The Development of an Australian Cobia Aquaculture Industry

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Non-Technical Summary

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PROJECT OBJECTIVES:

- Develop reliable and robust controlled spawning methods for Cobia, utilising hormonal, social and/or environmental manipulation
- Produce sufficient fingerlings to enable Pacific Reef Fisheries (PRF) to on-grow commercial quantities of Cobia for market
- Develop pilot-scale Cobia fingerling production by the PRF hatchery
- Formulate diets designed to meet the specific nutritional and energetic requirements of Cobia
- Develop and field test new farmed Cobia product(s) with high market acceptance.

OUTCOMES ACHIEVED

1. A reliable and robust supply of Cobia juveniles to support the commercial production and future growth of Cobia aquaculture in Australia
2. A cost-effective pond-based production system for Cobia producing 20T per ha and an industry value of $1M per year
3. An optimum position for farmed Cobia products in the market place.

LIST OF OUTPUTS PRODUCED

1. Sufficient fingerlings were produced by the Department of Agriculture and Fisheries (DAF) to enable PRF to on-grow commercial quantities of Cobia for market, and to support pilot commercial Cobia fingerling production by the PRF hatchery
   - A total of 142,000 fingerlings were produced by DAF for on-growing by PRF during the project
   - In addition, 81,000 larvae were sent to the PRF hatchery for preliminary larval-rearing trials.
2. Controlled spawning methods for Cobia, utilising hormonal, social and/or environmental manipulation

- An extended spawning season, running from September to March, has been achieved through photothermal and hormonal manipulation
- Protocols for broodstock maturity assessment were developed, leading to an improved assessment of reproductive state and an increase of spawning success to >70% of fish induced, compared to <25% using previous methods.

3. Methods for assessing a suite of seven previously described microsatellite markers for Cobia were optimised for use in pedigree testing.
   - Preliminary studies showed accurate parental assignment of >90% of progeny
4. New farmed Cobia products with strong retailer acceptance and commercially competitive pricing.
   - An expert consumer taste panel rated cooked fresh Cobia equivalent to Atlantic Salmon, and superior to Yellowtail Kingfish, in terms of overall appeal, flavour and texture
   - Consumer panels also indicated a preferred price point for fresh Cobia between Atlantic Salmon and Yellowtail Kingfish
   - Skin and modified-atmosphere packaging significantly extended the shelf life of fresh Cobia to 17 and 21 days respectively
   - A hot-smoked Cobia product displayed sensory characteristics as good as any currently available hot-smoked fish product
   - Fresh Cobia received wide acceptance and favourable reviews by chefs in several high-end restaurants with descriptors such as "*waygu of the sea*" (Tom Walton,chef, Bucket List, Bondi) and "*the Rolls Royce of fish"* (Josh Niland chef, FishFace, Double Bay).
5. Formulation of diets designed to meet the specific nutritional and energetic requirements of Cobia
   - Vastly improved performance, in terms of growth, survival and feed conversion, was demonstrated for Cobia fed a commercial diet containing additional taurine, in comparison to those fed a similar diet without additional taurine.

ABSTRACT

The current project was conceived as a "*hatchery to plate"* project across the whole production chain. It was aimed at moving Australian Cobia aquaculture from pilot towards commercialisation. The project was undertaken in conjunction with a large, successful prawn farming company, Pacific Reef Fisheries (PRF) based in Ayr, north Queensland. The strategy therefore involved the integration of Cobia aquaculture into an existing prawn aquaculture business, and addressing some specific research outcomes related to this. There was also a need to address some generic research questions related to pond-based Cobia production, as well as to investigate aspects of post-harvest product development and consumer acceptance of this emerging species.

A new method of broodstock maturation assessment was developed, based on the quantification of all stages of oocytes in an ovarian biopsy sample. This approach provided an improved measure of readiness to spawn in Cobia broodstock and a substantial improvement in the success of hormone-induced spawning from <20% to >80%. The use of photothermal manipulation of broodstock resulted in broodstock being brought successfully

into spawning condition in October, three months earlier than previously observed for Cobia held under ambient conditions. Early spawning, and thence fingerling production, were key to the inclusion of Cobia within PRF's overall farm production strategy.

An essential part of the project was to facilitate the development of PRF's hatchery, nursery and grow-out production capabilities through the provision of stock, training of staff and development of on-farm expertise. Over the course of the project more than 140,000 fingerlings were provided to PRF to enable commercial-scale production of Cobia in ponds, and PRF harvested up to 100T per year of Cobia >5 kg. Additionally, 81,000 larvae were provided for a series of hatchery production trials. In order to improve on-growing efficiency, a comprehensive assessment of the effectiveness of two commercially aquafeeds was also completed.

An expert consumer panel was commissioned to undertake a detailed assessment of the flavour and textural characteristics of fresh Cobia, and to compare Cobia with other similar products. This demonstrated Cobia to be equivalent to Atlantic Salmon and superior to Yellowtail Kingfish in terms of overall appeal, flavour and texture. A hot-smoked Cobia product was also developed, and this had a number of favourable characteristics as good as, or better than similar Atlantic Salmon products. Fresh Cobia was also shown to have excellent shelf life qualities, with a shelf life of up to 21 days in modified atmosphere packaging, and 17 days in skin packaging.

Acknowledgements

The commitment over several years of Pacific Reef Fisheries (PRF) to Cobia aquaculture, in particular the ongoing support of Nick and Maria Mitris, is gratefully acknowledged. Alistair Dick, former General Manager of PRF, was instrumental in the establishment of this project. Brad Calcott, Farm Manager at PRF, and Bastien Finet, Hatchery Manager at PRF, have both been strong drivers for developing Cobia production capability at PRF.

Thanks to Ido Bar for a great deal of work in optimisation, analysis and interpretation of the microsatellite marker work.

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Finally thanks to the Seafood CRC for providing the opportunity to undertake Cobia aquaculture research in Australia, and for setting the foundations of a successful new aquaculture industry.

1. Introduction and Background

Cobia, *Rachycentron canadum*, is a large benthopelagic species endemic to all tropical and subtropical waters across the globe with the exception of the eastern Pacific (Shaffer & Nakamura 1989). They belong to the order Perciformes and are the only species within the family Rachycentridae. The phylogenetic positioning of Rachycentridae within Perciformes has been open to conjecture by several authors. Cobia have been assumed to be closely related to remoras (Echeneidae) due to similarities in form, colour and the fin shape of juveniles. However, osteological examination suggests a greater likelihood of relatedness between the Rachycentridae and Coryphaenidae (Mahi Mahi) (Johnson 1984).This has more recently been challenged by a study by O'Toole (2002) which also used a range of osteological features, and concluded the Rachycentridae to be closer to Echeneidae than Coryphaenidae.

Cobia can attain a length of up to 2 metres and exceed 60 kg weight (Franks, et al., 1999). They have an elongate 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 predominantly white ventral surface. During juvenile and adolescent stages a prominent white stripe runs the entire length of the mid-section, becoming less obvious as the fish ages. Cobia are opportunistic carnivores, feeding on cephalopods, fish and crustaceans (Salini, et al., 1994).

Cobia are 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 small: less than 30 tonnes per annum (van der Velde, et al., 2010).

1.1 Cobia Aquaculture

Growth rates that exceed 5 kg per year, adaptability to commercially available aquafeeds, excellent palatability and temperature and salinity tolerance are some of the attributes that make Cobia an exceptional candidate for aquaculture (Liao, et al., 2007; Shiau, 2007; Weirich, et al., 2007).

Commercial Cobia aquaculture began in Taiwan in the late 1990s and has since been adopted by several nations throughout the Asia-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 30,000 of the global annual production of 40,000 tonnes (FAO, 2014) Cobia production in Vietnam has increased rapidly in recent years to exceed 1000 tonnes using a similar production model to that of Taiwan (Nhu, et al., 2011). Pilot-scale production using submersible sea cages has also shown promising results in the Caribbean (Benetti, et al., 2010; Stone, 2014; GLOBALG.A.P., 2015).

1.2 Cobia Aquaculture in Australia

Cobia research and development began in Australia in 2007, focused largely on introducing Cobia as an alternative and off-season crop for prawn farms in Queensland (Dutney, et al., 2010).

Trials conducted at the Bribie Island Research Centre (BIRC) demonstrated the successful production of juvenile Cobia using hatchery infrastructure and techniques similar to those used to produce other marine and estuarine species at the site. 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 were 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 technical feasibility of producing Cobia, the development of commercial aquaculture in Australia has been relatively limited, due in part to low or inconsistent supply of seed stock for grow out.

1.3 Project Aims

In 2009 the Seafood CRC issued an invitation for new industry investment in the area of finfish production. Good Fortune Bay Fisheries, based in Queensland, responded with a proposal to develop Cobia aquaculture. Subsequently, further industry interest in Cobia aquaculture was canvassed, with the result that Pacific Reef Fisheries (PRF) (Qld), Marine Produce Australia (WA) and Ridley Aquafeeds (Qld) also committed, in principle, to join a Cobia research consortium. Following a series of discussions between all parties in late 2009/10, the consortium dissolved, but DAF continued to work with PRF in a collaborative research program, continuing to investigate the feasibility of pond rearing of Cobia. The collaborative research effort between PRF and DAF highlighted both the high potential of Cobia as a candidate species for production in ponds in north Queensland, as well as some problems in the hatchery phase of production, in particular spawning regularity and control. In 2010 Pacific Reef Fisheries indicated interest in expanding and pursuing the existing collaborative research project under the auspices of the Seafood CRC. After a series of preparatory discussions, PRF and DAF agreed to participate in a research project with the aims of: achieving regular, controlled spawning of Cobia; transferring hatchery technology to PRF, developing and testing Cobia-targeted diet formulations.

Collaborative research undertaken by DAF in 2007-09 identified the supply of fingerlings as a potential major bottleneck to an Australian industry. A major aim of this project was therefore to establish broodstock husbandry and spawning methods to enable hatcheries to reliably supply large numbers of high-quality fingerlings to on-growing operations. In order to facilitate commercialisation of Cobia, increased industry capability in the area of Cobia hatchery production was targeted through technology transfer and training of PRF staff.

Inconsistencies in Cobia reproductive performance both at BIRC and commercially highlighted the importance of better understanding the nutritional requirements of farmed Cobia in Australia. The project utilised links with an ACIAR-funded project developing feeds and bioenergetic models for several finfish species, including Cobia (“Improving feed sustainability for marine aquaculture in Vietnam and Australia”, Project leader Dr Brett Glencross, CSIRO; http://aciar.gov.au/project/FIS/2006/141) to facilitate information transfer to the Australian aquafeed industry regarding Cobia diets.

A marketing study undertaken under the auspices of the Seafood CRC in 2009 (Project No. 2010/715, Freeman, J. (2010) Cobia Market Analysis) outlined the extent of world Cobia production, which currently extends to 30 countries. The study also noted that the market for Cobia is highly variable, driven by low production and underdeveloped markets. Although the majority of Cobia is consumed in Asia, it is also recognised as a fish readily accepted by Western palates, as evidenced by its position in high-end markets and restaurants in the USA. The study noted an opportunity for Australian producers to develop products for both the specialised Asian restaurant and broader retail sectors, offering in Australia a local alternative to imported white flesh fish such as Nile Perch. This project aimed to develop a more thorough understanding of the consumer preferences for Cobia. This also considered product preferences, taking into account such factors as fish size, degree of processing, flesh composition/quality and shelf life.

1.4 Need

Aquaculture in Queensland is dominated by pond-based production of prawns and Barramundi, which collectively account for 90% of industry value (Heidenreich, 2014). Expansion of the Queensland aquaculture sector has been significantly constrained by regulatory factors, with no new farms licenced in Queensland since 2002. However, during that time, industry productivity has continued to increase, through improved efficiencies and production strategies.

The development of alternative species for pond aquaculture is one element in maintaining a robust, efficient and profitable pond-based aquaculture sector. Such developments should also be readily capable of expansion should the regulatory landscape change. Cobia fulfil these criteria, with demonstrated efficient production in ponds and acceptable financial returns (Dutney, et al., 2010).

Broodstock management and hatchery production represent bottlenecks both to Cobia production per se, but more specifically to pond-based Cobia production integrated within prawn production. Under these conditions, the production of fingerlings must be highly controlled and predictable, enabling tight scheduling of stocking ponds. The development of a robust Cobia aquaculture sector is also dependent on a reliable commercial supply of fingerlings. In addition, the market potential of Cobia in Australia is poorly understood, and this needs to be better defined to enable Cobia aquaculture to fulfil its potential.

There is therefore an opportunity to develop a collaborative, whole-of-chain approach to launching this new species so that the market is carefully developed and supplied with fish of consistent quality at economically sustainable prices. This project provides an avenue for DAF to work with the Queensland aquaculture sector to bring another species, suited to pond aquaculture, to farm- and market-ready state. For PRF it represents an opportunity to lead the market in Australian Cobia production and marketing, and to establish the company as a vertically integrated producer of both prawns and Cobia.

1.5 Objectives

1. Develop reliable and robust controlled spawning methods for Cobia, utilising hormonal, social and/or environmental manipulation
2. Produce sufficient fingerlings by DAF to enable PRF to on-grow commercial quantities of Cobia for market
3. Develop pilot-scale Cobia fingerling production by the PRF hatchery
4. Formulate diets designed to meet the specific nutritional and energetic requirements of Cobia
5. Develop and field test new farmed Cobia product(s) with high market acceptance.

2 Methods

Refer to the Methods section in the appendices dealing with individual objectives.

3 Results

Refer to the Results section in the appendices dealing with individual objectives.

4 Discussion

Refer to the Discussion section in the appendices dealing with individual objectives.

5 Benefits and Adoption

The close relationship between DAF and PRF has ensured immediate benefits and where appropriate, adoption of, new technology and production methods. Advances in broodstock photothermal management led directly to the out-of-season provision of fingerlings to PRF, in order to integrate into PRF's production schedule. As a result of this project, PRF has developed a robust, efficient, integrated production strategy for both prawns and Cobia. This has led to the production of approximately 100T per year of Cobia at PRF.

Regular communication between PRF's senior hatchery staff and DAF staff, together with short-term training placements at BIRC have ensured that PRF staff have been able to develop appropriate skills for the ongoing development of Cobia hatchery facilities. These skills have been put into practice, with the provision of early-stage larvae to the PRF hatchery, which have been successfully reared through to fingerlings by PRF staff. PRF intends to build on this improved capacity in order to further their development of Cobia hatchery facilities. This will not only secure PRF's future fingerling supply, but potentially enable PRF to supply to other industry players.

Frequent dialogue between research and commercial participants has helped PRF to develop and improve farm production systems and management approaches 'on the ground' leading to improvements in Cobia survival and growth, particularly in the post-transfer period. The development of production and management strategies which result in fingerling transfer at a size and time suited to both Cobia and prawn farming operations has been a key element of the project, as has the adoption by PRF of a nursery cage system for post-transfer rearing of Cobia fingerlings.

Product development by DAF has also been undertaken in close association with PRF. As a result, the information generated has been immediately usable by PRF in their marketing activities. PRF is using the information on product development and consumer acceptability to further develop market options for farmed cobia. Current strategies, utilising project findings to market Cobia as a high-quality product attractive to the restaurant sector, have achieved a strong market position and price for the product. The product evaluation data will also provide a valuable basis for future development of a retail market.

6 Further Developments

DAF and PRF are both committed to continuing R&D into Cobia aquaculture, with the goal of establishing a 1000T farmed Cobia industry in the medium term (3-5 years). DAF is continuing engagement with other potential investors in Cobia aquaculture, with the aim of establishing another collaborative industry-based research group. Plans are in place for the establishment of a new Cobia research and production consortium involving PRF and at least one new industry partner.

DAF will continue to act as a fingerling supplier to the Cobia industry in the short term. This provides both PRF and new entrants to the industry with some surety for this period while commercial hatchery facilities are being developed. However, the transfer of responsibility for fingerling production to industry will continue, and DAF will continue to directly support this through ongoing information sharing, staff training and technology transfer.

Research into issues related to hatchery production, in particular the emergence of intersex individuals will continue as a key part of any new research project. Research will also focus

on farm production issues, such as pathogen and predator control, feeds and feeding, and DAF will continue to work with PRF and other industry partners in these areas.

7 Planned Outcomes

7.1 Public Benefit Outcomes

- Commercial viability of pond-based Cobia production in north Queensland together with the establishment of a pilot commercial supply of high-quality, fresh farmed Cobia to the Australian marketplace. This sets the base for the future development of a 1000+T Cobia industry by 2020.
- The provision of high-quality Cobia products to the Australian market, in particular, the restaurant sector
- Improved performance of Cobia on diets supplemented with taurine.

7.2 Private Benefit Outcomes

- Technology and techniques to maximise Cobia performance post-transfer can enable private farms to at least raise Cobia from larvae, reducing dependence on public hatchery/nurseries
- Pilot-scale hatchery production of Cobia fingerlings demonstrating viability and reliability of seed supply
- Commercial scale pond-based production of Cobia can be envisaged given that seed is available and PRF have been able to demonstrate commercially viable production in ponds
- Cobia is recognised by consumers as high quality product and is likely to receive premium prices in the marketplace if successfully marketed, for example to high end restaurants in Australia supporting profitable production.

7.3 Linkages with CRC Milestone Outcomes

The project has successfully developed production systems for a new aquaculture species, identified researchable constraints to the production of a new species and successfully addressed these constraints. It therefore links to all three milestones on **Output 1.1** (*Technically verified new aquaculture production systems on a commercial scale*), namely:

1.1.1 Milestone	Pilot-scale systems operational in at least two new production systems
1.1.2 Milestone	Key researchable constraints identified and characterised in at least two new production systems
1.1.3 Milestone	Key researchable constraints successfully addressed in at least two new production systems.

8 Conclusions

The project has successfully met the overarching goal of achieving commercial Cobia production in ponds in Australia. The current production of up to 100T per year of high-quality product for the high-end restaurant market appears feasible and bodes well for the industry's future.

The consistency of production remains dependent on a reliable hatchery sector, and significant steps have been taken towards this. The development of improved reliability of

spawning both in and out of season, together with the demonstration of Cobia larval production in existing prawn hatchery facilities are major achievements that meet prerequisites for future reliable fingerling production.

The study of Cobia diets has again underlined the importance of appropriately formulated diets, particularly for fast-growing species such as Cobia.

Cobia has been shown to be a product with excellent traits for the marketplace, both in terms of its flavour profile and other characteristics, and its shelf life. There is clearly a place for fresh locally produced Cobia in the Australian marketplace, but the challenge will remain as always to maintain price as production rises.

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Appendices

Appendix 1

Objective 1: Develop reliable and robust controlled spawning methods for Cobia, utilising hormonal, social and/or environmental manipulation

Appendix 2

Objective 2: Produce sufficient fingerlings by DAF to enable PRF to on-grow commercial quantities of Cobia for market

Objective 3: Develop pilot-scale Cobia fingerling production by the PRF hatchery

Appendix 3

Objective 4 Formulate diets designed to meet the specific nutritional and energetic requirements of Cobia

Appendix 4

Objective 5 Develop and field test new farmed Cobia product(s) with high market acceptance

Appendix 5

Media outputs

10 Appendix 1

Objective 1: Develop reliable and robust controlled-spawning methods for Cobia utilising hormonal, social and/or environmental manipulation

10.1 Methods

Capture and General Husbandry

Broodstock Cobia ranging in size from 2-10 kg were sourced from the outer reaches of Moreton Bay in 2011 and 2012. The fish were held in quarantine at BIRC for approximately five weeks, when they were allowed to recover from any injuries resulting from capture and then given three treatments of 200 ppm formalin for 1 hour 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 to allow fish to be individually identified. Selected G1 individuals produced during the 2009/2010 and 2010/2011 production seasons were also used as broodstock in some studies, as described below.

The broodstock maturation system at BIRC consists of four 35,000L fibreglass tanks each with a separate enclosed recirculating system consisting of 500µm pre-filter screens, dual 1kW transfer pumps, zeolite media filter, 150W UV steriliser, 13kW heat/chill unit, foam fractionator and moving bed bioreactor. All tanks are fitted with internal bottom drains, and overflow outlets that facilitate the collection of eggs post-spawn. Each tank is fitted with a vinyl cover to exclude natural light and prevent escape. Lighting is provided by two twin 37W fluorescent lights. Fish were fed to satiety 5 days per week on a mixture of squid, prawns and pilchards. Pilchards were supplemented with a vitamin premix . 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. Prophylactic disease treatments were conducted monthly, using a bath of 200ppm formalin for 60 minutes.

Sedation

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

Blood sampling

Individuals were placed ventral side up in a supportive cradle and 0.5ml blood samples were extracted from the caudal vein using a heparinised 21G x 38mm needle and 1ml syringe. Blood was then transferred to two 1.5ml heparinised Eppendorf tubes and embedded in shaved ice. Plasma was separated by centrifugation at 4000 rpm for 20 minutes at 4°C. Five 200µL aliquots of plasma from each sample were then held at -80°C until required. Estradiol (17β-estradiol) and 11 ketotestosterone (11KT) concentrations in the plasma were quantified using EIA competitive assay kits (Cayman Chemical ACE ™).

Oocyte assessment

Ovarian samples were taken by inserting 1mm diameter plastic cannula through the gonopore and approximately 5-10cm into the ovary. Oocyte samples were 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.

A factor contributing to the limited success for hormone induction in Cobia may be related to the methods used to assess ovarian development of broodfish. If the sampling strategy is not providing an accurate measure of ovarian development it could belie the readiness for hormone-induced spawning. Hormone therapy applied to induce spawning in immature fish or prematurely to adult fish are is generally inefficient or ineffective (Mylonas, et al., 2010).

Cobia do not show obvious external signs of advancing sexual maturity that may occur in other fish, such as pronounced external changes in body shape, alterations in jaw shape or other secondary sex characteristics (Crim & Glebe, 1990).

The current method of assessing the ovarian development of Cobia in commercial or research aquaculture facilities is via cannulation (gonad biopsy). This involves inserting 1mm diameter plastic tubing into the urogenital opening and applying gentle suction to extract a small amount of gonad material. The material is then expelled onto a microscope slide for examination under a light microscope. Initially, based on standard methods for quantifying ovarian development, development was assessed by the average diameter of the 10 largest or examine the most advanced group of oocytes present in the sample Studies on wild Cobia populations conducted by van der Velde et al. (2010) employed a similar method, using histological examination of the gonads.

It is generally accepted that marine teleosts, including Cobia, are ready for hormone induction when the oocytes attain a diameter of 500µm or larger (Benetti, et al., 2007; Weirich, et al., 2007). However, our experience at BIRC was at odds with this: despite a large proportion of fish with oocyte diameters of 500µm or above, hormonal induction often failed to achieve spawning (Table 1).

Table 1. Spawning history of Cobia at BIRC 2007 to 2010.

Month	Total No. mature (Wild caught)	No. with oocytes >500µm	No. hormone Inductions	Spawns	Fertilisation rate (%)
2007/08 Summer Season					
Nov	6	0	0	0	
Jan	6	2	3	2	75, 82
Feb	6	1	1	0	
2008/09 Summer Season					
Nov	6	1	0	0	
Dec	6	1	0	0	
Jan	6	3	3	1	81.5
Feb	6	3	0	0	
Mar	6	3	1	0	
2009/10 Summer Season					
Dec	17	4	0	0	
Jan	17	6	5	0	
Feb	17	6	6	1	90

Observations of gonad samples collected by cannulation showed high variability in oocyte size, indicative of asynchronous development. The typical variation observed in samples from Cobia at BIRC is demonstrated in Figure 1. Figure 1A shows a variety of oocyte stages from a single sample, with a mix of previtellogenic through to late-stage, well-developed oocytes. In contrast, the sample image in Figure 1B shows an almost unimodal distribution of late-stage oocytes.

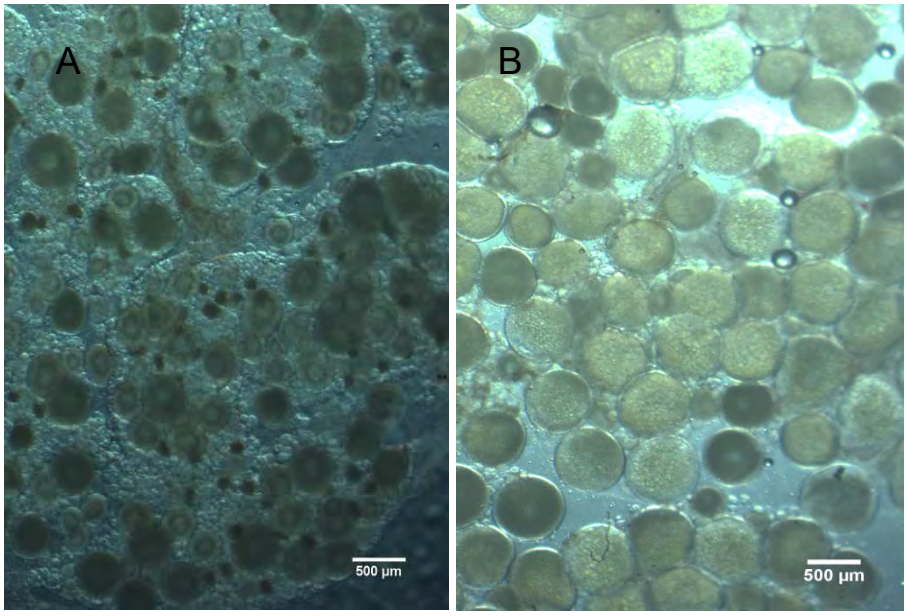
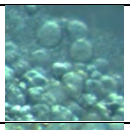
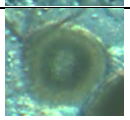
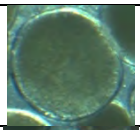
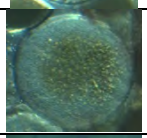
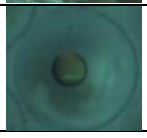


Figure 1. Cobia cannula sample showing a oocytes from two different fish, at differing developmental stages, but with similar maximum oocyte size.

A single figure descriptor, to quantify the oocytes in Figure 1 based on the size of the largest oocytes, indicated readiness for induction; however, as a function of asynchronous or perhaps dysfunctional development, the ovary was not sufficiently developed for hormone induction to be effective. There was a need to assess the entire cannulated sample to provide a frequency distribution of the various oocyte stages to accurately assess the development of female Cobia, rather than providing a single point quantification.

An alternative assessment method was used, following Morehead et al. (1988), in which oocytes were classified into five developmental stages based on the size and physical parameters of the oocyte (Table 2).

Table 2. Cobia oocyte stages and description (adapted from Morehead, et al. (1998)).

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	

Ovarian development was then classified using a frequency distribution of each oocyte stage, in order to more accurately assess overall development of the ovary. The images obtained from the cannulation were analysed using NIS Elements software. An image area of 4mm² was selected; any void area was then outlined, measured and subtracted from the selected sample area. The area occupied by each oocyte stage was outlined and measured to provide a percentage of area occupied by each of the oocyte stages in each sample.

DNA marker assessment

Microsatellite markers are recognized as being well suited to the analysis of wild population studies and aquaculture breeding programs (Garcia de Leon, et al., 1998). Thirty-five microsatellite markers have been developed for Cobia (Pruett, et al., 2005; Renshaw, et al., 2005) and seven of these were investigated for their suitability to discriminate within Australian populations of cultured Cobia (Table 3). PCR methods were optimised for these markers, and they were used to genotype a group of fifteen wild-caught Cobia broodstock. Markers showed significant variation in both the number of alleles present, as well as the level of heterozygosity in the fish examined. Marker F06 was highly polymorphic, and all fish tested were heterozygous. In contrast, markers E02 and B12 had only 3 alleles, and B12 was largely homozygous (Table 3).

The markers were then used in a pedigree study of offspring from mass spawnings. In a sample of 6 individuals from one spawning, 100% were assigned to parents. Based on these very preliminary results , the current suite of markers holds promise for future pedigree testing.

Table 3. Summary data for microsatellite markers used in the current study.

Marker label (Pruett *et al.* (2005)	Marker label (current study)	Expected size range[1]	Actual size range	Number of alleles	Heterozygosity
Rca 1B-EO2	E02	298 – 314	320-326	3	0.538
Rca 1-CO4	C04	223 – 253	229-279	8	0.769
Rca 1-A11	A11	167 – 201	190-207	6	0.923
Rca 1B-FO6	F06	260 – 300	252-357	14	1.000
Rca 1-B12	B12	177 – 193	190-196	3	0.154
Rca 1-E11	E11	167 – 181	181-202	5	0.583
Rca 1B-H09	H09	176 - 224	179-215	8	0.923

[1] From Pruett *et al.* (2005)

10.1.1 Experiment 1. Effect of low-dose hormone treatment on oocyte maturation

Historical data from BIRC have shown that while individuals with well-developed gonads may be successfully induced to spawn with hormone implants, a substantial number of broodstock fail to initiate any level of gonadal development. This study was undertaken to investigate the capacity of treatment with low-dose luteinizing hormone releasing hormone analogue (LHRHa) to initiate the onset of ovarian development in captive Cobia. LHRHa is the hormone routinely used to induce spawning in captive Cobia and numerous other finfish species.

The trial consisted of two groups of four female Cobia matched by size and developmental status. This design provided approximately equal start point and body weight of fish in the treatment and control groups. Fish in the treatment group were sedated as described above, weighed, blood and oocyte samples collected, then injected with 15µg/kg LHRHa in a cholesterol pellet (Lee, et al., 1986). Control fish underwent the same procedures of sedation, weight check, blood sampling without receiving the hormone injection.

All fish were placed in a 35,000L recirculating broodstock system at maximised day length (14L:10D) and temperature at 25.5°C. Temperature was slowly increased to 28°C by the conclusion of the trial. Three naïve, wild-caught male fish of similar size were added to the system. Fish were fed to satiety 5 days/week as above.

The first hormone injections were given to the treatment fish immediately after the baseline weight, blood and cannula sampling was conducted. A second injection was given two weeks later, immediately after the second examination. The fish were examined at 4 and 8 weeks after the trial commenced.

- Start (T0): weight check, blood sample, cannulation, hormone induction
- Week 2 (T1): weight check, cannulation, hormone induction
- Week 4 (T2): weight check, blood sample, cannulation
- Week 8 (T3): weight check, blood sample, cannulation

The developmental state of gonads was quantified as described above and estradiol (17β-estradiol) concentrations in the plasma were quantified using an EIA competitive assay kit (Cayman Chemical ACE ™). Data of both oocyte stage distribution and serum estradiol levels were analysed by ANOVA for repeated measures, using Genstat 14th Edition.

10.1.2 Experiment 2. The effect of photothermal regime on oocyte maturation in Cobia

Temperature and day length (photothermal) profiles are a major factor in the onset of maturation in fishes (Pankhurst & Porter, 2003). The effects of temperature and photoperiod on oogenesis in Cobia were examined in a series of trials from 2011 to 2013.

In 2011 two tanks of Cobia broodstock each with 7 females and 5 males were subjected to an advanced photothermal regime, while control populations had ambient conditions (Figure 2). Under the advanced regime, both day length and temperature were increased from July until October, when typical mid-Summer conditions were reached. The trial was repeated in 2012 and ovarian biopsies were undertaken at four-weekly intervals, as described above, to assess the impact of the photothermal regime on oocyte development.

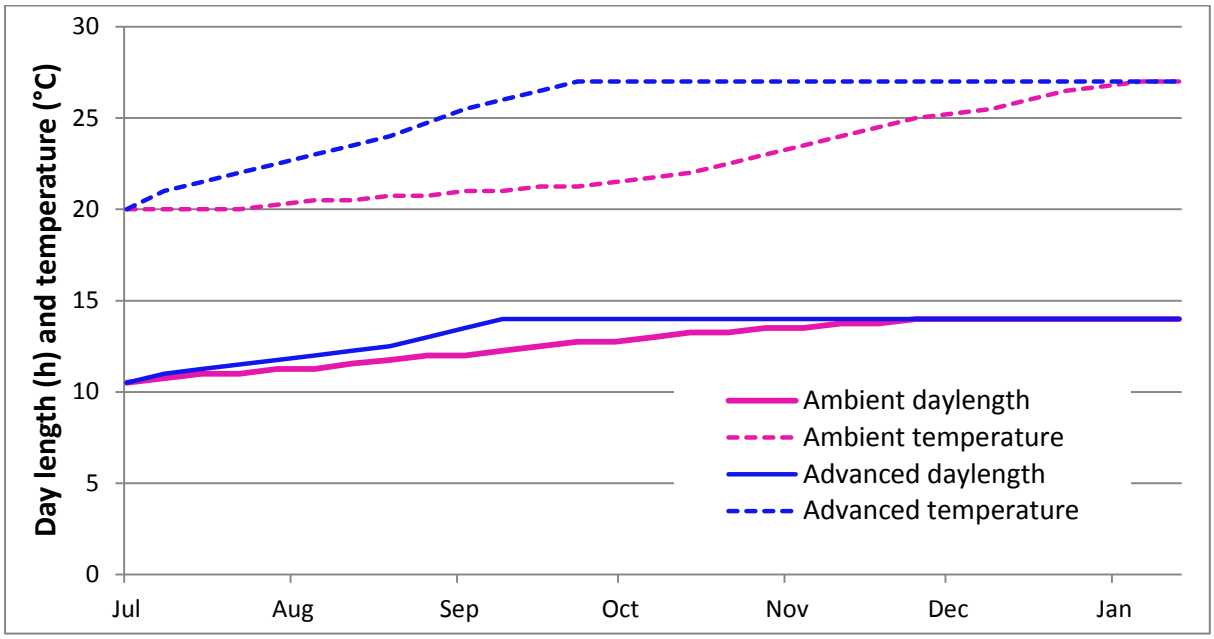


Figure 2. Ambient and advanced photothermal regime used in both the 2011/12 and 2012/13 production seasons.

10.1.3 Experiment 3. Investigation of sexually dimorphic growth in Cobia

There are conflicting reports in the literature regarding sexually dimorphic growth in Cobia. Shaffer, Nakamura (1989) reported that female Cobia grew faster whereas van der Velde el al. (2010) reported no differences in growth rates between the sexes. Anecdotal evidence from aquaculture facilities suggests that female Cobia grow faster than males (D. Benetti, pers. comm.); however, this has not been quantified or documented.

The occurrence of sexually dimorphic growth can impact both broodstock management and commercial grow-out of a species. If a species exhibits sexually dimorphic growth and the largest fish of a cohort are selected as future broodstock, the resultant population may be biased towards a single sex. Therefore, in order to ensure a sufficient sex mix a larger group of randomly selected fish are required to be held until their sex can be positively determined. Sexually dimorphic growth can have a significant impact on the economics of a grow-out operation. The identification of significant economic gains could justify further investigation into the development of single sex culture of Cobia. This study has investigated the occurrence of sexually dimorphic growth in Cobia, so that, if it does exist, the point at which the divergence becomes significant can be determined and an assessment made of the potential economic impact for commercial producers.

Juvenile fish

Two cohorts of juvenile Cobia were produced at the Bribie Island Research Centre in semi-intensive green water tank systems for two weeks post hatch. The larvae were then transferred to extensive pond production for weaning and on-growing to approximately 10 g. 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 contributed to cohort 1. The relative contribution of each of the brood fish was not determined.

Sampling strategy

One hundred fish were randomly selected from each of the two Cobia cohorts and individually identified using T bar tags. Cohort 1 was weighed every two months over a one-year trial period. Cohort 2 was weighed monthly for the first four months and every second month for the remainder of the trial. Once the fish reached sexual maturity, the sex of each individual was positively identified via gonadal biopsy and confirmed for each individual post mortem. This information was then fitted to the weight data collected at each time point to complete the data set (Figure 3).

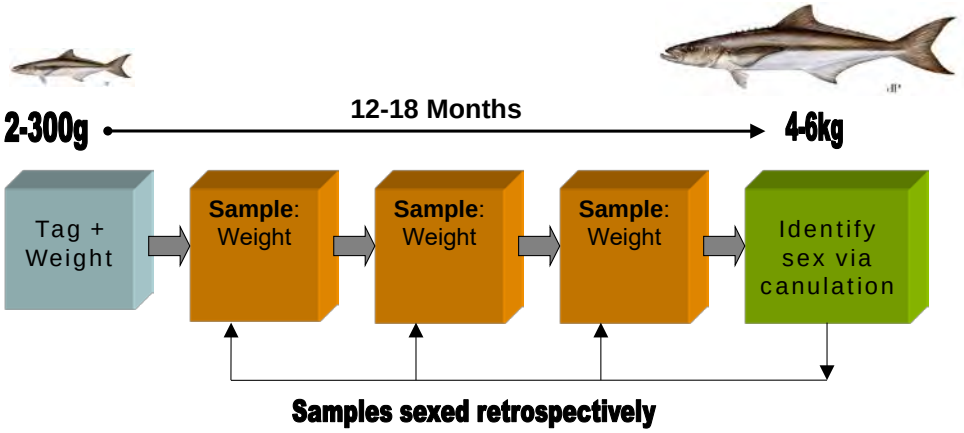


Figure 3. The sampling model used to examine sexually dimorphic growth in Cobia.

Husbandry

The average weight of cohort 1 was approximately 350g when the trial commenced on 06/09/2012. The fish were grown in a single 30,000 L tank fitted with an independent recirculating aquaculture system (RAS). The average weight of the fish was 3.8 kg on 20/02/2013. At this point the biomass was approaching the carrying capacity of the system. The population was split evenly between two 30,000L tanks with identical RAS fitted, where they were maintained until the completion of the trial.

Cohort 2 was tagged on 27/02/2013 (average weight 202 g) and maintained in a single 10,000L circular tank with flow-through seawater. In order to maintain suitable water temperature through the winter period, the fish were transferred on 27/5/2013 to a single 10,000L tank that was part of a 50,000L RAS at an average weight of 627g. The population was split and returned to two 10,000L flow through systems on 18/09/2012 when the average weight was 1.3 kg, where they remained until the completion of the trial.

Although the population of each cohort was divided toward the end of the study, the growing conditions were similar for each group of fish within each cohort. The final densities in cohort 1 tanks were 2.4kg/m³ and 2.6kg/m³ and in cohort 2 tanks were 17.2kg/m³ and 15.8/m³. Temperature, salinity, dissolved oxygen and pH were measured and recorded daily using YSI Professional Plus multi-parameter meter. Total ammonious nitrogen (TAN) was measured weekly when stocking densities exceeded 5 kg/m 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.

Analysis

The data were analysed by ANOVA, using Genstat (VSN-International, 2007). 71% and 88% of the fish in cohort 1 and 2 respectively were used in the final assessment due to low-level mortality and lost or broken tags.

10.1.4 Experiment 4. Evaluation of the effectiveness of plasma and tissue levels of 11-ketotestosterone to identify male Cobia

The androgen 11-ketotestosterone (11KT) plays a major role in spermatogenesis in male teleosts. The concentration of 11KT in the blood plasma is an effective indicator of the sex of individuals. However, the extraction of blood to provide a plasma sample is a significantly invasive process that requires heavy sedation, skilled technicians and carries an inherent risk to animal health. Using readily available, sensitive analytical techniques, androgens and

other steroid hormones can also be measured in other tissues, which may be collected non-invasively (Hutchinson, et al., 2012). Although it is present at significantly lower levels than within the plasma, 11 KT concentrations can be determined from fin clips, a minimally invasive sampling technique to identify the sex of individuals.

As with most reproductive hormones, 11KT concentrations fluctuate within individuals depending on the reproductive stage of fish and the season. This study aimed to identify if the size and season influence the accuracy of using plasma 11KT concentration to determine sex of Cobia. The second aspect of the study was to determine how effectively the measure of tissue 11KT can predict the concentration in the plasma, in order to use fin clip analysis to determine sex.

Sampling strategy

A preliminary analysis was conducted using blood and tissue samples from four large, mature, known-sex Cobia to act as a positive control to test the validity of measuring 11KT concentration in fin clips to serve as an indicator of plasma levels.

Subsequent samples were obtained from a population of approximately 100 Cobia that were individually identified and maintained in a 30,000L recirculating aquaculture system (the same population used as cohort 1 in the dimorphism trial, above). Blood and tissue samples were taken every two months from 20 randomly selected fish. The sex of the sampled fish was determined at the end of the trial via macroscopic post mortem examination and added retrospectively to the data. Table 4 shows the samples that were analysed to best incorporate the influence of fish size/maturity and season.

Table 4. Sampling details of fish assessed for 11KT levels.

Cohort	Average Weight	Season
12A	1.8kg	Early Summer
12A	2.8kg	Late Summer
12A	5.8kg	Winter

Sampling methods

Blood samples were collected and processed as described above. Fin clips were then taken from the tip of the ventral lobe of the caudal fin using single round hole punch to provide a standardised 5mm diameter tissue sample. Fin clips were placed into 1.5ml polypropylene Eppendorf tubes and placed in dry ice. The fin clips were 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 associated instruments used for tissue samples were rinsed with 100% ethanol between samples.

Hormone measurement

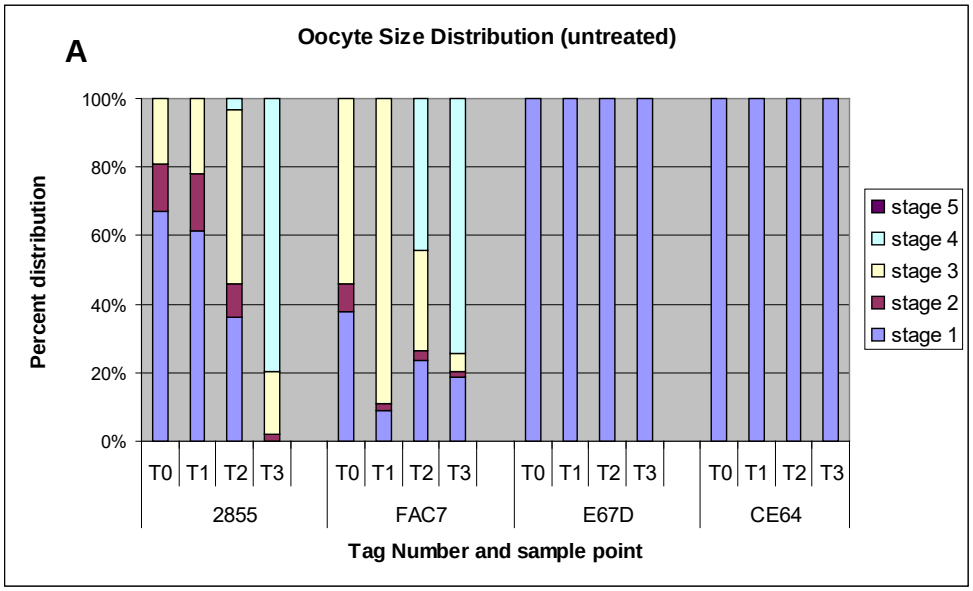
11-ketotestosterone concentrations in the plasma samples were determined as described above. Diethyl ether (1.5ml) was added to the tubes containing fin clips, and tubes were then vortexed three times, each for 30 seconds. The solvent was decanted to a clean tube and allowed to evaporate overnight at room temperature. Buffer from the 11KT EIA kit was then added to the tube and pulse vortexed three times. The sample was then processed according to the EIA kit methods. The amount of 11KT present was then calculated and

expressed per ml of plasma. Data for 11KT in fin clips is presented as either pg per standardised sample or corrected for sample weight and presented as ng per g of tissue.

10.2 Results

10.2.1 Experiment 1: Effect of low-dose hormone treatment on oocyte maturation

Oocyte development was highly variable in both the treatment and control animals. Two fish in each treatment group failed to show any change in oocyte 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 distribution of oocytes of stage 3 or higher (Figure 4). There was no significant difference in the size distribution of oocytes between the control and treatment fish at any of the sampling periods. The inclusion of fish weight or initial proportion of Stage 3 oocytes as a covariate in the analysis did not affect the significance of the results.



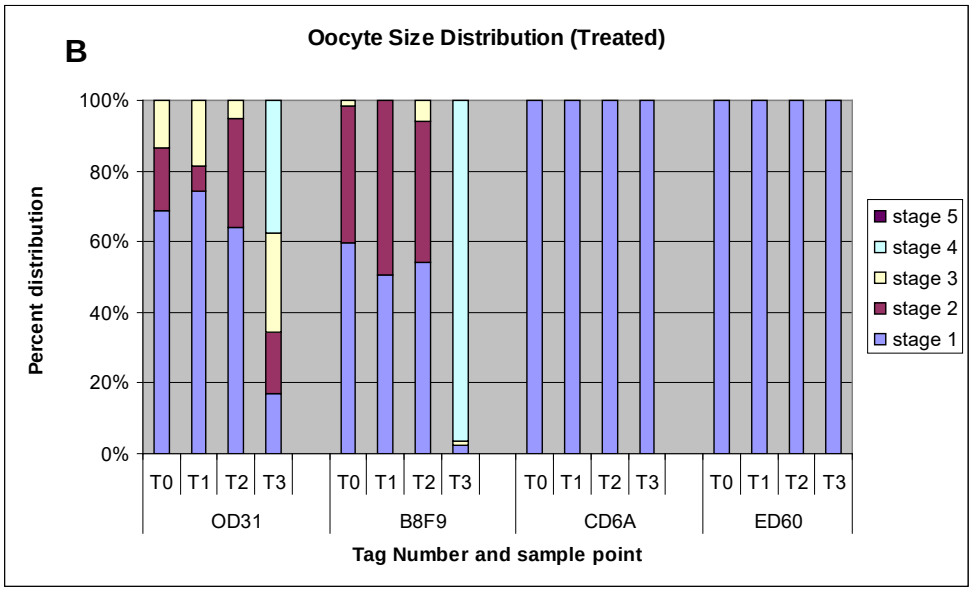


Figure 4**.** Percent distribution of oocyte stages at four sampling times in control (A) and treated (B) fish. See text for details of sample points.

The presence of stage 4 oocytes is considered to be a prerequisite for spawning of Cobia at BIRC. The proportion of Stage 4 and 5 oocytes in each group over the course of the experiment is shown in Figure 5. Stage 4 oocytes were first observed two weeks after the commencement of the trial in the treated fish, and after four weeks in the control fish. The proportion of Stage 4 and 5 oocytes in individuals within each treatment group was highly variable (Figure 5) and did not differ significantly between groups.

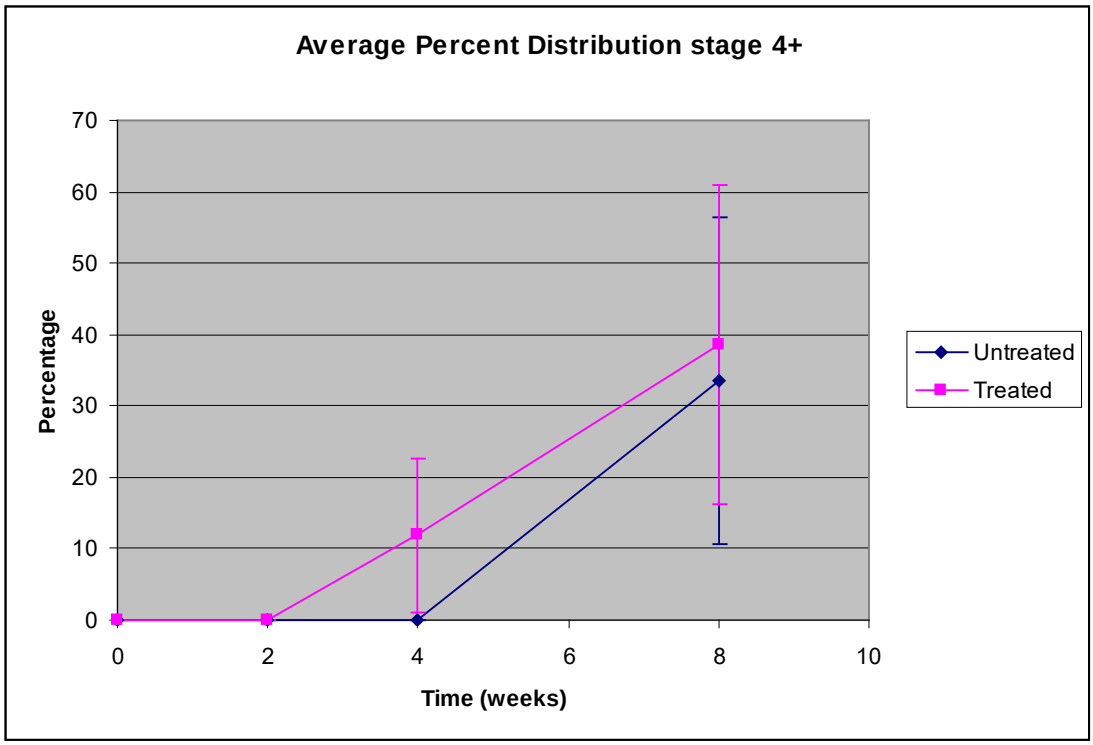


Figure 5**.** Percentage (mean ±SE) of stage 4 and 5 oocytes in treated and control fish, at four sampling times. See text for details of sample points.

Estradiol concentrations were highly variable within control and treatment animals at each sample point and showed no pattern or trends throughout the sample period. There were no significant differences between the estradiol concentrations of treated and untreated fish at any of the three sampling times (Figure 6).

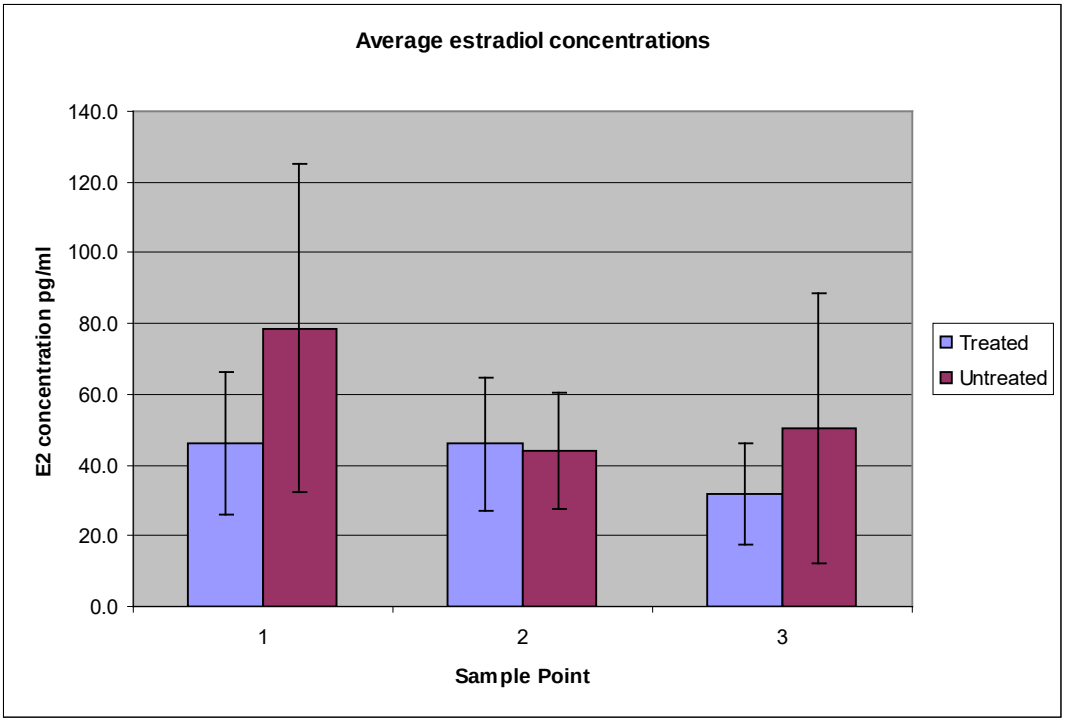


Figure 6. Average plasma estradiol concentrations of LHRHa treated or control (untreated) female Cobia at each sample point (± SE). See text for details of sample points.

10.2.2 Experiment 2: The effect of photothermal regime on oocyte maturation in Cobia

In 2011 the advanced photothermal regime was successful in bringing broodstock into sufficiently advanced reproductive condition for hormonal induction. In October 2011 two female fish were identified in each of two tanks as being sufficiently developed for induction, and these were injected with 30µg kg^{-1} GnRHa. Spawning occurred in both tanks, with high fertilisation and hatch rates (see Appendix 2).

In 2012 the results of gonadal biopsies showed no effect of the advanced photothermal regime on reproductive development in female Cobia by October, and the experiment was terminated (Figure 7).

10.2.3 Experiment 3: Investigation of sexually dimorphic growth in Cobia

All water quality parameters were maintained within the tolerance limits for Cobia for each cohort throughout the trial. The water temperature profile for the production systems for each cohort is presented in Figure 8.

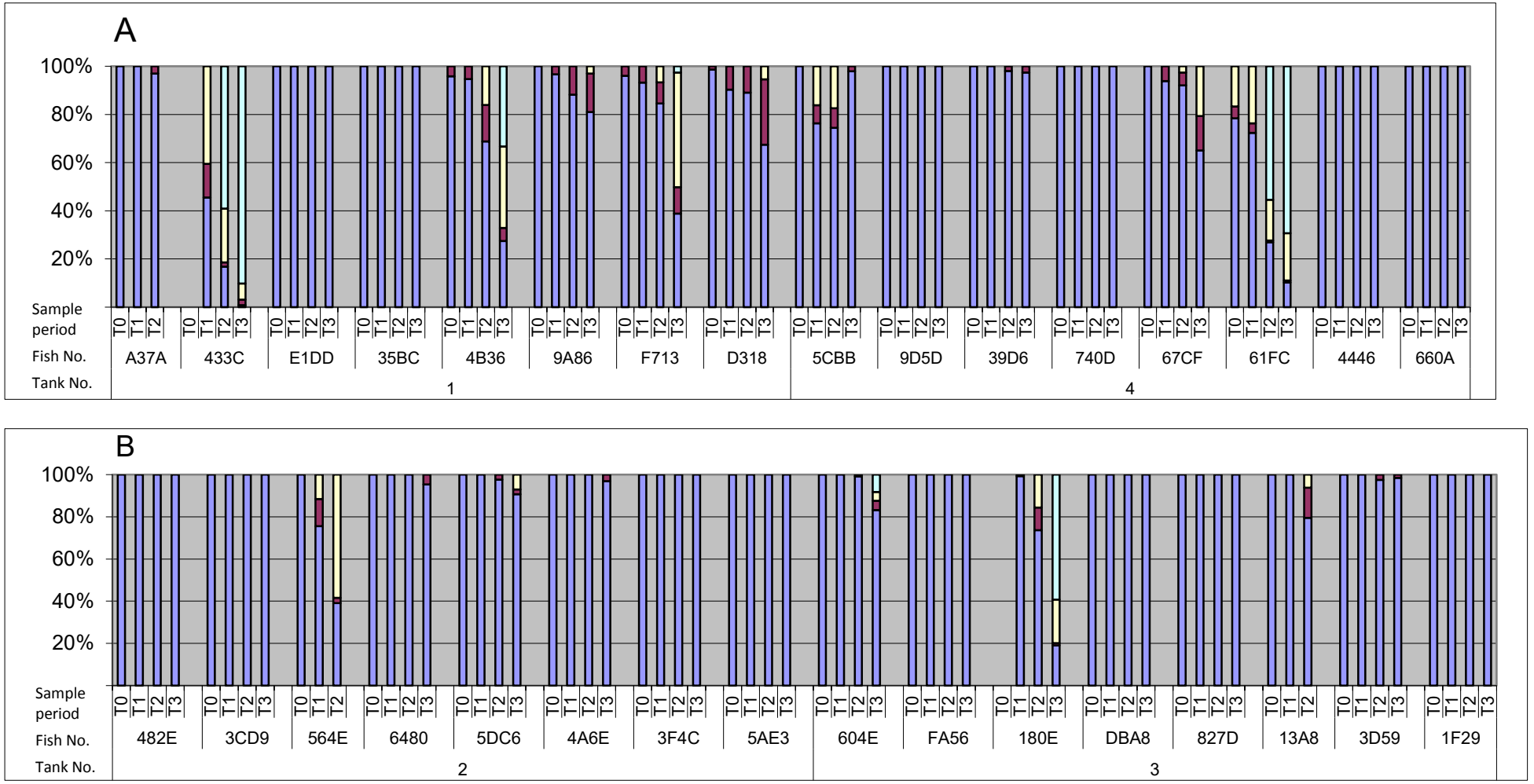


Figure 7. Percent distribution of oocyte stages at four sampling times in ambient (A) or advanced (B) fish. See text for details of sample periods. Oocyte stages as for Figure 4.

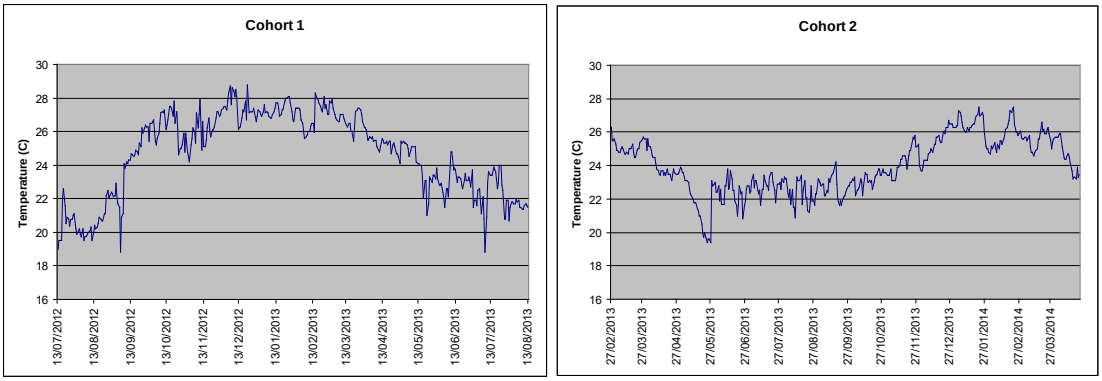


Figure 8. Water temperature for two cohorts of Cobia used in the study of sexual dimorphic growth.

The average weight of fish in each cohort was approximately five kilograms at the conclusion of the sampling period (Table 4). There was no significant difference between the weight of male and female Cobia in either cohort at any stage throughout the sampling period. In August 2013 the weights (mean±SE) of fish in cohort 1 were: male 4250±277g; female 4039±160g; intersex 3660±188g (Figure 9). Intersex fish sampled in November 2013, February 2014 and April 2014 were significantly smaller than male and female fish in cohort 2 (Figure 9). In April 2014 the weights (mean±SE) of fish in cohort 2 were: male 4771±327g; female 5124±187g; intersex 3365±735g.

Table 5. Sex of Cobia in cohort 1 and cohort 2.

Cohort 1	Number of individuals	Cohort 2	Number of individuals
Female	38 (53.5%)	Female	58 (65.9%)
Male	21 (29.6%)	Male	24 (27.3%)
Intersex	12 (16.9%)	Intersex	6 (6.8%)

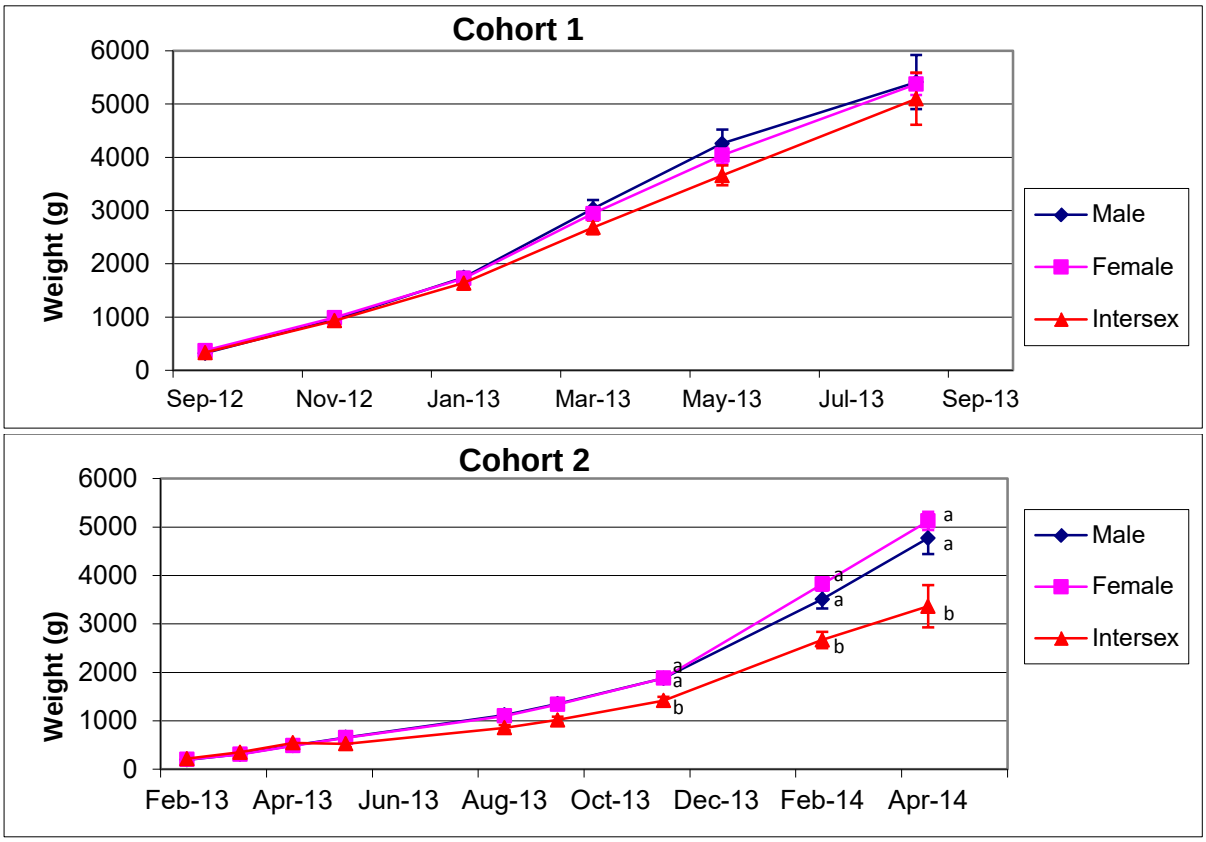


Figure 9. The growth rate of male, female and intersex Cobia in cohort 1 and cohort 2. Within each sampling period, absent or common superscripts indicate no significance between means ($p>0.05$).

Reproductive abnormalities

Several fish in each cohort were identified as intersex fish, in which both sperm and oocytes were observed in the biopsy samples (Table 5). The incidence of intersex fish was higher in cohort 1 (17%) than in cohort 2 (7%). The sex ratio in cohort 1 was approximately equal whereas in cohort 2 it was skewed at 66% female.

Post mortem examination of the fish confirmed the presence of both ovarian and testis tissue in the intersex individuals (Figures 10-12). Intersex gonads were also observed in fish that were not part of this experiment but originated from the same batches as cohorts 1 and 2. Intersex gonads were recognisable at the same stage that the gonads could be discerned macroscopically; when the fish were around 300g (Figure 10). On each occasion the intersex gonads comprised of distinct ovarian tissue anteriorly and testis tissue posteriorly (Figure 11). There was a distinct junction between ovary and testis tissue with limited or no mixing of tissues beyond the junction. The proportions of each tissue varied between intersex individuals (Figure 6). Both ovarian and testis tissues were generally misshapen. Individuals showed varying stages of gonadal development, in most cases both tissues appeared capable of producing gametes. Active sperm and developing oocytes were observed, however gamete quality was not examined.

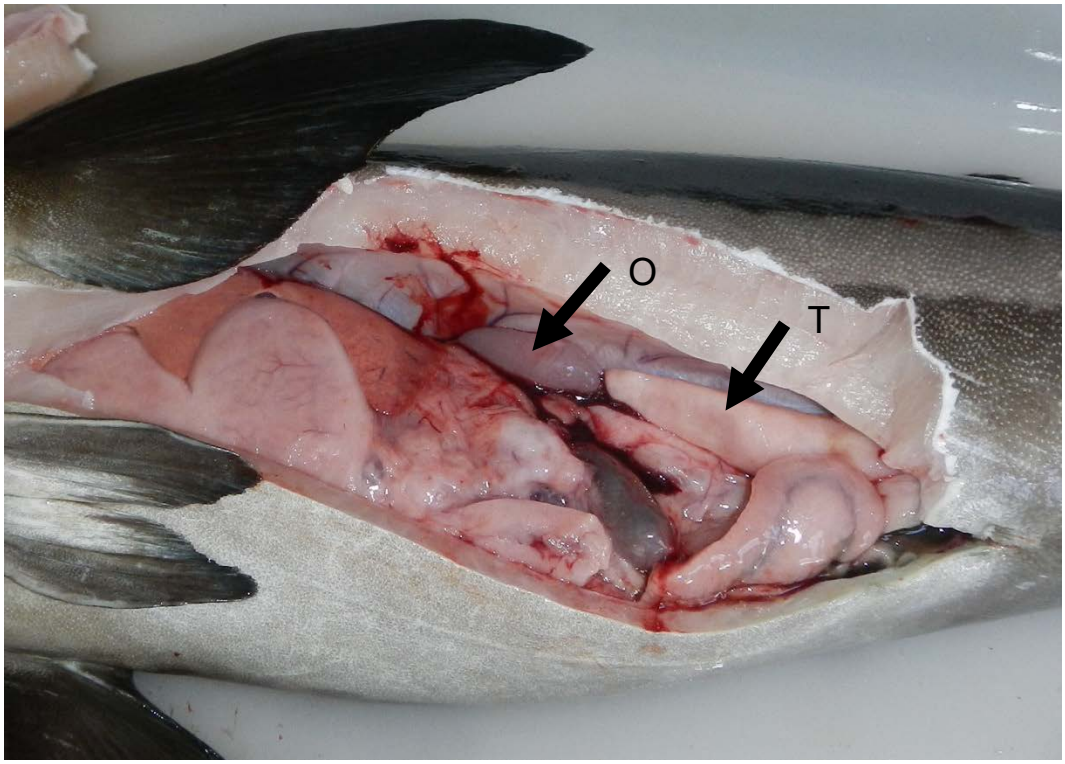


Figure 10. Cobia peritoneal cavity showing the orientation of the intersex gonad. In all intersex fish the anterior section was ovary (O) and posterior section is testis (T).

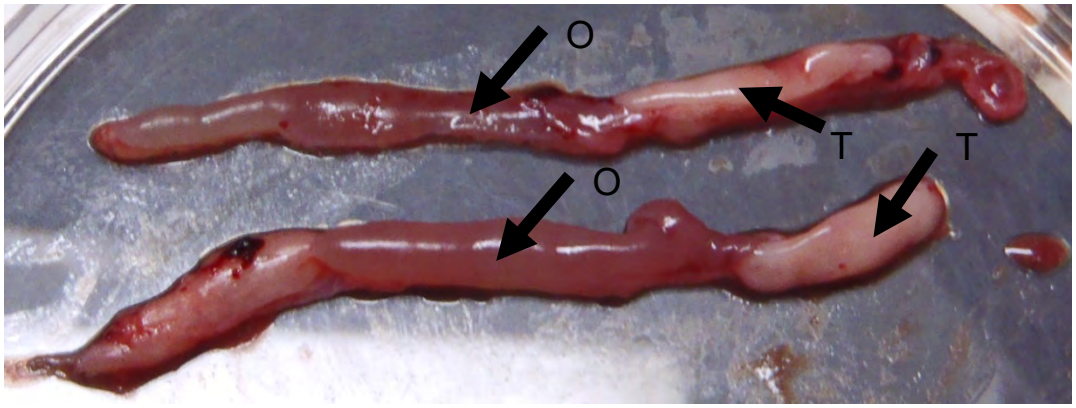


Figure 11. Intersex gonad from 400g Cobia with both ovarian (O) and testis (T) tissues. Intersex gonads were identifiable shortly after gonads could be determined macroscopically.

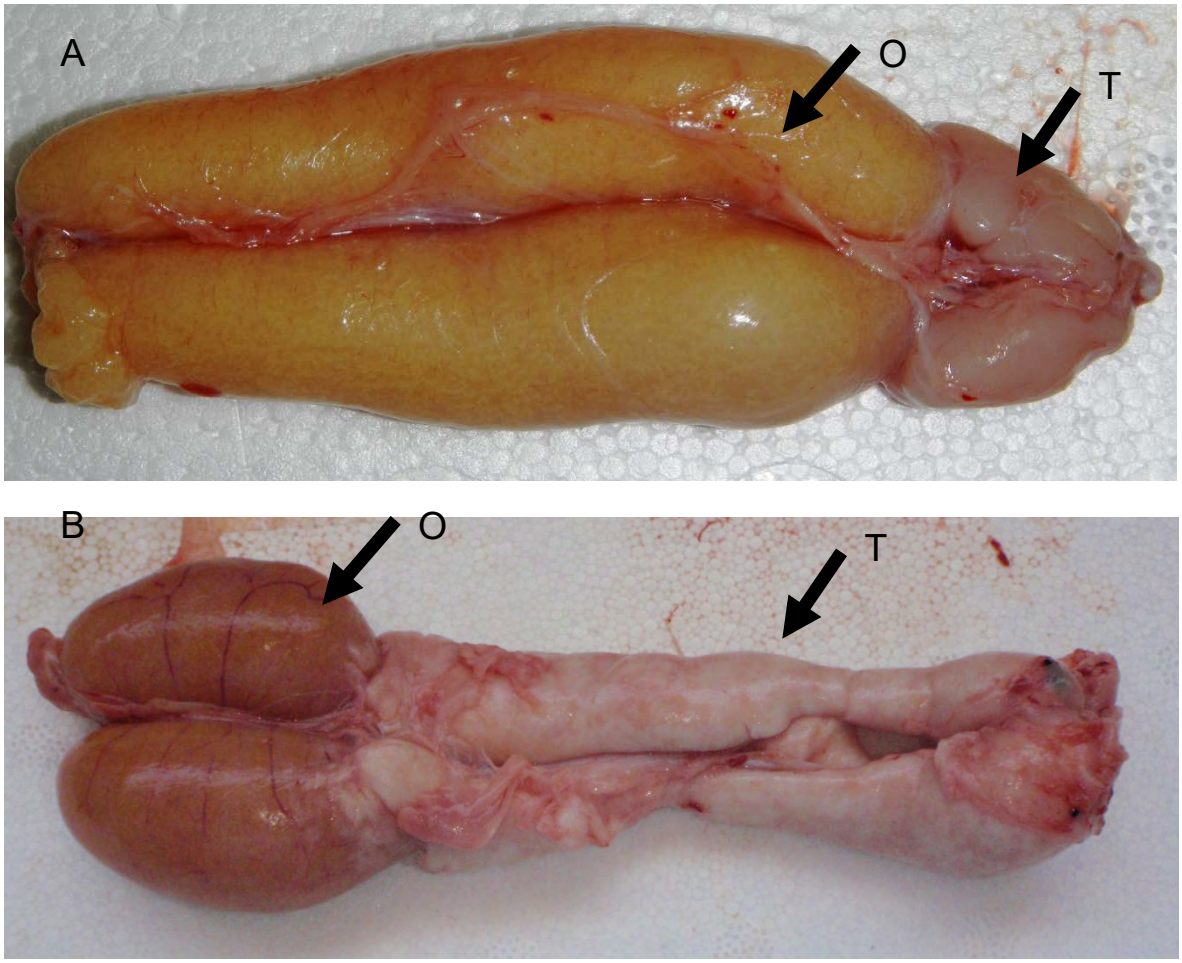


Figure 12. Intersex Cobia gonads showing: A. Predominantly ovarian tissue (O) and B. predominantly testis tissue (T)

10.2.4 Experiment 4: Evaluation of the effectiveness of plasma and tissue levels of 11-ketotestosterone to identify male Cobia

There were significant changes in circulating 11KT levels throughout the sampling period, with levels declining from Summer through to Spring (Figure 13). At each sampling period, mean circulating levels of 11KT differed significantly between sexes. However, the analysis of individual 11KT levels did not demonstrate a clear delineation between males and females at all sampling times.

In fish sampled in mid-Summer with a mean weight of 1.8kg, individuals with plasma 11KT levels less than 10pg/ml were all female, and those with levels greater than 20pg/ml were all male (Figure 14). However, a mixed-sex group comprising nearly half of all fish sampled had intermediate levels. Data from the same population sampled in late Summer are shown in Figure 15. All females had plasma 11KT levels less than 8pg/ml, while all males had levels greater than 14pg/ml. In Winter, all fish with levels less than 4pg/ml were female and those with levels above 10pg/ml were male, but again there was a significant mixed-sex group with intermediate levels (Figure 16).

The preliminary study of mature fish demonstrated a significant linear relationship ($F=0.012$) between 11KT levels detected in the plasma and fin clips (Figure 17). In

contrast, analysis of the data collected across a range of fish sizes and seasons found no significant relationship (Figure 18).

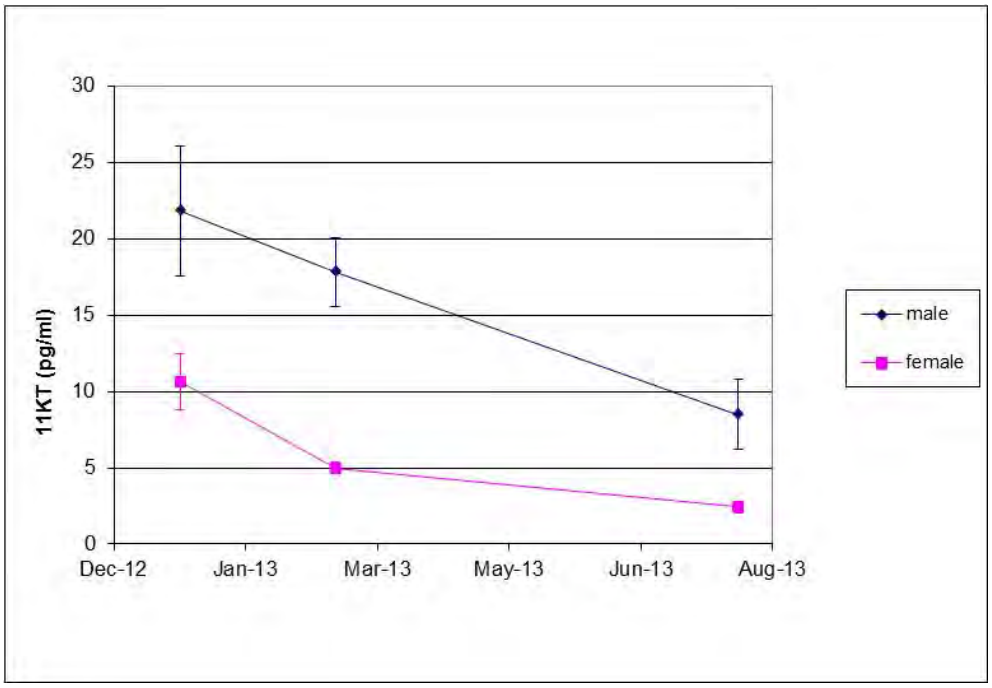


Figure 13. Mean (±SE) 11KT levels in the plasma (pg/ml) of Cobia sampled periodically over an 8-month period.

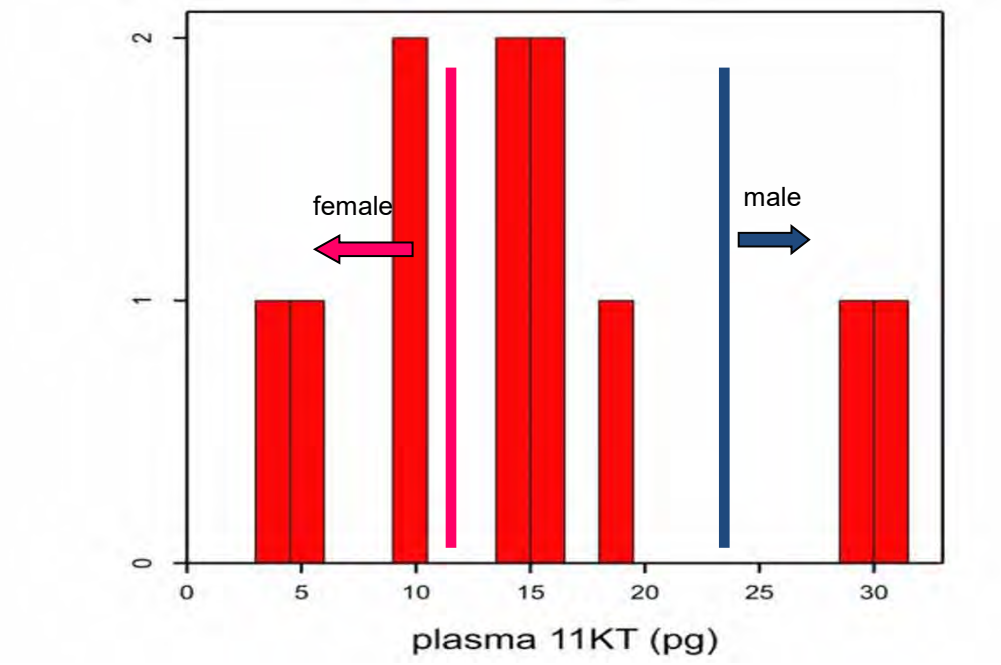


Figure 14. Frequency distribution of plasma 11KT levels (pg/ml) in individual Cobia, mean weight 1.8kg, sampled in early Summer.

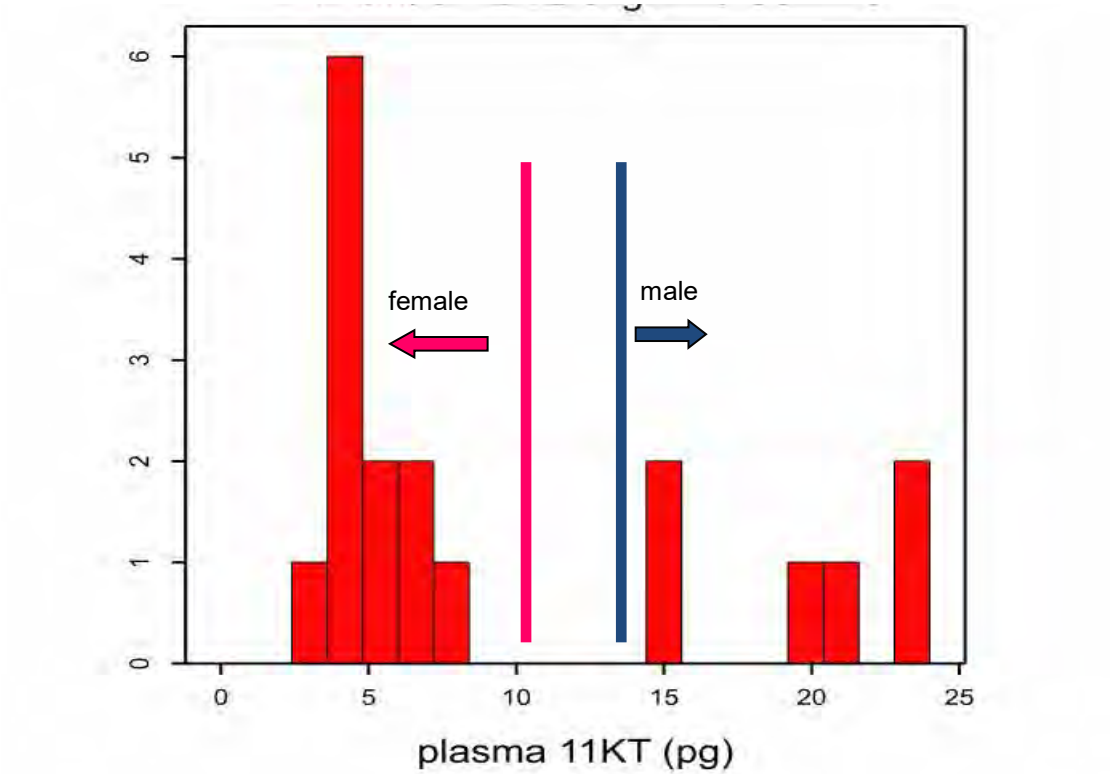


Figure 15. Frequency distribution of plasma 11KT levels (pg/ml) in individual Cobia, mean weight 2.8kg, sampled in late Summer.

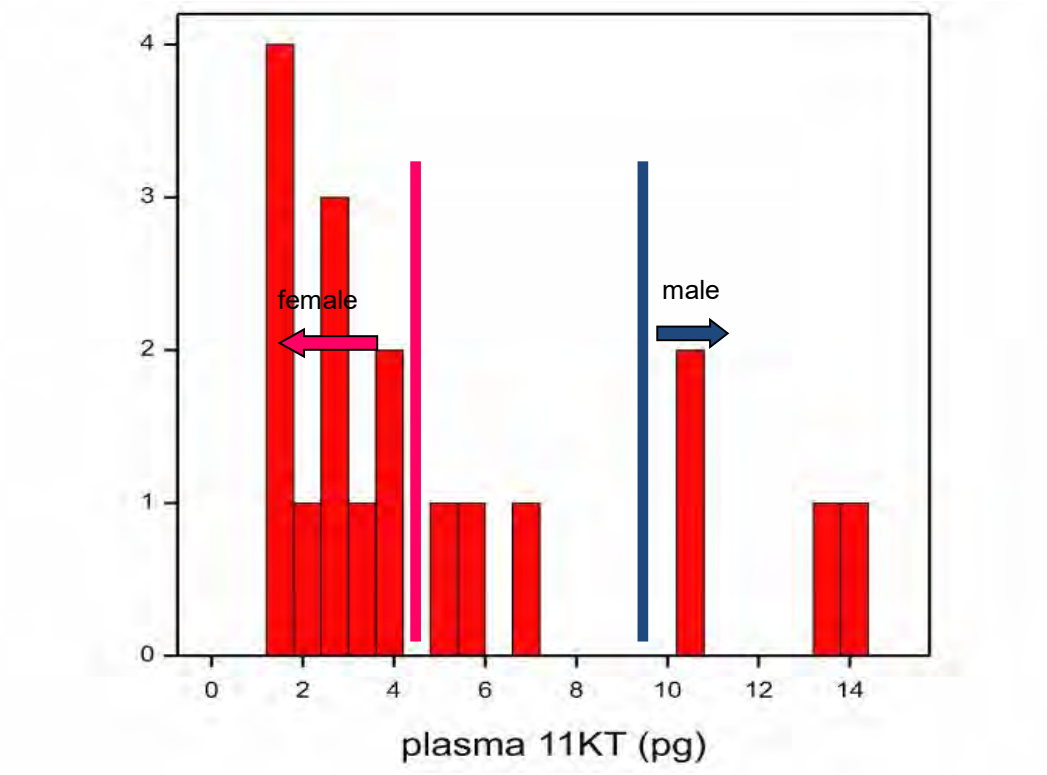


Figure 16. Frequency distribution of plasma 11KT levels (pg/ml) in individual Cobia, mean weight 5.8kg, sampled in Winter.

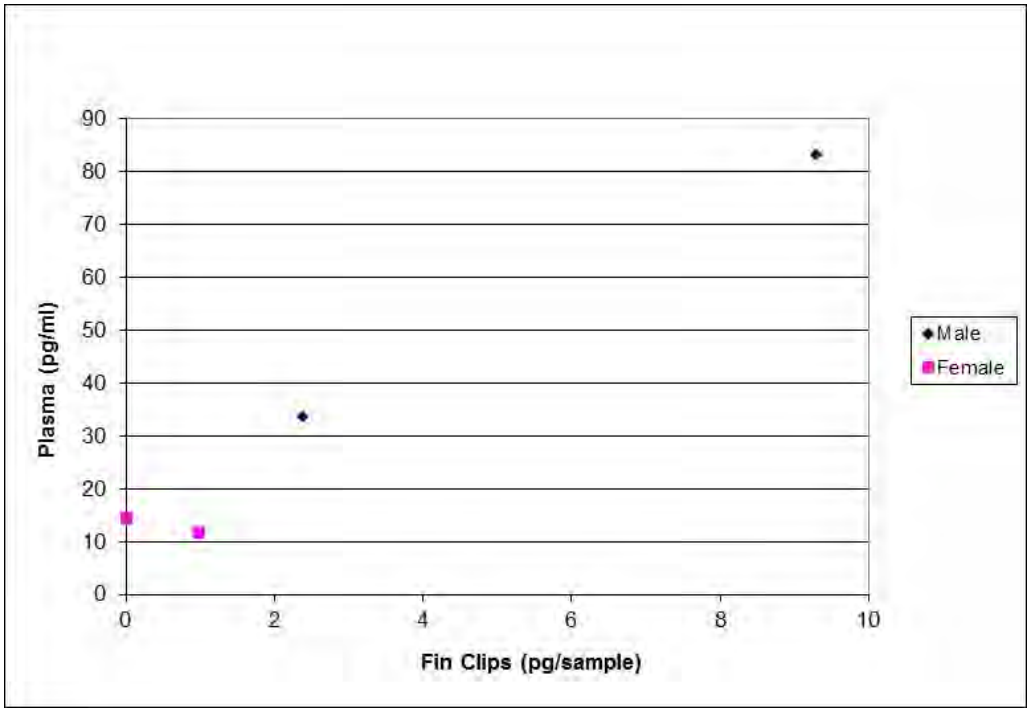


Figure 17. 11KT levels in plasma (pg/ml) and fin clips (pg/sample) from large mature fish, during mid-Summer.

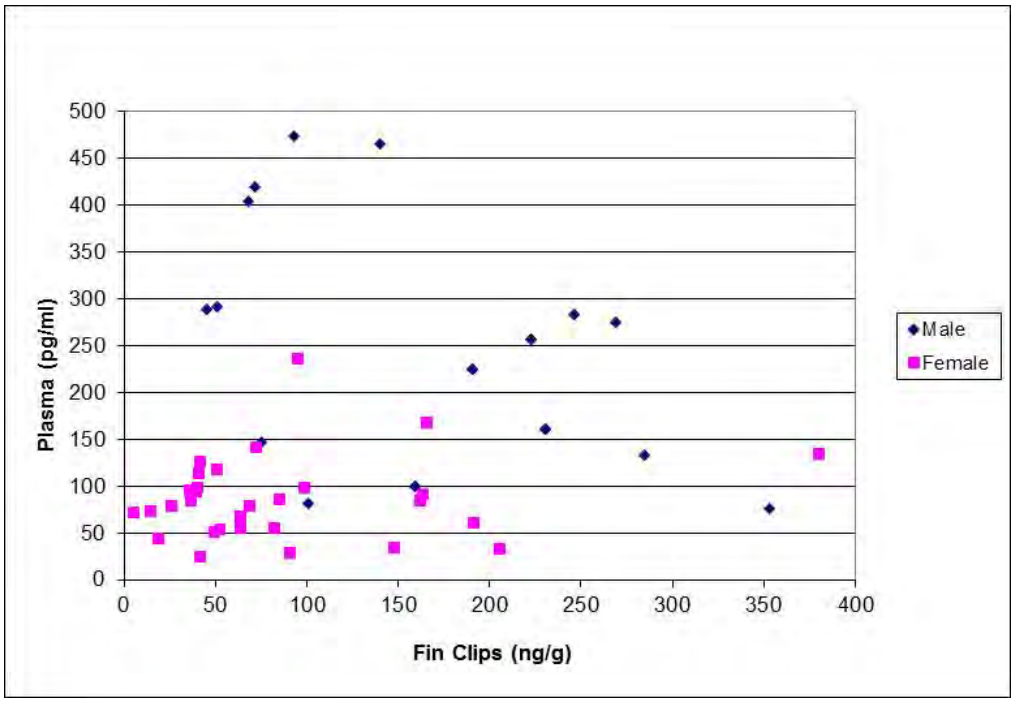


Figure 18 11KT levels in plasma (pg/ml) and fin clips (ng/g) from fish ranging from 1.8kg to 5.8 kg, sampled in early and late Summer, and Winter.

10.3 Discussion

Spawning in unmanipulated Cobia broodstock maintained at BIRC typically commences in January and may continue through until March (Table 1). In the current study, photothermal manipulation did advance maturation in Cobia, with broodstock successfully spawned in October 2011. This is consistent with the successful manipulation of spawning of a wide variety of fish species, through photothermal manipulation (Pankhurst & Porter, 2003). However, in the present study, the effect was not consistent across seasons, with poor results in 2012 and 2013. While such a result may reflect limitations of this method to control Cobia maturation, the results are confounded by impaired reproductive development in these cohorts, including the development of intersex individuals. Based on the reported effects of intersex from other studies described below, it is proposed that the results for 2012 and 2013 are the result of external factors affecting reproductive development, rather than an inherent lack of response to the photothermal manipulation.

The cohorts of Cobia investigated in 2012 and 2013 displayed generally poor reproductive performance (see Appendix 2) as well as structural gonadal abnormalities, including intersex. Individuals in these cohorts also failed to reach spawning condition over Summer, the peak ambient spawning period for Cobia at BIRC. Other studies of induced intersex development in fish and of fish with other facets of impaired reproductive development have also demonstrated reduced reproductive performance in these individuals (Tetreault, et al., 2011; Gerbron, et al., 2014; Fuzzen, et al., 2015).

Potential underlying causes of intersex development in fish include environmental factors such as temperature (Baroiller, et al., 1995; Patiño, et al., 1996; Pavlidis, et al., 2000), endocrine-disrupting chemicals (Purdom, et al., 1994; Jobling, et al., 2002) and genetic factors such as inbreeding (Su, et al., 1996; Gallardo, et al., 2004). There is no clear indicator of a potential cause for the observed intersex and gonadal abnormalities seen in Cobia at BIRC; however, any future Cobia research will investigate this phenomenon further if it arises.

The approach to Cobia oocyte assessment described in this study provides an effective means of quantifying oocyte development in this species. The approach enables the reproductive state to be more accurately described, and may be readily adapted for use in commercial hatcheries.

The study of microsatellite markers has shown that some of the existing markers may be readily applied to Cobia aquaculture for progeny testing. However, the suite of seven markers used in this study has not proven adequate for unequivocal determination of pedigrees, and further markers should be added to the existing group. Renshaw, et al. (2005) recommended that 26 of the 35 markers identified in their study may be most suitable tools for use in aquaculture, and additional markers may be selected from this group.

Based on various investigations of Cobia oocyte development in this study, generally, fish that initiated development continued through to maturity (Stages 4 and 5); however, those fish that did not initiate any form of development failed to develop at any level. However, increasing the proportion of individuals reaching this stage has not been achieved by the use of low-dose LHRHa and there is no evidence that low-dose LHRHa induction had any effect on initiating or supporting continued ovarian development in Cobia.

This is the first documented study examining the progressive growth of individuals to identify and determine the extent of sexually dimorphic growth in Cobia. The absence of sexually dimorphic growth supports the findings of a study of wild Cobia

on the east coast of Australia (van der Velde, et al., 2010). Cobia is a widely distributed species, and although considered migratory (Shaffer & Nakamura 1989), there may be population differences between those fish found in the western Pacific compared with those in the Gulf of Texas, where sexually dimorphic growth has been reported. This may explain the contrasting results reported by Shaffer and Nakamura (1989) suggesting female Cobia grow faster than males. Based on the evidence of the current study, Cobia broodstock selection can be based on size through until maturity, without the risk of creating a biased sex ratio. Furthermore, there is no reason to pursue monosex culture of Cobia, unless genetic protection is sought.

The measurement of 11KT in Cobia broodstock will have limited application in a hatchery. The inability to detect males at a small size limits the effectiveness of this technique to optimise the number of broodstock retained. Furthermore the poor correlation between plasma and fin clip 11KT levels means that studies of 11KT will need to refer to plasma in most cases, although fin clips may be suitable for larger, mature fish.

The integration of Cobia into prawn production systems will always rely on the timely supply of fingerlings. In order to achieve this, a high level of control over broodstock maturation and spawning will be required. The current study has demonstrated the effectiveness of environmental manipulation to provide control over Cobia maturation, albeit subject to other factors which may impede normal reproductive development.

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11 Appendix 2

Objective 2: Produce sufficient fingerlings by DAF to enable PRF to on-grow commercial quantities of Cobia for market

Objective 3: Develop pilot-scale Cobia fingerling production by the PRF hatchery

11.1 Methods

A reliable supply of high-quality seed is the basis of any successful aquaculture industry. In the current project, the supply of fingerlings, fish aged approximately 45 days and weighing approximately 5 g, was essential for PRF to develop pond-based production methods, and to provide product for market evaluation. It was also recognised that the long term viability of the industry would also require a commercial hatchery to take up fingerling production, as ongoing fingerling production was not in the remit of the DAF hatchery. To that end, PRF committed to developing their cobia hatchery capability under the auspices of the project.

Broodstock capture and general husbandry

Broodstock Cobia were sourced from the outer reaches of Moreton Bay. 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 treatments of 200ppm formalin for 1 hour 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 to allow fish to be individually identified. The broodstock maturation system at BIRC consists of four 35,000L fibreglass tanks each with a separate enclosed recirculating system consisting of 500µm pre-filter screens, dual 1kW transfer pumps, zeolite media filter, 150W UV steriliser, 13kW heat/chill unit, foam fractionator and moving bed bioreactor. All tanks are fitted with internal bottom drains, and overflow outlets that facilitate the collection of eggs post-spawn. Each tank is fitted with a vinyl cover to exclude natural light and prevent escape. Lighting is provided by two twin 37W fluorescent lights. Fish were fed to satiety 5 days per week on a mixture of squid, prawns and pilchards. Pilchards were supplemented with a vitamin premix . 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. Prophylactic disease treatments were conducted monthly using a bath of 200ppm formalin for 60 minutes.

Oocyte assessment

Broodstock were regularly assessed for reproductive development by ovarian sampling. Ovarian samples were taken by inserting 1mm diameter plastic cannula through the gonopore and approximately 5-10cm into the ovary. Oocyte samples were 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. The method of oocyte assessment is detailed in Appendix 1.

Spawning induction and larval rearing at BIRC

Broodstock identified as being ready to spawn were sedated then injected with 30-50µg/kg LHRHa in a cholesterol pellet (Lee, et al., 1986). Spawning typically occurred within 40 hours of induction. Larvae were reared in semi-intensive green water tank systems for two weeks post hatch. The larvae were then transferred to extensive pond production for weaning and on-growing to approximately 10 g. Fingerlings were freighted by road from BIRC to Pacific Reef Fisheries farm in Ayr. The numbers of fingerlings sent to PRF are shown in Table 1.

Larval transport and larval rearing at PRF hatchery

The provision of larvae to the PRF hatchery was essential to developing Cobia hatchery techniques and experience. Given the cost and logistical constraints of establishing a broodstock facility at PRF within this project, the PRF prawn hatchery at Guthalungra was instead supplied with cobia larvae which were then reared on

site. While the primary aim was to provide staff at the PRF hatchery with experience in Cobia larval rearing, other factors were also considered. Previous transport of newly hatched Cobia larvae from BIRC had been successful but this required the receiving hatchery to undertake the entire larval rearing cycle. Transport of later stage larvae, already weaned onto *Artemia* as a live feed, presented logistical difficulties. However, it would allow the PRF hatchery to commence larval production without the need to culture additional live feed, with *Artemia* already in routine use for prawn larval culture. An additional key consideration in the provision of larvae to the PRF hatchery was to maintain existing hatchery dry-out periods. In general the hatchery is shut down from January to April, to enable facilities to be thoroughly cleaned and dried, and maintenance carried out. The potential windows for Cobia transfer were therefore either in January or April

Larvae were collected from hormone-induced spawnings, conducted as described above and in Appendix 1. Initial trials conducted in 2011 were based on the transfer of mid- and late-stage larvae, which had been moved on from rotifers as a live feed. In April 2011, approximately 20,000 mid-stage larvae were sent 13 days after hatch (dah) by airfreight to Townsville for transfer to the PRF hatchery at Guthalungra (Table 1). Fish were placed in doubled heavy plastic bags and filled with 10L of clean seawater and 20L of pure oxygen. Bags were sealed using heavy duty rubber bands and placed in air transport-approved 40L polystyrene boxes. Given the limited information on the tolerance of advanced Cobia larvae to transport conditions, fish were freighted at several densities to ensure that at least some survived. A total of 5 boxes of larvae were air freighted with a fish density ranging from 2,400/box to 6,600/box. In October 2011 approximately 11,000 late-stage larvae, at 19 dah were also sent to PRF hatchery at Guthalungra (Table 2). Larvae were packed as previously and stocked at approximately 2,500/box.

Based on the equivocal results of transporting mid- to late-stage larvae, it was decided to target the transfer of early larvae. In March 2013 larvae were produced for transport, but were considered to be of too poor quality to transport. This was considered to be due to the late-season spawning, and a January 2014 window was targeted for the next transfer. In February 2014, 50,000 newly hatched larvae were packed and shipped as previously described, at 17,000 per box. (Table 2).

Staff training

Staff transfers were also undertaken as part of the capability development for the PRF hatchery. In January 2012 Tony Maddison and Jessica Henderson of PRF travelled to Bribie to work alongside DAF staff during the spawning season. Tony and Jess got hands-on training and experience in live feed production, broodstock management and larval rearing.

Bastien Finet, PRF Hatchery Manager, visited Bribie in March 2013. He was involved in a Cobia spawning induction, as well as receiving training in live feed production and broodstock handling.

11.2 Results

Spawning induction and larval rearing at Bribie

Table 1 Fingerling transfers from Bribie Island Research Centre to Pacific Reef Fisheries, 2011-2014.

Transport date	Number
July 2011	12,000
November 2011	23,000
March 2012	49,000
January 2013	38,000
December 2013	20,000

In total, 142,000 fingerlings were transferred to PRF over the course of the project. This comprised 84,000 in 2011/12, 38,000 in 2012/13 and 20,000 in 2013/14 (Table 1). This is less than the anticipated target of 160,000 over the course of the project. The decline in the 2013/14 fingerling production was a direct result of poor reproductive health and performance of Cobia broodstock at BIRC. This has been attributed to the high incidence of intersex individuals and reproductive malformations in these cohorts, discussed in Appendix 1. These broodstock were the first cohorts of F1 stocks that were able to be crossed, as each cohort had been produced from different wild-caught parents. The reasons for this intersex condition is not yet clear, and will be the subject of ongoing research.

Substantial mortality (>20%) occurred within some ponds of fingerlings in 2011/12 due to Amyloodinium infection. This led to the development of more rigorous on-farm water quality and heath monitoring methods and to the use of a nursery cage system for the 2012/13 and subsequent seasons. Nursery cages allowed for more detailed fingerling observation and sampling, and if necessary, treatment.

Larval transport and larval rearing at PRF hatchery

Table 2. Cobia larval transport from BIRC to PRF.

Spawning date	Transport date	Number	Age (days after hatch)	Fingerlings produced
15/04/2011	28/04/2011	20,000	13	1000
22/09/2011	12/10/2011	11,000	19	0
20/02/2014	21/02/2014	50,000	1	2000

Approximately 50% of the mid-stage larvae survived transport, with evidence of higher mortality in the more densely stocked boxes. Hatchery staff commenced larval rearing according to the methods based on those outlined above. Approximately 1000 fingerlings were produced by late May 2011. No late-stage larvae survived the transport in October 2011.

The early stage larvae transferred in February 2014 had high survival, and were reared at the PRF hatchery using methods based on those outlined above. A total of approximately 2000 fingerlings were produced from the 50,000 larvae. Larval development proceeded normally, with no evidence of physiological or developmental abnormalities.

11.3 Discussion

The provision of over 140,000 fingerlings to PRF has enabled the company to make considerable advances in pond-based Cobia aquaculture, as well as developing commercial markets for the product. Steady improvements in performance resulting from improved technologies, farm practices and staff experience have resulted in harvests of up to 20T per hectare and value of production nearing $1M.

There has been significant success in rearing Cobia through from newly hatched larvae to weaned fingerlings at the PRF hatchery. The Cobia larval rearing practices used at BIRC have mostly translated well to the PRF hatchery, with some modifications. The timing of Cobia spawning has also proven to be adaptable to suit the production cycle of a prawn hatchery. However, it is recommended to spawn the Cobia at the cessation of the prawn spawning season, rather than prior to its commencement, as broodstock and egg quality are generally higher in January/February than March/April.

Although both early- and mid-stage larvae were successfully transportable, early-stage larvae can be transported more cost effectively and successfully than later stages. A number of factors may have contributed to the mortality event during the transport of late-stage larvae. The most likely cause is adverse water quality conditions as a result of the high fish density during transfer. The negative effect on Cobia survival caused by suboptimal water quality is compounded due to Cobia larvae undergoing metamorphosis from 15-25 dah. During metamorphosis larvae undergo significant changes within body systems, including changing from cutaneous to gill respiration, as well as circulatory and digestive system developments. The impact of this major transformation is further compounded in aquaculture as it also coincides with the beginning of weaning onto manufactured diets. Such physiological stressors may be reason that the larvae are unable to withstand the physical and environmental challenges that arise during a high-density transfer. In contrast, an optimal physical environment can be more readily maintained as newly hatched larvae are sustained on endogenous energy reserves and have a significantly low biomass. It may be possible to improve survival of advanced larvae by further reducing stocking density for transport. However, in doing so the logistical costs would significantly outweigh the production gains, especially when considering that the long term goal is to close the life cycle of Cobia within the PRF hatchery.

11.4 References

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

Appendix 3

Objective 4: Formulate diets designed to meet the specific nutritional and energetic requirements of Cobia

11.5 Methods

The development of specialised feeds for a species is likely the ideal outcome for many production parameters. However, in a practical sense, the feed options for emerging species will be limited by available feeds from aquafeed suppliers. This project aimed to resolve this issue by seeking to facilitate information transfer from an established project which was investigating improved feeds for a variety of species, to the Australian aquafeed sector. Unfortunately, information was not readily forthcoming to this project, so an alternative approach was developed. A trial was designed to quantify the performance of cultured Cobia marine finfish diets produced by Ridley Aquafeeds: “Marine Float” and “Pelagica”. Marine Float is primarily formulated as a floating Barramundi diet suited for tank and pond culture. This diet had been used for pond and tank production of Cobia at BIRC and PRF since 2008. In 2011 and 2012 some issues of poorer performance of cobia were recorded at both PRF and BIRC. Pelagica was developed to provide a diet better suited to pelagic marine species, and was formulated to contain elevated levels of taurine and amino acids shown to be essential in diets for several species, including Cobia (Lunger, et al., 2007; Watson, et al., 2013) and *Seriola* spp. (Takagi, et al., 2008; Khaoian, et al., 2014).

The trial was established in February 2013. Experimental units consisted of 1000L tanks fitted with a centre drain and a single weighted airstone. The tanks were arranged in two blocks of four tanks, with two replicates of each diet contained in each block. Each tank was stocked with fifteen 200g Cobia and all fish were fitted with a PIT tag. Tanks were arranged in two groups of four tanks, and within each group, two tanks were randomly assigned to receive Pelagica and two the Marine Float.

Fish were initially fed at 2% body weight (BW) per day, with feeding quantities incremented weekly. All individuals were weighed monthly. Each tank was provided with flow-through sea water set at approximately 5 L/min for the first 14 weeks of the trial. Reduced temperatures resulted in a decrease in appetite, at which point the fish were moved to a similar set of 1000L tanks connected to a temperature-controlled recirculating aquaculture system (RAS). The fish remained in the RAS for 15 weeks before being returned to the original flow-through system. As the biomass increased in the flow-through tanks, the exchange rate was increased to a maximum of 12 L/min. There was also a noticeable difference in feeding response from late August (T6), from which point all fish were fed to satiety and this continued until the end of the trial.

Pellet size for each diet started at 4mm and increased for each diet in concert according to the growth of the fish to a final size of 10mm. The indicative composition of the diets is shown in Table 1.

	Marine Float[1]	**Pelagica**[2]
Crude protein	45%	49%
Crude fat	20%	13%
Fibre	2%	2.5%

Table 1. indicative composition of Marine Float and Pelagica diets (for pellet sizes 8mm and above).
[1]source: https://www.primo.net.au/shop/Ridley-AquaFeed/marine-float-range-manufactured-by-ridley-aquafeed; [2]source: manufacturer’s labelling

The weights of individual fish were recorded upon stocking and at approximately four-week intervals. Average weight, specific growth rate (SGR) and food conversion ratio (FCR) were calculated and analysed. SGR was calculated as follows (Priestley, et al., 2006):

$$SGR\ (\%/day) = \frac{\ln(W_f) - \ln(W_i)}{\text{time (days)}} \times 100$$

where W_f and W_i were the final and initial wet weights of the fish, respectively. FCR, also termed economic FCR, was calculated as biomass increase divided by the total amount of food added (Tacon, 2004). Mortalities were not replaced in the tanks and not considered in the calculation of FCR.

Water quality samples were taken from one tank in each block on a weekly basis to measure dissolved oxygen, salinity, pH and total ammonious nitrogen (TAN). Temperature observations of the flow-through seawater and the RAS were recorded six days per week.

Statistical Analysis

Average weight, SGR and survival data were subjected to randomised block analyses of variance (ANOVA). All analyses were conducted in GenStat (VSN-International, 2007).

11.6 Results

The water temperature was moderately influenced by cool ambient temperatures in May and June (Figure 1); following transfer to a recirculating system, the temperature remained greater than 20°C. In previous seasons, Cobia has been reared at BIRC and experienced a similar temperature regime.

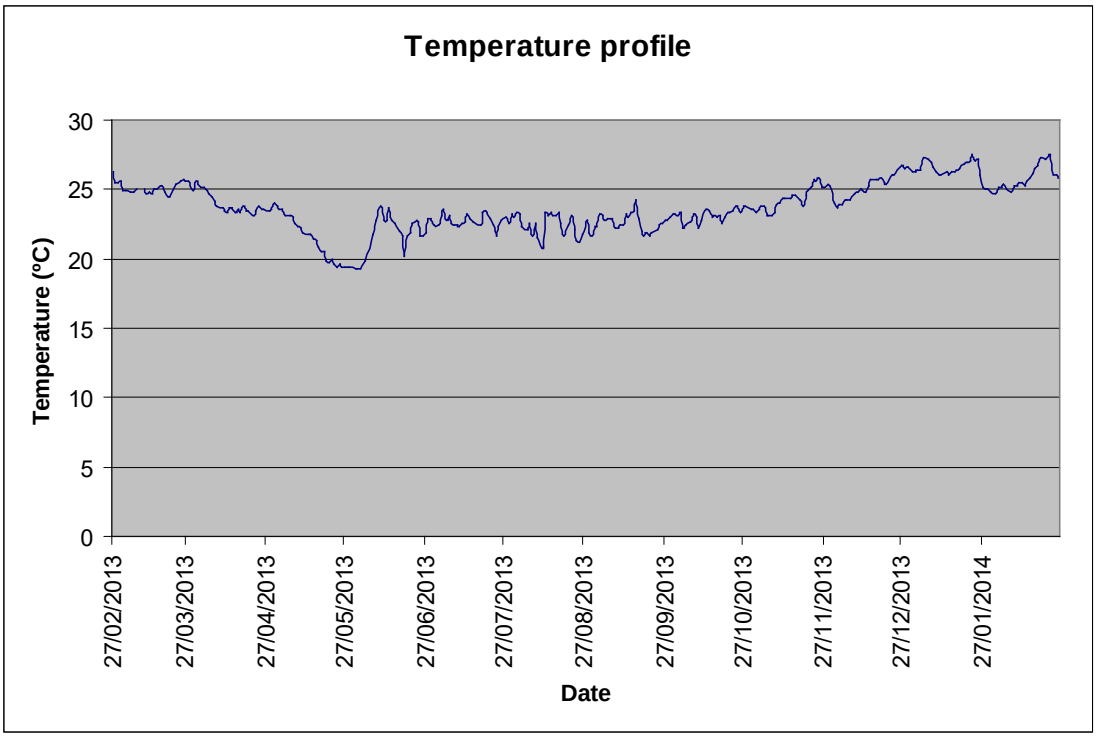

Figure 1. Temperature profile of feed trial tanks between February 2013 and February 2014.

Both mean weight and survival were similar for both groups until October (T8) when significant differences ($p<0.05$) in both factors were found. These significant differences continued until the end of the trial as both weight and survival continued to diverge (Figures 2 and 3).

Similarly, there was no significant difference in growth rate from February to September (T7) between the two diets, but the growth rate of fish fed Pelagica was significantly higher than those fed Marine Float from October 2013 (T8, $p=0.02$) to the end of the trial in March 2014 (Figure 4).

The mean (±SE) feed conversion ratio at the conclusion of the trial differed significantly between groups, and was 2.27±0.16 for fish fed Pelagica, and 13.94±4.65 for fish fed Marine Float. There is clearly a strong influence of differences in mortality levels on these results, as the calculation of economic FCR does not include the biomass of mortalities. The reduction of feeding response and increased mortality observed in the fish fed Marine Float was the basis for the change from feeding a fixed ration to feeding to satiety from August (T6).

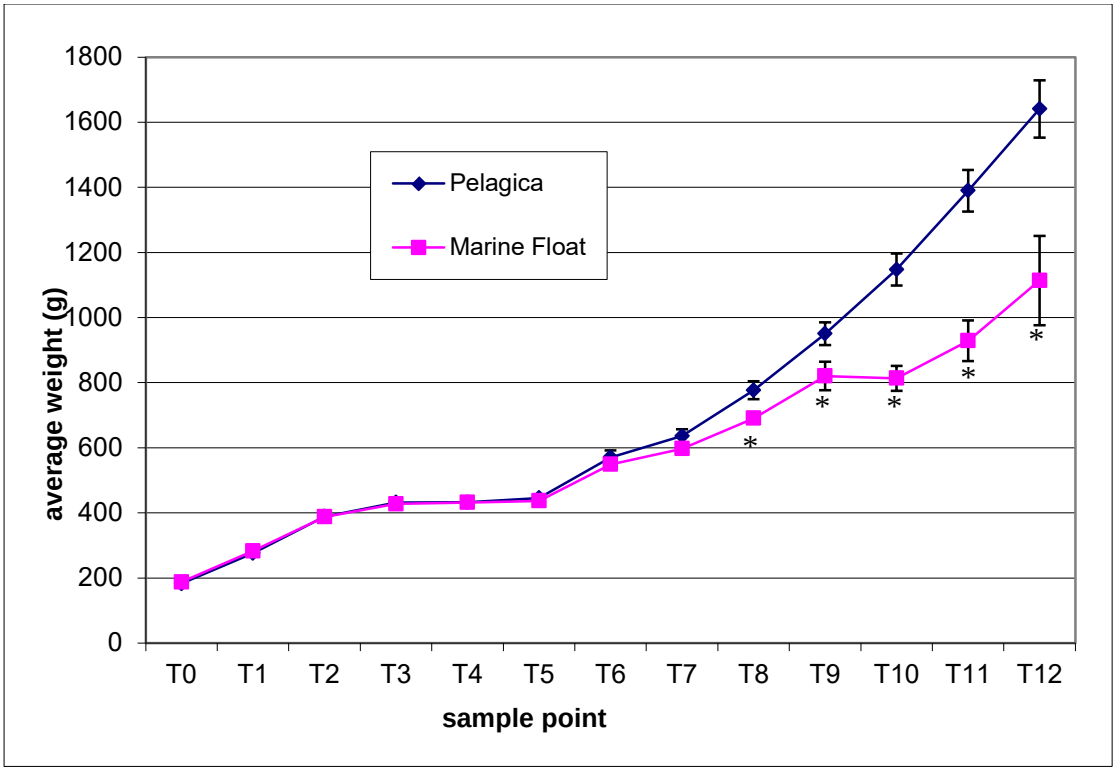

Figure 2. Mean weight of Cobia fed Pelagica and Marine Float (± SE) at 4-week intervals starting from 27 February 2013 (T0). Asterisks indicate a significant difference ($p<0.05$) between Pelagica and Marine Float mean weight at the sample point.

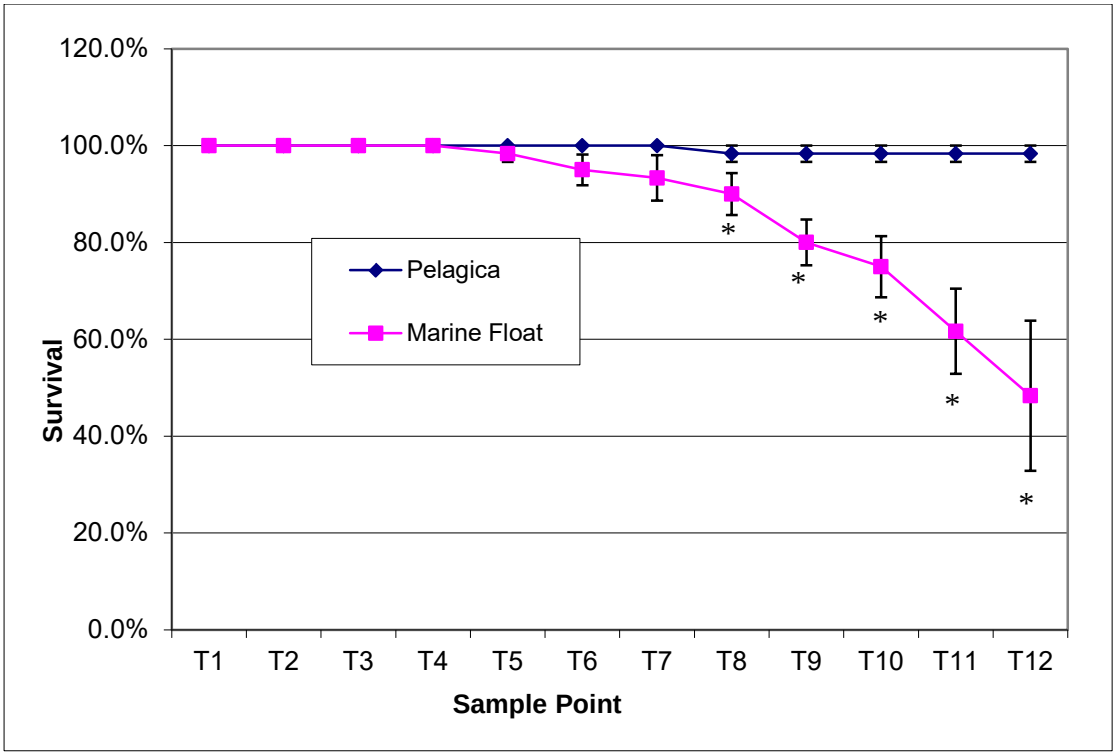

Figure 3. Mean percent survival of Cobia fed Pelagica and Marine Float (± SE) at 4-week intervals starting from 27 February 2013 (T0). Asterisks indicate a significant difference ($p<0.05$) between Pelagica and Marine Float mean survival at the sample point.

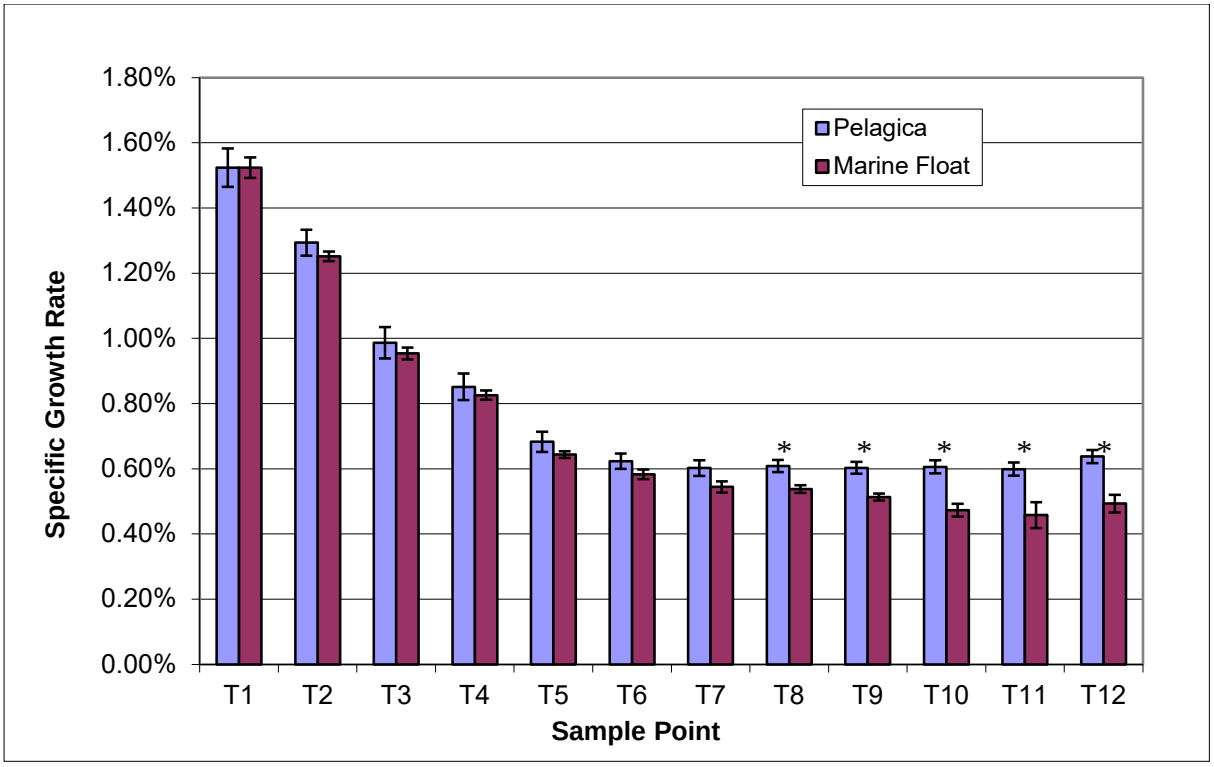

Figure 4. Specific growth rate of Cobia fed Pelagica and Marine Float (± SE) at 4-week intervals starting from 17 February 2013 (T0). Asterisks indicate a significant difference between Pelagica and Marine Float SGR at the sample point.

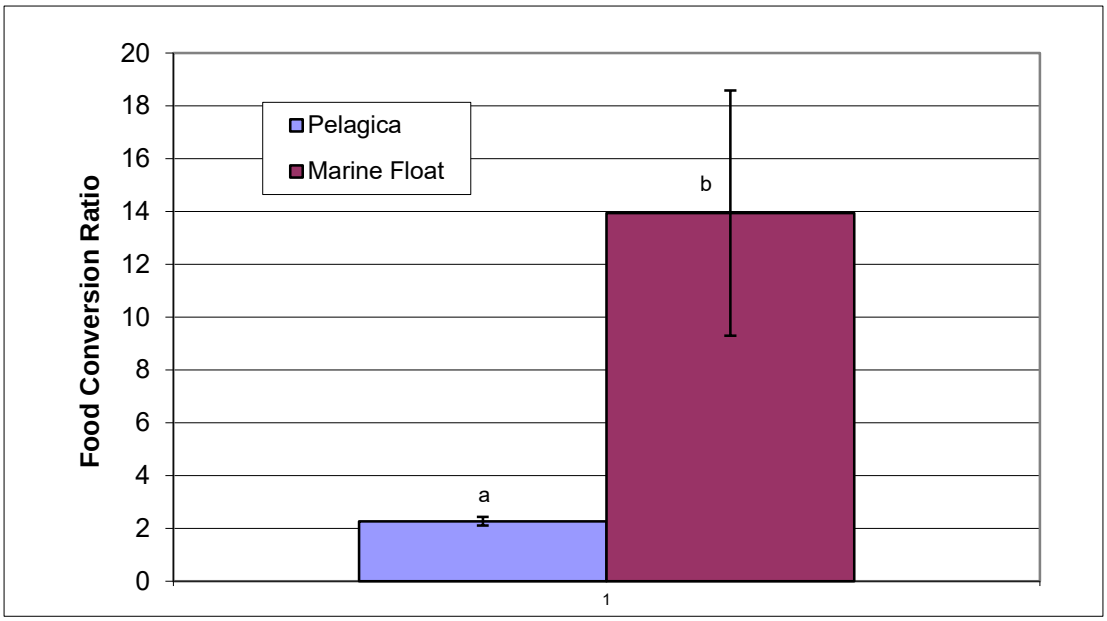

Figure 5. Overall feed conversion ratio of Cobia fed Pelagica or Marine Float (± SE) for 13 months. Different subscripts indicate significant differences between means.

11.7 Discussion

The present study has demonstrated some key factors in relation to Cobia feeds and husbandry. Pelagica clearly performed better than Marine Float, in terms of both growth and, importantly, survival. The improvements in growth occurred in spite of the fact that the fish fed on Pelagica were reared at a higher density than those fed Marine Float for the bulk of the study.

Interestingly, there were no significant differences between the performance parameters of fish fed either Pelagica or Marine Float feeds for approximately seven months. During that period the fish had a growth increment of approximately 300%. This is certainly within the parameters of time and growth increment that nutritional studies on Cobia (e.g.Chou, et al., 2001; Trushenski, et al., 2012; Watson, et al., 2013) use to draw their conclusions. The results of the present study suggest that basing conclusions on a negative result at that time may not always be the correct approach.

The diets do differ in their proximate composition, in particular in the ratio of protein to energy. The Marine Float is a higher energy diet with lower protein in comparison to Pelagica. Studies on juvenile Cobia have determined optimum levels of dietary protein and lipid to be 45 and 5-15% respectively (Chou, et al., 2001; Fraser & Davies, 2009), and the results of the present study are consistent with this, for Cobia up to 1.5kg.

One additional feature of the Pelagica diet is the supplementation with taurine. This has been shown to be an important nutrient in the diets of marine fish including Cobia (Lunger, et al., 2007). Taurine supplementation is of greatest importance when plant proteins are used to replace fish proteins in diets (Lunger, et al., 2007; Watson, et al., 2013; Khaoian, et al., 2014) and this is likely to be exacerbated in fast-growing species such as Cobia or Yellowtail Kingfish.

This study has demonstrated the importance of adequate nutrition for Cobia production. The research base on specific nutritional requirements has grown rapidly over recent years. As such studies continue to address specific issues, better performing diets should be the result. The Australian Cobia industry will take some time to develop the critical mass needed to drive feed production; however the industry is fortunate in having feed companies that are generally responsive to industry's needs and requirements.

11.8 References

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12 Appendix 4

Objective 5: Develop and field test new farmed Cobia product(s) with high market acceptance

(double click the image below to open file)

13 Appendix 5

Media outputs

Videos:

- DAF staff at Bribie Island Research Centre were featured in a YouTube video, produced as part of a series on fishing and fisheries issues by local fishing media identity, Dave “Nugget” Downie: (http://www.youtube.com/watch?v=YOAT7t7wHGY). The video outlines achievements in past and present cobia research including aims and achievements of the current project.

- DAF staff at BIRC were featured in an episode of Scope TV, a children’s science show, presented by CSIRO scientist, Dr Rob Bell. The program, which aired on 4th October 2012, featured aquaculture, and included a segment on the present cobia project: http://ten.com.au/video-player.htm?movideo_p=41450

Industry publication:

- Preston, N, Glencross, B & Lee, P (2012) Cooperation grows Australian aquaculture: Projects include feed technology, cobia culture. Global Aquaculture Advocate 15(2): 76-79.

Conference presentations:

- Dutney, L., Callcott, B., Moloney, J,. Borchert, T., Nixon, D., Lee, P. (2014) Towards the commercial production of cobia in Australia – an update. Presentation at World Aquaculture 2014, Adelaide June 2014.
- Dutney, L., Elizur, A., Lee, P. (2014) Investigations into sexually dimorphic growth and early identification of gender in cobia. Presentation at World Aquaculture 2014, Adelaide June 2014.