a significant correlation between surface mucus and plasma 11KT concentration. Improved predictability of plasma 11KT levels may be obtained by analysing surface mucus in cobia. The concentration of 11KT in the fin clip samples in the current trial were at, or near, the lower limit of detection of the test kit, which may have impacted on the accuracy of the measurements provided. The use of larger tissue samples or perhaps refining the extraction technique may increase the quantity of 11KT and facilitate a more accurate measure of 11KT in the tissue to better predict plasma levels. The improved correlation obtained when analysing the March data, which had the highest concentration of the points sampled, suggests that a more accurate prediction of plasma level might be obtained by examining fin clips, when the fish were 7 kg or larger and during the spring or summer when plasma 11KT was higher still. As with plasma steroid analysis, the ability to predict gender using surface mucus is often reliant upon sampling mature age fish, often during the reproductive season (Schultz et al. 2005, Schultz et al. 2007, Barkowski and Haukenes 2014). However; the efficacy and commercial application of such procedures in cobia is questionable. The objective of the

study was to examine the use of fin clip analysis to predict of gender of cobia at an early age. At a later stage, the gender of cobia can be readily identified at this stage using canulation, which remains a more cost effective and commercially applicable strategy.

The current study demonstrated that the gender of individual cobia can be identified by analysing plasma 11KT during the reproductive season, provided that the population mean weight is greater than 2 kg. The relatively low levels of steroid hormones observed in this and previous studies on cobia warrants further investigation, in order to gain a better understanding of the reproductive physiology of the species. The absence of a relationship between circulating and tissue 11KT prevents the application of this less invasive method for cobia. Whilst likely to remain a crucial element in the management of certain broodstock species that may be excessively large or difficult to anaesthetise, it is not essential to the management of cobia broodstock. The relative cost, infrastructure and equipment required to conduct steroid analysis limits the application of this methodology in commercial cobia production.

5.6 Acknowledgements

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5.7 References

Barkowski, N. A. and A. H. Haukenes (2014). "Investigating the utility of measuring 11α-Ketotestosterone and vitellogenin in surface mucus as an alternative to plasma samples in assessments of the reproductive axis of white bass." North American Journal of Aquaculture **76**(2): 112-118.

Benetti, D. D., M. R. Orhun, B. Sardenberg, B. O'Hanlon, A. Welch, R. Hoenig, I. Zink, J. A. Rivera, B. Denlinger, D. Bacoat, K. Palmer and F. Cavalin (2008). "Advances in hatchery and grow-out technology of cobia *Rachycentron canadum* (Linnaeus)." Aquaculture Research **39**(7): 701-711.

Benetti, D. D., M. R. Orhun, I. Zink, F. Cavalin, B. Sardenberg, K. Palmer, B. Denlinger, D. Bacoat and B. O'Hanlon (2007). Aquaculture of cobia (*Rachycentron canadum*) in the Americas and the Caribbean. Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan**:** 57-78.

Chu-Koo, F., R. Dugue, M. Alvan Aguilar, A. Casanova Daza, F. Alcantara Bocanegra, C. Chavez Veintemilla, F. Duponchelle, J. F. Renno, S. Tello and J. Nunez (2009). "Gender determination in the Paiche or Pirarucu (*Arapaima gig*as) using plasma vitellogenin, 17beta-estradiol, and 11-ketotestosterone levels." Fish Physiology and Biochemistry **35**(1): 125-136.

Crim, L. W. and B. D. Glebe (1990). Reproduction. Methods for Fish Biology. C. B. Schreck and P. B. Moyle. Bethesda, American Fisheries Society**:** 529-553.

Devlin, R. H. and Y. Nagahama (2002). "Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences." Aquaculture **208**(3–4): 191-364.

Dutney, L. and P. Palmer (2008). Farmed cobia making progress at BIARC. Queensland Aquaculture News, Queensland Government Department of Primary Industries and Fisheries**:** 6-7.

Ellis, T., M. B. Sanders and A. P. Scott (2013). "Non-invasive monitoring of steroids in fishes." Wiener Tierärztliche Monatsscgrift - Vetinary Medicine Austria **100**: 255-269.

Feist, G., J. P. Van Eenennaam, S. I. Doroshov, C. B. Schreck, R. P. Schneider and M. S. Fitzpatrick (2004). "Early identification of sex in cultured white sturgeon, *Acipenser transmontanus*, using plasma steroid levels." Aquaculture **232**(1–4): 581-590.

Holt, J. G., C. K. Faulk and M. H. Schwarz (2007). "A review of the larviculture of cobia *Rachycentron canadum*, a warm water marine fish." Aquaculture **268**(1-4): 181-187.

Hutchinson, W., G. J. Partridge and J. Hutapea (2012). Achieving consistent spawning of captive yellowfin tuna (*Thunnus albacares*) broodstock at Gondol Research Institute for Mariculture, Bali, Indonesia. A. Government. Canberra, Australia, Australian Centre for International Agriculture Research. **Final Report:** 36.

Kohn, Y. Y., P. M. Lokman, A. Kilimnik and J. E. Symonds (2013). "Sex identification in captive hapuku (*Polyprion oxygeneios*) using ultrasound imagery and plasma levels of vitellogenin and sex steroids." Aquaculture **384–387**(0): 87-93.

Liao, I. C., T.-S. Huang, W.-S. Tsai, C.-M. Hsueh, S.-L. Chang and E. M. LeaÃ±o (2004). "Cobia culture in Taiwan: current status and problems." Aquaculture **237**(1-4): 155-165.

Liao, I. C., E. M. Leano, C. T. Hsu and C. C. Ku (2007). Marine cage culture of cobia in Taiwan. Cobia Aquaculture: Research, Development and Commercial Production. I. C. Liao and E. M. Leano. Keelung, Taiwan, Asian Fisheries Society, World Aquaculture Society, The Fisheries Society of Taiwan, National Taiwan Ocean University**:** 131-145.

Moncaut, N., F. L. Nostro, M. Maggese, amp, x and C. a (2003). "Vitellogenin detection in surface mucus of the South American cichlid fish *Cichlasoma dimerus* (Heckel, 1840) induced by estradiol-17β. Effects on liver and gonads." Aquatic Toxicology **63**(2): 127-137.

Mylonas, C. C., N. Mitrizakis, M. Papadaki and I. Sigelaki (2013). "Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity I. Description of the annual reproductive cycle." Aquaculture **414–415**(0): 309-317.

Nagahama, Y. (1994). "Endocrine regulation of gametogenesis in fish." Int J Dev Biol **38**(2): 217-229.

Nhu, V. C., H. Q. Nguyen, T. L. Le, M. T. Tran, P. Sorgeloos, K. Dierckens, H. Reinertsen, E. KjÃ¸rsvik and N. Svennevig (2011). "Cobia *Rachycentron canadum* aquaculture in Vietnam: Recent developments and prospects." Aquaculture **315**(1-2): 20-25.

Partridge, G. J., G. I. Jenkins and K. R. Franklin (2002). Manual for the hatchery production of snapper (*Pagrus auratus*) and black bream (*Acanthopagrus butcheri*). Fremantle, Fisheries Research and Development Corporation and the Aquaculture Development Unit, WA Maritime Training Centre, Challenger TAFE.

Purdom, C. E., P. A. Hardiman, V. V. J. Bye, N. C. Eno, C. R. Tyler and J. P. Sumpter (1994). "Estrogenic effects of effluents from sewage treatment works." Chemistry and Ecology **8**(4): 275-285.

Redding, J. M. and R. Patiño (1993). Reproductive Physiology. The Physiology of Fishes. D. H. Evans. Boca Raton, CRC Press**:** 503-534.

Sampaio, L. A., C. B. Moreira, K. C. Miranda-Filho and A. N. Rombenso (2011). "Culture of cobia *Rachycentron canadum* (L) in near-shore cages off the Brazilian coast." Aquaculture Research **42**(6): 832-834.

Schultz, D. R., N. Perez, A. J. Mendez, D. Snodgrass, J. E. Serafy, E. D. Prince, W. A. Crow and T. R. Capo (2007). "Tracking gender factors in fish surface mucus: temporal patterns in individual Koi (*Cyprinus carpio*)." Journal of Applied Ichthyology **23**(2): 184-188.

Schultz, D. R., N. Perez, C. K. Tan, A. J. Mendez, T. R. Capo, D. Snodgrass, E. D. Prince and J. E. Serafy (2005). "Concurrent levels of 11-ketotestosterone in fish surface mucus, muscle tissue and blood." Journal of Applied Ichthyology **21**(5): 394-398.

Scott, A. P., V. J. Bye, S. M. Baynes and J. R. C. Springate (1980). "Seasonal variations in plasma concentrations of 11-ketotestosterone and testosterone in male rainbow trout, *Salmo gairdnerii* Richardson." Journal of Fish Biology **17**(5): 495-505.

Shaffer, R. V. and E. V. Nakamura (1989). Synopsis of biological data on the cobia *Rachycentron canadum* (Piscies: Rachycentridae). U. D. o. Commerce. Washington DC USA, FAO Fisheries synopsis **153:** 21.

Stieglitz, J. D., D. D. Benetti, R. H. Hoenig, B. Sardenberg, A. W. Welch and S. Miralao (2012). "Environmentally conditioned, year-round volitional spawning of cobia (*Rachycentron canadum*) in broodstock maturation systems." Aquaculture Research **43**(10): 1557-1566.

Takemura, A. and M. Oka (1998). "Immunochemical sexing of living yellowfin tuna, *Thunnus albacares* (Bonnaterre), using a vitellogeninlike protein." Aquaculture Research **29**(4): 245-249.

Van Veld, P. A., B. J. Rutan, C. A. Sullivan, L. D. Johnston, C. D. Rice, D. F. Fisher and L. T. Yonkos (2005). "A universal assay for vitellogenin in fish mucus and plasma." Environmental Toxicology and Chemistry **24**(12): 3048-3052.

Chapter 6: Summary and Final Conclusions

This PhD study focused on several aspects of broodstock management in order to improve the efficiencies of cobia production.

6.1 Sexually dimorphic growth

The current study demonstrated that captive-reared cobia exhibit sexually dimorphic growth in the relative absence of the intersex anomaly. Female cobia were larger than males when average population weight exceeded 2 kg and were almost 30% heavier than males when the average weight of the population was approaching market size of 5 kg. The significant difference in growth between male and female cobia suggests that there would be significant productivity and economic gains made from the development of single sex female populations. Furthermore, sexually dimorphic growth should be considered when allocating broodstock, as selection of fish based solely on size will result in a female-biased population.

6.2 Occurrence of intersex fish

An unexpected outcome of this research was the discovery of intersex cobia. The occurrence of the intersex anomaly within a population was also associated with an impact on the growth dynamics and reproductive development of phenotypically male or female fish. Cobia is a gonochoristic species, and the presence of intersex individuals has not previously been reported, despite numerous studies investigating cobia reproduction. This was also the first reported occurrence of intersex gonads from a gonochoristic fish species from Australian waters.

Significantly skewed sex ratios of up to 2.4 female: 1 male were associated with those cohorts with a high incidence of intersex fish. In the absence of the intersex anomaly, sex

ratios were not significantly different from the expected 1:1. The relatively low number of phenotypic males compared to females in the current study suggests that the intersex fish are possibly male fish symptomatic of a demasculinising and/or feminising influence. The occurrence of the intersex condition was shown to impact on the growth dynamics of cobia, evidenced by a lack of sexually dimorphic growth in the two cohorts with a relatively high incidence of intersex.

Fish in the 2013 photothermal manipulation trial showed sporadic development in which ovarian samples contained low numbers of late stage oocytes amongst a large percentage of previtellogenic oocytes. Furthermore, a majority of fish in this cohort showed limited ovarian development. Ovarian samples from the 2014 fish showed more regular progression in development and a large percentage of fish developed to reproductive maturity. The apparent reproductive dysfunction observed in 2013 was associated with the higher incidence of intersex, with an apparent impact on the ovarian development of phenotypic females in that cohort. Gonadal malformations in phenotypic males or females were also only observed in the affected populations.

The skewed sex ratios and intersex condition were most likely induced by endocrine disruption during the early life stage of the fish. Environmental pollutants classified as endocrine disrupting chemicals (EDC) are capable of interfering with the action and/or synthesis of steroid hormones, resulting in an imbalance of male and female hormones which may manifest as reproductive abnormalities. The impact of EDC is heightened at critical developmental stages, such as during sexual differentiation, and the impacts are often irreversible. The source or mechanism behind the phenomena observed in this study remains unknown.

6.3 Development of an oocyte assessment strategy

Ovarian development in marine fish is typically quantified by calculating the average diameter of the ten largest oocytes or by examining the most advanced group of oocytes present in a sample. However, an evaluation based on only the largest oocytes in the sample does not provide true indication of ovarian development, especially when partial maturation or asynchronous development of the ovary occurs, as observed in cobia. A simple, commercially applicable, assessment method was developed to provide a more accurate measure of the overall development of the ovary. This approach was based on a measure of the relative abundance of all oocyte stages within an ovarian biopsy sample, to provide accurate quantification of ovarian maturation that incorporates the various oocyte stages often present in ovarian biopsy samples from cobia.

6.4 Hormonal manipulation

There was no evidence to suggest that either luteinising hormone releasing hormone analogue (LHRHa) or human chorionic gonadotropin (hCG) were effective in stimulating or supporting ovarian development in cobia. 17β-Estradiol (E2) concentrations were low in cobia, relative to other fish species, and were found to be highly variable between individual fish within and across treatments. There was no change in E2 concentrations as a result of LHRHa administration. There was an increase in relative E2 concentration subsequent to the third hCG administration, however this was not associated with a measurable change in oocyte development. Those fish that initiated vitellogenesis continued to develop regardless of treatment with exogenous hormones. While hormonal therapy is effective for the induction of spawning in cobia, the results suggest that hormone therapy is not an effective approach to initiating or supporting early stage ovarian development in cobia.

6.5 Photothermal manipulation

A different response to photothermal manipulation was observed between two cohorts of cobia, possibly related to the observed levels of gonad malformation. In the 2013 trial, which had a higher incidence intersex fish within the cohort, there was no significant difference in development between the compressed and ambient phototherm regime. Likewise, there was no significant difference in ovarian development between the compressed and ambient phototherm in 2014 at the completion of the trial. However, in 2014, the compressed phototherm induced development earlier in the season. The results of the 2014 trial demonstrated that ovarian development could be advanced with the use of photothermal manipulation, with the most rapid development occurring when temperature was 24-27 °C and photoperiod was 12.5-14.0 hrs.

Oocyte integrity was maintained in post vitellogenic oocytes for up to three months in a large percentage of fish in the 2014 trial, suggesting that cobia could be maintained in a state suitable for hormone induction for a number of months during the reproductive season. 17β-Estradiol concentrations have been shown to be a predictor of maturation and ovarian development in a number of fish species; however, the current study was unable to demonstrate a relationship between E2 concentration and ovarian development. Oogenesis appears to proceed in cobia in the presence of relatively low level sex steroids.

6.6 Identification of gender using steroid analysis

The concentration of plasma 11KT in cobia was influenced by season and the size and sex of fish. As such, the capacity of plasma 11KT concentration to accurately identify the gender of an individual was also influenced by both fish size and season. The current study demonstrated that cobia gender could be identified using the analysis of plasma

11KT, provided that the fish weight was 2 kg or greater and sampling coincided with increased reproductive development in the species. The study investigated the potential of using a less invasive method for identifying gender, by analysis of 11KT concentrations in fin clips. However, there was no correlation between 11KT concentration in the plasma and that measured from fin clips. As such, fin clip analysis was considered ineffective for predicting gender. The relative cost, infrastructure and equipment required to conduct steroid analysis limits the application of this methodology in commercial cobia production.

Overall cobia were found to have relatively low level sex steroids, evidenced in both hormonal and photothermal manipulation experiments that examined E2, and when examining 11KT for gender identification.

6.7 Future research

The source and type of endocrine disrupting chemicals and the potential mechanism for the disruption observed in this study remain unclear. It does, however, warrant further investigation due to the potential impact on commercial production, broodstock management, wild fish stocks and the wider aquatic environment.

The production of monosex populations of cobia offers significant commercial benefits. Monosex production has been demonstrated in a number of cultured fish species; however, it is often a complicated task that first requires the identification of the sex determining mechanism of the species, followed by the development of suitable methodologies to produce single-sex populations. The development of such techniques would also be advantageous in the protection of genetically improved stocks of cobia.

6.8 Commercial implications

Outcomes of this PhD study that can be implemented directly to the management of cobia in both commercial and research facilities include:

- Broodstock selection can be based on size until approximately 2 kg, with a low risk of skewed sex ratio.
- The allocation of cobia broodstock over 2 kg should be based on positive identification of gender through gonad biopsy or by assess gender specific sex steroids such 11-ketotestosterone.
- The use of 11KT analysis to identify gender requires that the fish are 2 kg or above and are sampled during the reproductive season.
- An oocyte assessment strategy that incorporates the various oocyte stages present in asynchronous developing ovaries, to provide an accurate measure of reproductive status.
- Demonstration that ovarian development of cobia is most rapid when temperature was 24-27°C and photoperiod was 12.5-14 hrs.
- The production of all female cobia production would lead to significant productivity and economic gains, warranting further research.

The research conducted in this PhD study formed a significant part of the Australian Seafood Cooperative Research Centre (CRC) funded project "The development of an Australian cobia aquaculture industry" (2001/724), which in turn, has led to the development of successful commercial production of cobia in Australia. The CRC project that was the recipient of Department of Agriculture and Fisheries (DAF) 2016 Australia Day Award, in recognition of its innovation and contribution to the development of commercial aquaculture in Queensland and Australia.