Studies towards advancing reproductive development in giant grouper (Epinephelus lanceolatus Bloch) using recombinant hormone manipulation

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Abstract

Groupers are an economically significant commodity owing to the major portion they occupy in the live reef fish trade (LRFT). The giant grouper *E. lanceolatus* is one of the most sought-after grouper species in the LRFT, and is also a favoured culture species due to its fast growth rate. Captive breeding of giant grouper, however, is challenging due to its large size at sexual maturation, where females mature at 20 kg while males can reach more than 100 kg body weight. Thus, handling is complicated and operational cost is more expensive due to larger facility and additional food and labour required. Giant grouper matures at a late age (~4 years) hence a long period of broodstock conditioning is required before spawning is achieved. In addition, being protogynous hermaphrodites, initially maturing as female and then changing into male, securing broodstock of both sexes is challenging. This thesis is aimed at inducing early sexual maturation in giant grouper using recombinant hormone manipulation so that captive breeding could be done with a younger and smaller broodstock.

In the first part of this thesis, the role of follicle stimulating hormone (FSH) in the reproductive development of groupers was examined. FSH is a pituitary glycoprotein hormone that is involved in the early stages of oogenesis and spermatogenesis as previously shown in gonochoristic fish species. A recombinant giant grouper follicle stimulating hormone (rggFSH) was produced using the yeast *Pichia pastoris* expression system. *In vitro* homologous receptor assay and ovarian tissue assay confirmed its receptor binding capacity and steroidogenic potency. Its effects on gonadal development was then examined using sexually immature tiger grouper *E. fuscoguttatus* injected weekly over a period of 38 weeks. Results have shown that rggFSH treatment stimulated oocyte growth and development but only up to cortical alveolar stage, after which it promoted female to male sex reversal. The rggFSH appeared to have initiated sex reversal via a steroid-independent pathway because androgenic activity was suppressed in the treated fish. Although the prolonged administration of rggFSH led to the onset of sex reversal, spermatogonial cells remained largely undifferentiated. This study has shown that rggFSH can be used to advance reproductive development and transition to male in immature grouper.

In the second part of this thesis, an oral delivery of FSH was assessed as an alternative administration method to induce early sexual maturation in grouper. A DNA expression construct encoding giant grouper FSH (pFSH) was synthesized. Single oral administration of liposome- and chitosan-encapsulated pFSH on sexually immature tiger grouper resulted in a significant increase in plasma FSH protein levels, indicating successful passage of pFSH through the digestive system and subsequent expression *in vivo*. Weekly oral administration of pFSH to sexually immature tiger grouper for 38 weeks initially resulted in increased oocyte size and more advanced developmental stage. Similar to the result in the first study, pFSH treatment promoted oocyte development only up to the cortical alveolar stage. Prolonged administration initiated sex reversal, which has also remained at the early transition phase. This study showed that pFSH can be orally delivered and yield the same effect as intramuscular injection of the recombinant FSH protein.

Results of this research demonstrates that in a protogynous hermaphrodite, FSH modulates both the process of sexual maturation and sex reversal. In the immature grouper, FSH promotes oogenesis up to the cortical alveolar stage, and then initiates sex reversal thereafter. These developmental changes can be induced either by intramuscular administration of recombinant FSH protein or through oral administration of encapsulated plasmid FSH. Knowledge obtained in this study can be applied towards obtaining younger grouper broodstock of both sexes for captive breeding.

Declaration of Originality

I hereby declare that this thesis is my own original work and has not been submitted in any form for another degree or diploma at any university or other institute of tertiary education. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made.

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The Lord is the strength of my life; of whom shall I be afraid?"

-Psalm of David 27:1

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List of Gene Acronyms

amh	Anti-Mullerian Hormone
bmp15	Bone morphogenetic protein 15
cog5	Conserved oligomeric Golgi complex subunit 5
cyp11b	Cytochrome P450 11B1; Steroid 11beta-hydroxylase
cyp19a1a	Cytochrome P450 19A1; Aromatase
dmrt1	Doublesex and mab-3 related transcription factor 1
figla	Factor in the germline alpha
foxl2	Forkhead box L2
fsh	Follicle stimulating hormone
fshr	Follicle stimulating hormone receptor
igf3	Insulin-like growth factor 3
lh	Luteinizing hormone
lhr	Luteinizing hormone receptor
sox9	Sex determine region Y box 9

Chapter 1 **General introduction and literature review**

1.1. Grouper aquaculture, fishery and biology

1.1.2. Grouper aquaculture and fishery

Groupers represent one of the most economically valuable fish in the market today occupying a major portion of the live reef fish trade (LRFT) where their annual trade value is estimated at over 100 Million U.S. Dollars (Pierre *et al*., 2008; Yin, 2014). Hong Kong, the main market of LRFT (Lee and Sadovy, 1998), imports live groupers mostly coming from Indo-Pacific countries including Indonesia, Thailand, the Philippines, Malaysia, and Taiwan (Pierre *et al*., 2008; Yin, 2014). Giant grouper (*Epinephelus lanceolatus*), Humpback grouper (*Cromileptes altivelis*), Red grouper (*Epinephelus akaara*) and Coral trouts (*Plectropomus* spp.) are among the prime species of grouper sought after in the LRFT (Lau and Parry Jones, 1999). The demand for these groupers persistently grew over the past decade (Sadovy de Mitcheson *et al*., 2013; Sadovy *et al*., 2003) driving the heavy exploitation of wild grouper stocks (Kirubasankar *et al*., 2013; Manojkumar, 2005; Yulianto *et al*., 2015b) and heightening the need to increase aquaculture production.

Among the cultured groupers, giant grouper has particularly gained much interest from fish farmers due to its superior growth rate, achieving the commercial size of 0.6 to 1.0 kg within six months compared to other grouper species which can take more than a year (Ho *et al*., 1997; Pierre *et al*., 2008; Sadovy *et al*., 2003). Aquaculture of giant groupers is currently being done in Taiwan (Liao *et al*., 2001), Hawaii (García-Ortega *et al*., 2016), Malaysia, Indonesia, Thailand, and Australia although production rate has remained relatively limited and unstable as in other cultured groupers (Liao *et al*., 2001; Marte, 2003).

Poor captive breeding technology for groupers accounts for a major gap in giant grouper aquaculture (Kiriyakit *et al*., 2011; Marte, 2003). The delayed sexual maturation of giant groupers, which can take three to four years, prolongs the brood stock conditioning period before spawning occurs. In addition, because of giant grouper's fast growth rate, size at sexual maturity is already about 20 kg. Their large size complicates handling during spawning induction, inflicting physical stress on the fish that can result in depreciated reproductive

performance (Schreck *et al*., 2001). Their large size also requires larger breeding facilities, more food, and additional labor, all of which ultimately correspond to increased financial input.

Although the total world production pattern of all groupers in past years has grown, majority of the produce is provided by wild capture fisheries while aquaculture contributes only about 20% (Tupper and Sheriff, 2008) with production rate yet to be stabilized (Rimmer *et al*., 2013). Groupers have been selectively overfished and their wild populations have been critically reduced in most countries involved in the export of live reef fish (Kirubasankar *et al*., 2013; Manojkumar, 2005; Yulianto *et al*., 2015a). The International Union for Conservation of Nature (IUCN) have included the giant grouper and other grouper species in the list of threatened fish species (Shuk Man and Wai Chuen, 2006). Hence, addressing the issue of captive breeding of giant grouper is crucial to the sustainability of both its aquaculture and fishery. It will not only provide seed for subsequent commercial production but also provide quality seed that can be used to enhance wild stocks.

1.1.3. General and reproductive biology of giant grouper

Giant groupers thrive in the tropical and sub-tropical waters of the Indo-Pacific region and, like most groupers, are associated with large coral reef areas, caves and wrecks (Madduppa *et al*., 2012). The giant grouper or *Epinephelus lanceolatus* Bloch 1793 (Serranidae) is one of the two largest known species of grouper (Heemstra and Randall, 1993). It can grow up to three kg in its first year (Sadovy *et al*., 2003) and can reach a maximum body size of 400 kg (Heemstra and Randall, 1993). They are characteristically sedentary and their diurnal movement is more or less restricted within their established territory (Sadovy de Mitcheson *et al*., 2013). They often settle at the sand bottom or hide in crevices from where they prey on fish or crustaceans.

During spawning season, giant groupers form aggregations in specific sites where they spawn communally. The disparity in female and male size dimorphism largely influence the sex ratio in the spawning aggregations (Sadovy *et al*., 1994). The aggregating spawners however have become the target of fishermen putting the whole grouper population at risk of recruitment by overfishing (De Mitcheson *et al*., 2008; Sala *et al*., 2001).

Like other species of groupers, giant groupers follow a protogynous hermaphroditic reproductive strategy where all individuals mature first as female and then, after a certain time, they change into male. In the Philippines and subtropical region of Australia, giant groupers attain sexual maturity at about 20 kg body weight and age of three to four years.

1.2. Reproductive physiology of fish

1.2.1. Brain-pituitary-gonad axis

Reproduction in fish is tightly regulated by the Brain-Pituitary-Gonad (BPG) axis through an endocrine mechanism that is highly conserved across vertebrates (Sower *et al*., 2009). The BPG axis involves coordinated endocrine effects in three organs – brain, pituitary gland and gonad – which together directs the major reproductive processes including steroidogenesis, spermatogenesis, vitellogenesis and gonadal maturation (reviewed by Taranger *et al*., 2010; Yaron and Levavi-Sivan, 2011).

The action of BPG axis starts with the synthesis of Gonadotropin-Releasing Hormone (GnRH) by the GnRH neurons distributed in the forebrain area including the ventral telencephalon, preoptic area and hypothalamus (González-Martínez *et al*., 2002). These neurons project from the brain and through the pituitary stalk innervating the pituitary adenohypophysis (Golan *et al*., 2015) where gonadotropic cells producing Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) are localized (Murata *et al*., 2012; Weltzien *et al*., 2004). GnRH input prompts the synthesis and secretion of gonadotropins into the circulation allowing interaction with their cognate receptors (FSH-R and LH-R), bound on the somatic cell layer of the gonad. FSH-R and LH-R are expressed in the granulosa cells in the ovary and in the Leydig and sertoli cells in the testis (Alam *et al*., 2010; Andersson *et al*., 2009; Miwa *et al*., 1994).

Activation of gonadotropin receptors induces steroid hormone production, primarily testosterone (T), which in females is absorbed by the granulosa cells where it is converted to estradiol-17β (E_2) by the enzyme aromatase (P450arom). In males, T is hydroxylated by P450-11β and oxidized by 11β-hydroxysteroid dehydrogenase (11β-HSD) converting it into 11-ketotestosterone (11-KT), the potent androgen in fish (Yaron and Levavi-Sivan, 2011). Production of E_2 and 11-KT is elevated during early stages of oogenesis and spermatogenesis, respectively, under the regulation of FSH. During gonadal maturation, the maturation

inducing steroid (MIS) (specifically 17,20β-dihydroxy-4-pregnen-3-one (DHP)) is produced by the gonad under the control of LH (Suzuki *et al*., 1988c).

1.2.2. Puberty

Puberty in fish is the process by which full reproductive competence is acquired allowing the gonad to undergo the first and complete reproductive cycle, starting from the entrance of oogonia and spermatogonia to meiosis and culminating at the production of the first batch of mature gametes (ova or spermatozoa) capable of fertilization (Okuzawa, 2002; Zanuy *et al*., 2001). Initiation of pubertal development requires fully functional BPG axis which includes expression of the receptors and their respective ligands in well-developed organs (Okuzawa, 2002).

Oogonia and spermatogonia are formed at the culmination of sex differentiation though they are mitotically-arrested (Murata *et al*., 2009). Elevated level of plasma DHP directs subpopulations of oogonia and spermatogonia to enter a meiotic division pathway leading to the formation of primary oocytes or spermatocytes marking the onset of puberty (Miura *et al*., 2007)

In females, initiation of meiosis is followed by the movement of pre-follicle cells around each oogonium creating a somatic cell layer known as granulosa. Outside, another somatic cell layer is formed consisting of theca cells where vasculature later develops. The oogonium and the somatic cell layers constitute the ovarian follicle also known as the primary oocyte. At the same time, vitellogenin, a yolk-precursor protein, is produced in the liver in response to estrogenic stimulation, which is regulated by FSH. Oocytes grow by accumulating vitellogenin. In the presence of social and hormonal cues, oocytes proceed to final maturation where the membrane of the germinal vesicle breaks down and oocytes absorb water, thus gaining a rapid increase in size. This step is accompanied by increasing levels of LH and DHP. The somatic follicle layer eventually ruptures releasing the ovum, an oocyte denuded of somatic cells (Lubzens *et al*., 2010).

In males, a supporting somatic cell layer, consisting of Sertoli cells, surround a subpopulation of spermatogonia while maintaining physical contact with each cell (Schulz *et al*., 2010). Spermatogonial cells within a subpopulation entering meiosis are interconnected by a

cytoplasmic bridge ensuring synchronized development (Schulz *et al*., 2010). Together, they undergo the following developmental stages: spermatogonium type A, spermatogonium type B, spermatocyte, spermatid and spermatozoa (Schulz and Nóbrega, 2011). Plasma levels of 11-KT and T increases gradually as spermatogenesis progresses until just before spawning, where a spike in DHP takes place (Schulz and Nóbrega, 2011).

The timing of the onset of puberty is species-specific and often sex-specific (Okuzawa, 2002; Zanuy *et al*., 2001). To date, the physiological mechanism that ultimately permits fish to advance into puberty is yet to be fully elucidated. Nonetheless, it is recognized that puberty is a product of a complex interplay between environmental and endogenous factors (Carrillo *et al*., 2009). Photoperiod and temperature are the primary environmental signals that strongly influence fish reproductive development (Cowan *et al*., 2012; Hermelink *et al*., 2011; Kanemaru *et al*., 2012). On the other hand, FSH along with the neuropeptide Kisspeptin, the gonadotropin releasing hormone and the sex steroid hormones are the endogenous factors holding critical roles in the initiation of puberty in fish (Carrillo *et al*., 2009).

1.2.3. Gonadotropins

The fish pituitary has two types of gonadotropic cells that produce two distinct gonadotropins, FSH and LH. The existence and functional characteristics of the two gonadotropins were first discovered and established in mammals (Bousfield *et al*., 1996)). In fish, it was initially difficult to prove the duality of gonadotropins until two forms of gonadotropins, named GTHI and GTHII, were chemically characterized in salmon (Suzuki *et al*., 1988a, 1988b, 1988c). These piscine gonadotropins resembled the biochemical and functional characteristics of their mammalian counterparts (Prat *et al*., 1996) hence the terms FSH and LH were eventually adopted (Li and Ford, 1998; Quérat *et al*., 2000).

Fish gonadotropins are heterodimeric glycoproteins consisting of non-covalently bound α and β subunits (Boime and Ben-Menahem, 1998; Suzuki *et al*., 1988b), each coded by separate genes (Pierce and Parsons, 1981). Within a species, FSH and LH share a common α subunit (GTHα) whereas the β subunit (FSHβ and LHβ) is hormone-specific, responsible for the unique physiological function of each gonadotropin through interaction with their specific receptors (Holdcraft and Braun, 2004; Levavi-Sivan *et al*., 2010). Binding of gonadotropins with their

receptors activates at least two intracellular signalling pathways including adenylate cyclase and phospholipase C, which in turn stimulate steroid production by activating the transcription of steroidogenic enzymes (Levavi-Sivan *et al*., 2010; Walker and Cheng, 2005; Yan *et al*., 2012).

During early stages of oogenesis, FSH regulates the expression of E_2, thereby controlling vitellogenesis (Yoshiura *et al*., 1999). FSH also enhances the capability of oocytes to take up vitellogenins (Tyler *et al*., 1991). In mummichog *Fundulus heteroclitus*, LH is co-expressed with FSH towards the end of vitellogenesis suggesting their complementary regulatory function at this stage (Shimizu *et al*., 2008). LH then regulates oocyte maturation and ovulation by stimulating the production of MIH. The timing of FSH and LH functional roles is also reflected in the appearance of FSH- and LH-producing cells and the expression of FSH-R and LH-R during early and late stages of ovarian development, respectively (Shimizu *et al*., 2008). This sequential expression of FSH and LH, and their cognate receptors is well pronounced in synchronous fish such as salmonids while the expression pattern is more complex in asynchronous fish such as groupers wherein several batches of oocytes simultaneously develop in the ovary (Lubzens *et al*., 2010).

The same temporal expression pattern of gonadotropins can be observed in males wherein FSH induces T and 11-KT synthesis directing spermatocyte development while LH incudes MIH synthesis hence promoting spermiation (Mazón *et al*., 2013; Ogiwara *et al*., 2013; Yoshiura *et al*., 1999). In sex-changing fish such as groupers, after female sexuality has been assumed for some time, FSH shifts from stimulating E_2 production to promoting 11-KT production causing oocyte degeneration and ovarian restructuring into testis (Kobayashi *et al*., 2010). Recent studies in European sea bass *Dicentrarchus labrax* (Moles *et al*., 2007), Medaka *Oryzias latipesI* (Murozumi *et al*., 2014) and zebrafish (Zhang *et al*., 2015) have implicated that FSH is more important in fish ovarian development than in testicular development.

1.3. Control of fish reproduction using gonadotropins

1.3.2. Fish recombinant gonadotropins

Earlier studies investigating functional characteristics of fish gonadotropins have been limited by the insufficient quantity of purified gonadotropin hormones. Initially, gonadotropins were

purified from fish pituitaries, a method that requires large number of fish and significant amount of time and labor (Levavi-Sivan *et al*., 2008; Slnha, 1971). The quality of pituitary extracts can be highly variable and prone to contamination with other pituitary hormones. The development of recombinant protein technology has allowed production of large amounts of pure gonadotropins (Aizen et al., 2016; Levavi-Sivan et al., 2008; Mazón *et al*., 2015; Meiri *et al*., 2000).

Recombinant proteins are made by transfecting DNA fragments coding for the desired protein into a biological expression system that can easily be manipulated in the laboratory in order to express the gene and consequently producing the protein of interest (Byrne, 2015; Fothergill-Gilmore, 1993). There are a number of biological expression systems available today including *E. coli,* mammalian and insect cell lines and the yeasts *Pichia pastoris* and *Saccharomyces cerevisiae* (Gräslund *et al*., 2008). For relatively large and dimeric proteins such as the gonadotropins, expression systems are limited to mammalian cells, insect cells, and yeast cells which are capable of accommodating large DNA sequence insert and perform necessary post-translational modifications (Wurm, 2004).

The first attempt to produce fish recombinant gonadotropin was that of common carp (*Cyprinus carpio*) GTHα produced in insect cells using baculovirus (Huang *et al*., 1991). The recombinant product stimulated testosterone production in gonadal (testis) tissue fragments proving its bioactivity (Huang *et al*., 1991). Succeeding work on recombinant fish gonadotropin produced single chain FSH and LH of Sea bream (*Sparus aurata*) by linking the beta with the alpha sequence (Meiri *et al*., 2000). Both recombinant gonadotropins elicited *in vitro* biological response. Further studies have demonstrated the production of bioactive fish recombinant gonadotropins in other species such as Channel catfish (*Ictalurus punctatal*) (Zmora *et al*., 2003), African catfish (*Clarias gariepinus*) (Vischer *et al*., 2003), Japanese eel (*Anguilla japonica*) (Kamei *et al*., 2003), Tilapia (*Oreochromis niloticus*) (Kasuto and Levavi-Sivan, 2005), and Manchurian trout (*Brachymystax lenok*) (Choi *et al*., 2005). However, the biological activities of these recombinant gonadotropins have only been tested *in vitro*.

Some studies have been carried out testing the biological activity of recombinant FSH *in-vivo*. In Goldfish (*Carassius auratus*), intraperitoneally administrated rFSH induced spermiation in males while LH induced ovulation in females (Kobayashi *et al*., 2006, 2003). In orange-spotted

grouper (*E. coioides*), intraperitoneal injection of rLH upregulated gene expression of both brain and gonad aromatase concurrent with increased sex steroid production (Cui *et al*., 2007). Immature orange-spotted grouper, after receiving intraperitoneal injection of rFSH, had increased T and E_2 circulating levels and developed primary oocytes (Chen *et al*., 2012). Japanese eel (*Anguilla japonica*) rFSH hastened oocyte growth in E_2 feminized Japanese eel (Kim *et al*., 2012). Few more studies have shown that recombinant FSH and LH have *in vivo* biological potencies comparable to their native counterparts, demonstrating that they can be used as an alternative to pituitary extracts in inducing gonadal development in aquaculture species (Kobayashi *et al*., 2003).

Recombinant gonadotropins have also been useful in developing immunoassays for measuring levels of circulating and pituitary fish gonadotropins. In tilapia, specific antibodies against FSH and LH were generated using rFSHβ and rLHβ, respectively, and used for establishing homologous enzyme-linked immunosorbent assay (ELISA) (Aizen *et al*., 2007). Using a similar approach, homologous ELISA was also established for Senegalese sole (*Solea senegalensis*) FSH (Chauvigné *et al*., 2015), European sea bass (*Dicentrarchus labrax*) FSH (Molés *et al*., 2012) and Greater amberjack (*Seriola dumerili*) FSH and LH (Nyuji *et al*., 2012).

1.3.3. Induction of sexual maturation using gonadotropin

The issue of late maturity in some aquaculture species, including the giant grouper, remains a bottleneck in the expansion of their culture production. As such, attempts have been made to advance early sexual maturation among these species. Although the current understanding on the mechanism ultimately responsible for initiating pubertal development in fish remains incomplete, stimulation of gonadal development or maturation have been possible through administration of gonadotropins (Levavi-Sivan *et al*., 2008).

In juvenile female rainbow trout (*Oncorhynchus mykiss*), intraperitoneal administration of rFSH (once at 100 µg/kg BW) resulted in the appearance of growing vitellogenic oocytes after 72 h (Ko *et al*., 2007). Sexually immature orange-spotted grouper (*E. coioides*) intraperitoneally injected with rFSH (200 µg/kg BW) five times every two days developed primary oocytes at the end of treatment (Chen *et al*., 2012). Vitellogenic oocytes were also found in juvenile female yellowtail kingfish (*Seriola lalandi*) after receiving intramuscular

injections of rFSH at 10-20 µg/kg BW every 10 days for two months (Sanchís-Benlloch *et al*., n.d.). Increased expression of E_2 within 6 h followed by increased vitellogenin mRNA expression were observed in immature cinnamon clownfish (*Amphiprion melanopus*) injected intramuscularly with either 100 or 1000 µg/kg BW rFSH (Kim *et al*., 2012). These studies demonstrate that treatment with gonadotropin, specifically FSH, can induce ovarian development in immature fish although evidences presented are only limited to oocytes in vitellogenic stage. Whether or not FSH-treatment can stimulate immature gonad to undergo full reproductive developmental cycle remains to be elucidated.

In males, though FSH positively affected spermatogenesis, effects were slower and less pronounced. In juvenile European sea bass (*Dicentrarchus labrax*), rFSH administration at 7.5 µg/kg BW twice within three days stimulated higher 11-KT secretion after 3 days and stimulated spermatogonial proliferative activity though spermatogenesis progressed only up to spermatogonia B stage (Mazón *et al*., 2014). Injection of rFSH at 5 mg/kg BW did not produce significant changes in the gonad of immature male Japanese eel (*A. japonica*) 18 days after treatment (Kazeto *et al*., 2008).

Recombinant gonadotropins have a relatively short half-life *in vivo* hence the need for multiple and regular treatments (Garcia-Campayo and Boime, 2001). One way to avoid the rapid clearance from the circulation is by fusing carboxyterminal peptide (CTP) of the human chorionic gonadotropin with the recombinant protein (Fares *et al*., 1992; Joshi *et al*., 1995). This technique not only prolongs the *in vivo* half-life of the recombinant protein but also enhances its bioactivity (Fares *et al*., 1992). Recent work has also explored DNA plasmid administration or direct gene transfer of rLH and demonstrated that circulating LH is significantly elevated compared with those injected with rLH protein (Mazón *et al*., 2013). The treatment with rLH DNA plasmid, twice with two days interval, also prolonged rLH expression for up to one month (Mazón *et al*., 2013).

Oral delivery of DNA incorporated in formulated feeds has been done in fish primarily for the purpose of delivering DNA vaccines (Ramos *et al*., 2005). This is done through encapsulation of DNA with liposome or chitosan which results in stability while inside the digestive tract (Balazs *et al*., 2010). Utilizing this technique for introducing recombinant gonadotropin

plasmids is one approach that merits further study and has potential application in both research and commercial operations.

1.4. Conclusion

A large and continuously growing market for live groupers faces an unstable supply from wild capture fishery due to the decline in wild grouper stock. To meet the market demand for grouper as well as to ease the pressure from wild stock, sustainable production of groupers through reliable breeding technology is of high priority. Giant grouper, one of the most in-demand species of groupers, has late maturation and a large size at maturity that presents technical difficulty in breeding activities as well as increased financial input. To circumvent this issue, hormonal manipulation, specifically administration of rFSH, can be employed. FSH, the pituitary hormone responsible for stimulating ovarian development, has been shown to induce early sexual maturation in other fish species. Administration of rFSH through intramuscular injection and oral rFSH DNA plasmid delivery are two approaches that merit investigation.

1.5. Objectives

This thesis is aimed at investigating approaches for inducing early sexual maturation in giant grouper using recombinant hormone manipulation so that captive breeding could be done with a younger and smaller broodstock. The specific objectives of this thesis are:

1. Produce a bioactive single chain form of recombinant giant grouper FSH (rggFSH) using yeast expression system.
2. Induce sexual maturation in sexually immature grouper through intramuscular administration of rggFSH.
3. Assess the oral delivery of giant grouper FSH in the form of an expression construct in inducing reproductive development in immature grouper.

Note

In preparing this thesis, the overall format and structure followed the USC Higher Degree by Research Thesis Guidelines while citation style followed the Elsevier (Harvard) using Zotero 5.0. Chapters 2 and 3 will be submitted for publication as full research article in scientific peer-reviewed journal thus the manner of writing.

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Chapter 2 **Induction of early sexual maturation in juvenile tiger grouper *Epinephelus* fuscoguttatus using recombinant follicle-stimulating hormone**

2.1. Abstract

The role of follicle stimulating hormone (FSH) in the reproductive development of protogynous hermaphroditic grouper (*Epinephelus* sp.) was investigated using recombinant giant grouper FSH (rggFSH) produced in yeast (*Pichia pastoris*). After confirming the binding capacity and steroidogenic potency of the rggFSH in *in vitro* homologous receptor assay and ovarian stimulation assay, respectively, its effects on gonad development was examined on sexually immature tiger grouper injected weekly over a period of 38 weeks. After 8 weeks of treatment (100 µg/kg body weight, BW), treated fish exhibited larger and more advanced oocytes (cortical alveolar stage compared to primary growth in control). Sustained treatment (20 to 38 weeks at 200 µg/kg BW) resulted in significant reduction in gonad size associated with degeneration of oocytes and proliferation of spermatogonial cells, which are indicative of female to male sex change. In contrast, control fish have shown ovarian development exhibiting cortical alveolar stage oocytes by the end of the trial. Gene expression analysis have shown that steroidogenic genes *cyp11b* and *cyp19a1a* were significantly suppressed among rggFSH-treated fish after 38 weeks, which prevented increase in the endogenous production of sex steroid hormones. Analysis of the expression profile of sex markers including *dmrt1*, *amh*, *figla* and *bmp15* provided some insights on mechanism underlying rggFSH-induced sex reversal. Based on our results, we propose that the process of female to male sex change in the protogynous grouper is initiated by FSH, rather than sex steroid and likely involves steroid-independent pathway. The cortical alveolar stage is the critical point after which FSH-induced sex reversal is possible.

2.2. Introduction

Vertebrate reproductive development is tightly regulated by the two pituitary gonadotropic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH). Gonadotropins are heterodimeric glycoprotein hormones consisting of a common α subunit and a unique β subunit (Boime and Ben-Menahem, 1998; Suzuki *et al*., 1988b), responsible for the specific physiological function of each gonadotropin through interaction with their cognate receptors (Levavi-Sivan *et al*., 2010). Unlike in mammals, where the function of gonadotropins is clearly defined and established, a considerable aspect of the roles of fish gonadotropins remains to be elucidated, one of the main reasons being the diverse reproductive strategies exhibited by fish. The current understanding on fish gonadotropins has been almost exclusively generated from studies on gonochoristic species.

Since the duality of gonadotropins was first demonstrated in salmon, LH has been extensively studied in fish and its indispensable role during the final gamete maturation has been consistently demonstrated. Its mRNA and protein expression levels exhibit a surge or are sharply elevated shortly before or at the onset of final oocyte maturation in females and spermiation in males (Hassin *et al*., 2000; Nyuji *et al*., 2012; Ogiwara *et al*., 2013; Prat *et al*., 1996). Gonads have also been shown to preferentially respond to LH rather than FSH stimulation in biosynthesizing and secreting the maturation inducing steroid, 17α,20β-dihydroxy-4-pregnen-3-one (DHP) (Kawauchi *et al*., 1989; Suzuki *et al*., 1988c). Recent report of *lh* gene disruption in female zebrafish resulted in failure of oocytes to undergo final maturation and ovulation despite normal follicular development (Chu *et al*., 2014). The role of LH in final gamete maturation has also been implicated in non-gonochoristic protogynous species (Cui *et al*., 2007; Kitano *et al*., 2011).

FSH, on the other hand, is primarily involved during the early stages of gametogenesis in fish. In females, FSH promotes oocyte growth through regulation of vitellogenin uptake (Tyler *et al*., 1991). In males, FSH stimulates spermatogonial proliferation and initiates spermatogenesis (Nóbrega *et al*., 2015). Genetic disruption of *fsh* in zebrafish interrupted the onset of both ovarian and testicular development (Zhang *et al*., 2015). This regulatory role of FSH is achieved through stimulation of gonadal steroidogenesis, particularly the synthesis of the key sex steroid hormones, 17β-Estradiol (E_2) and 11-Ketotestosterone (11KT). Specific

effects of FSH could also be mediated independent of steroids as shown recently in *in vitro* trials using salmon and zebrafish testis, wherein expression of a suite of FSH responsive genes were not suppressed in the presence of trilostane, an inhibitor of 11KT synthesis (Nóbrega *et al*., 2015; Sambroni *et al*., 2013). Generally, fish exhibit a sustained expression of FSH in order to support the process of sexual maturation, which is a more prolonged process relative to final oocyte maturation and ovulation (Molés *et al*., 2011; Nyuji *et al*., 2013; Suzuki *et al*., 1988a).

A few studies in sequentially hermaphroditic teleosts, which mature as one sex and later change into the opposite sex, have noted the sexually dimorphic expression of FSH (Y. Kobayashi *et al*., 2010; Meiri *et al*., 2004; Ohta *et al*., 2008). The involvement of FSH in the process of protogynous sex reversal (female to male) has been demonstrated in honeycomb grouper (Y. Kobayashi *et al*., 2010). With the existing literature being fragmentary, the role of FSH in the reproduction of hermaphroditic fish remains unclear. For instance, it is not understood how FSH supports one sex, and then shifts that function to promote sex change, or whether FSH supports the maintenance of a particular sex.

Groupers are protogynous hermaphrodites, maturing first as females and then reversing to males, and have been used as a model species to study the biology of sex differentiation and sex change in teleosts (Zhou and Gui, 2010). In the present study, we report the production of a recombinant giant grouper FSH and provide evidence of its *in vitro* bioactivity by a homologous receptor binding assay and ovarian sex steroid stimulation assay. We also demonstrate its *in vivo* bioactivity via administration to sexually immature tiger grouper (*E. fuscoguttatus*) and describe the effects of chronic treatment on gonadal development and gene expression. Our results provide a potential hypothesis regarding the role of FSH during gonadal maturation and sex change in a protogynous hermaphrodite. Our long-term goal is to advance sexual maturation and control sex change in giant groupers (E. lanceolatus), a high-value aquaculture species, which is now classified as vulnerable by the IUCN (Shuk Man and Wai Chuen, 2006).

2.3. Materials and methods

2.3.1. Isolation of gene sequences

The sequences encoding for genes relevant for this study were obtained from transcriptomic data generated in-house. Total RNA was extracted from giant grouper (*E. lanceolatus*) and tiger grouper (*E. fuscoguttatus*) tissue samples (brain, hypothalamus, pituitary gland, and gonad) using RNeasy Mini Kit (Qiagen). The quality of RNA extracts was evaluated using RNA 6000 Nano Kit for the 2100 Bioanalyzer instrument (Agilent). RNA with RIN >7 were sent to Novogene, Hong Kong for library preparation and RNA-Seq on an Illumina HiSeq platform, producing 150 bp paired end reads. Transcriptome assembly and annotation were done as described previously (Bar *et al*., 2016). Briefly, raw reads were processed through Trimgalore (v0.4.4) to remove Illumina adapters followed by quality checking using FastQC (v0.11.5). *De novo* transcriptome assembly was then done using Trinity (v2.5.0) while subsequent annotation was done by sequence alignment similarity search (BLAST v2.2.30).

The transcriptomic analysis of giant grouper was done by Dr. Ido Bar while that of tiger grouper was done with significant assistance of Dr. Kelli Anderson. Both researchers are members of the Genecology Centre, USC.

2.3.2. Production of rggFSH

Production of rggFSH in yeast (*Pichia pastoris*) expression system was done following previously described methods (Aizen *et al*., 2007; Kasuto and Levavi-Sivan, 2005). The expression cassette designed to produce a single chain peptide consisted of the mature protein-coding sequences of giant grouper FSHβ- and α-subunits linked with a histidine tag (Fig. 2.1.). The histidine tag is also utilized as the basis for purification and antibody detection. This was codon-optimized according to *P. pastoris* coding preference prior to ligation into pPIC9K expression vector. Following electroporation of the *Sal*I-linearized construct, yeast transformants were plated onto histidine-deficient agar plates (2% agar, 1 M sorbitol, 2% dextrose, 1.34% yeast nitrogen base, 0.4 ppm biotin, and 0.005% L-glutamic acid, L-methionine, L-lysine, L-leucine, and L-isoleucine). To screen for colonies containing high copy number of the construct, colonies were grown in YPD broth with increasing dose of G418 sulphate antibiotic (1-2 mg/ml; Sigma-Aldrich). Three surviving colonies were further tested

for recombinant protein expression following a published method (Aizen *et al*., 2007). Yeast colonies were separately cultured in 50 ml BMM (100 mM potassium phosphate buffer, 1.34% yeast nitrogen base, $4x10^{-5}$% biotin) by the shake flask method and induced by adding 1% methanol every day for 72 h. Recombinant protein was purified from the medium using Ni-NTA Superflow beads (Qiagen) following manufacturer's instruction and were subjected to western blot analysis. One colony was then selected for large scale production.

Purified recombinant protein was subjected to SDS-PAGE and western blot analysis as previously described (Sanchís-Benlloch *et al*., 2017). Briefly, recombinant protein products were treated with *N*-glycosidase (PNGase F; New England Biolabs) according to manufacturer's instruction. Proteins were then resolved in 12% polyacrylamide gel (Mini-PROTEAN TGX Precast Gels; BioRad) at 150 V for 1 h and then transferred onto nitrocellulose membrane (Trans-blot turbo transfer system transfer pack; BioRad). Western blot was done as follows: (1) the membrane was blocked with blocking buffer consisting of Tris-buffered saline pH 7.5 (TBS; 50 mM Tris-Cl, 150 mM NaCl), 3% BSA and 0.1% Tween-20 overnight at 4°C; (2) washed twice with TBS containing 0.05% Tween-20 and 0.2% Triton X-100 (TBS-TT) and once with TBS for 10 mins at room temperature; (3) incubated with penta-his antibody (1:2,000; Qiagen) diluted in blocking buffer for 1 h at room temperature; (4) washed twice with TBS-TT and once with TBS, and; (5) incubated with secondary antibody (IRDye 800 CW 1:5,000; Li-Cor). Protein bands were then visualized using Odyssey CLx Infrared Imaging System (Li-Cor).

To produce large quantity of rggFSH, yeast was induced in a biofermentor (BioFlo 115; New Brunswick) following *Pichia* Expression Kit Manual (Catalog no. K1710-01, 2010; Thermo Scientific). The fermentation basal salts medium was supplemented with 1% casamino acid. Temperature was reduced from 30°C to 22°C after yeast has adapted to methanol (about 18 h after methanol feed started). The recombinant protein was purified from the medium using Ni-NTA Superflow beads (Qiagen) following manufacturer's instruction and then dialysed in 10kDa molecular weight cut off dialysis cassette (ThermoFisher Scientific) against 0.1x PBS (PBS Tablet; Sigma) for 4 h at 4°C. The amount of rFSH was quantified using NanoOrange™ Protein Quantitation Kit (Invitrogen). Recombinant proteins were then lyophilized.

2.3.3. *In vitro* activation of giant grouper FSH Receptor

To confirm that the recombinant protein is bioactive, a receptor binding activity was conducted using COS-7 cells transiently expressing ggFSHR and luciferase reporter gene that is controlled by a cAMP responsive element (CRE-Luc in pGL4.29; Promega, USA). For this, an expression construct of the ggFSHR was designed using the pcDNA3.1 (Fig. 2.1). The assay was based on earlier studies (Biran *et al*., 2008; Sanchís-Benlloch *et al*., 2017) with some modifications. Briefly, 3 µg ggFSHR and CRE-Luc was transfected into COS-7 cells (15 ml culture in Petri dish) using TransIT-LT1 Transfection Reagent (MirusBio) according to manufacturer's instruction. After 18 h, cells were then divided equally and seeded into 6 well plate wherein they were starved with serum-free medium for 18-24 h. Starving medium consisted of Dubelcco Modified Eagle Medium (Gibco) containing 0.5% BSA, 1% antibiotics (Pen-Strep; Gibco) and 1% Nystatin. This was followed by treatment with increasing doses of rggFSH (0-1,000 ng/ml) and incubation for 6 hours. Cells were then lysed using Luciferase Cell Culture Lysis 5X Reagent (Promega) according to manufacturer's instruction. Luciferase activity was read using EnSpire Multimode Plate Reader (PerkinElmer).

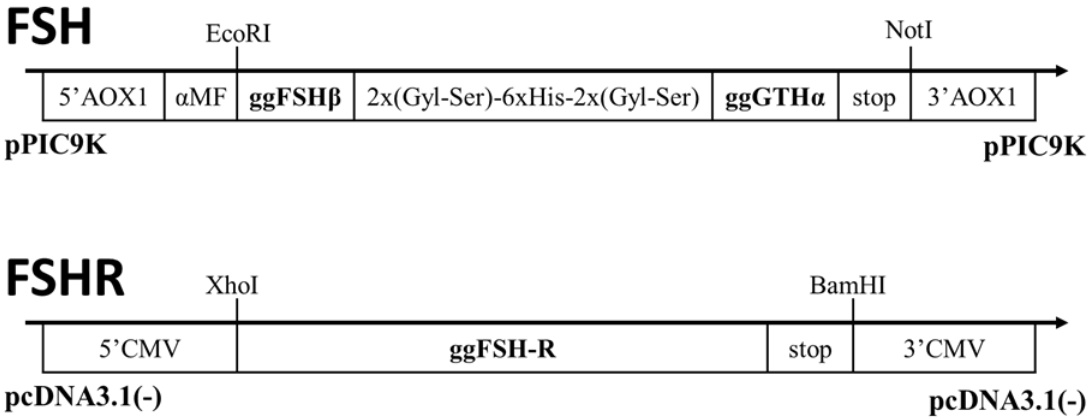

Fig. 2.1. Construction of the expression vector for the giant grouper FSH and FSHR. FSH: The expression cassette consisting of mature giant grouper FSHβ, a linker sequence and GTHα were ligated in the EcoRI-NotI restriction sites of pPIC9K. Flanking the cloning site at the 5' end is the promoter sequence of alcohol oxidase gene (*AOX1*) and an α yeast mating factor which directs the recombinant protein into the secretory pathway. FSHR: Mature coding sequence of ggFSHR was inserted into XhoI-BamHI restriction sites. Expression is driven by cytomegalovirus promoter.

2.3.4. *In vitro* stimulation of ovarian steroidogenesis

The potency to stimulate the steroidogenic pathway by rggFSH was tested using ovarian tissue following methods described previously (Aizen *et al*., 2007; Sanchís-Benlloch *et al*., 2017) with few modifications. Briefly, about 80 mg tissue fragments were prepared from vitellogenic ovary of tiger grouper *E. fuscoguttatus* (2.2 kg body weight, bw; 1.5 % GSI) and distributed in 24-well plate with 1 ml basal eagle medium. Tissue fragments were preincubated for three hours changing the medium every hour followed by treatment with increasing doses of rggFSH (0 to 800 ng/ml) for 18 hours at 28°C. The medium was then collected and the steroids were extracted with 5 ml diethyl ether per sample. Steroid extracts were kept in -80°C until analysis for E_2.

2.3.5. rggFSH-treatment of immature grouper

Sexually immature tiger groupers (*E. fuscoguttatus*; 720±50 g BW; 34±1 cm total length, TL) were kept in concrete tanks equipped with flow-through water system. Each fish was implanted with a PIT tag (Biomark HPT9; Biomark, USA) for identification. Groupers were fed with fresh fish and squid and were also allowed to acclimate in the set up for about two months before the hormonal treatment.

Fish were given weekly intramuscular injections of 0 (Control, N=10) or 100 µg FSH/kg BW (rggFSH, N=10) in the first eight weeks and then the dose was increased to 200 µg/kg BW in rggFSH group until 38 weeks. Three fish from each group were sacrificed after 8 and 20 weeks, and the remaining fish were sacrificed after 38 weeks of treatments. Anaesthetics were applied before handling (200 ppm 2-phenoxyethanol; Sigma, Singapore). Fish were sacrificed by anaesthetic overdose followed by decapitation. For gene expression analysis, tissue samples were immediately stabilised in RNALater and then kept at -80°C until analysis.

2.3.6. Sample analyses

Estradiol and testosterone were quantified through competitive enzyme immuno-assay as described by Rahman *et al* (Rahman *et al*., 2000) using commercially available reagents: steroid standards (E_2 and T; Sigma, Singapore), T and E_2 antibody-tracer sets (CosmoBio,

Japan), and mouse anti-rabbit IgG (Sigma, Singapore). For 11-KT ELISA, a commercial kit was used (Cayman Chemicals) following manufacturer's instruction.

Plasma FSH levels were determined using a competitive ELISA developed for yellow tail kingfish (ytk; *Seriola lalandi*) FSH. The primary antibody was a rabbit antiserum raised against ytkFSHβ subunit while the standard was a purified full length recombinant ytkFSH (Sanchís-Benlloch *et al*., 2017). Parallelism of cross reactivity was confirmed between serially diluted recombinant ytkFSH and rggFSH. Details of the development of the competitive FSH ELISA will be published elsewhere.

For histological examination, tissues were fixed in Bouin's solution and subsequently processed using standard histological techniques, sectioned at 5 µm, and stained with hematoxylin and eosin. Sections were examined under Leica DM5500 microscope. Oocyte sizes were measured using ImageJ 1.51j8.

2.3.7. Gene expression analysis

Total RNA was extracted from each gonad sample and reverse-transcribed using commercial kits (RNeasy Mini Kit, QuantiTect Reverse Transcription Kit; Qiagen) following manufacturer's specifications. The quality of RNA extracts was confirmed through gel electrophoresis and using 2100 Bioanalyzer (Agilent). Quantitative real-time PCR was performed with 10 µl total reaction volume consisting of 5 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 0.4 µl each of forward and reverse primers (10 µM), 3.2 µl water and 1 µl template. Assays were run on Corbett Rotor-Gene 6000 (Qiagen) in Rotor-Disc 72 using the following cycling conditions: 50°C for 2 min, 95°C for 2 min, 40 cycles of 95°C and 60°C for 15 and 20 sec, respectively. Each sample was assayed in duplicate. Each assay included a no-template control which showed no amplification over 40 cycles, and two standard dilutions ($1.0x10^7$ and $1.0x10^4$ copies/µl). Specificity of the products were further examined by their melt curves. Copy number were calculated by comparing the sample threshold cycle (C_T) values to a standard curve (89-105% efficiency; Table 2.2). For each gene, a standard curve was constructed from seven concentrations ($1.0x10^8$ to $1.0x10^2$ copies/µl) of serially diluted PCR-amplified gene fragments (400-450 bp product size; Table 2.1). Primers used to amplify 110-150 bp-fragment of genes from the samples are shown in Table 2.2. The calculated copy

number of the samples were presented relative to that of the reference gene (conserved oligomeric Golgi complex subunit 5, *Cog5*). Cog5 was chosen as the reference gene for this study as recommended from previous study (Wang *et al*., 2017) showing its stable expression in gonadal tissue of another grouper species *E. akaara*.

2.3.8. Statistical analysis

All data are presented as the mean ± SEM. Values were subjected to one-way analysis of variance followed by multiple comparison using Tukey test. To compare control and rggFSH values within each time point, independent samples t-test was performed. The differences were considered significant at $P<0.05$.

2.3.9. Ethics

This study was approved by the Animal Ethics Committee of the University of the Sunshine Coast (approval no. AN/A/16/102).

Table 2.1. Nucleotide primers used to create standard concentrations.

Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Product size (bp)
fshr	GCACCTCAGGAAACTCATCATA	CATTTCTGGTGAGCCGTATCT	416
foxl2	CAACACGACCAAGGAGAAAGAG	GTCTCCTCCGAACAAGGATTTC	430
cyp19a1a	CAATGGAGAGGAGACACTCATAC	CTCTGGTGAATCCAGTCAAACT	450
bmp15	CAGTACAACCTCGACACTCTTC	CCCTTTCCTCCTCCAGGTATAA	430
amh	GGAGGAATCAGAGGTGTCAAAG	GAAGAAGGAGGTGTGTGAAGAG	420
cyp11b	CAGGTGTTGGAAAGAAGGGTAT	TCCATCGCAACCTCATCAAG	430
sox9	GGGTCAAGACTACAAGAAGGAG	CATTTGGAGAGATGTGGGTTTG	434
figla	AGGAGAGACTGAGAATCAGGAA	AGCAGGGTTGAGACATCAAAG	420
igf3	CACTGAAGGTGTTCTGTGTTTG	GTCTGAGAGAAGGCTGTGTT	450
dmrt1	CCATCCAAAGGCCACAAATC	GACGGCTGGTAGAAGTTGTAATA	449
cog5	AGCTTCCACGAATTCACTTCT	CCTCTTGCTCAGGTACAAGATAC	406

Table 2.2. Nucleotide primers used to amplify genes from the samples and to construct standard curve. These primers amplify sequence within the ~400 bp sequence amplified by their respective primer in Table 1.

Gene	Primer Sequences (5'-3')		Product size (bp)	Standard curve efficiency
	Forward	Reverse		
fshr	CGACATGCTGGAGAGTATCAC	GGGAGATCAGAGAAAGCAGAAG	120	1.00
foxl2	GAAAGGTTGGCAGAACAGTATCAGA	TGGGTCGAGTGTCCAGTAATTC	112	0.92
cyp19a1a	GTTTGGGTCATGTGGATGTCCT	AACACAGTCTGCCATGTGTCAA	142	1.00
bmp15	CAGAGCCTCATTCATCCATCTC	CAAAGCTCTCTTTGCCCAGT	143	0.90
amh	TCGTGTTGACCTTTGAACTCT	CGACTGACTCGTGAAAGTGATA	119	0.97
cyp11b	CTGACATCGCTGAGCTGTTT	CGTTCTTGAGGAACACTCCTTT	117	0.89
sox9	CTGGAGATTGCTCAACGAAGTA	TTGCCCGTTCTTGACAGATT	133	0.89
figla	GAGCGCATTGAGGATGGATT	GGAGCTACTTGGATGATGAACTG	122	1.01
igf3	GCTGTGGTTCTGATCTCCTTAG	ACTGGTCAACAATACCCTTCC	123	1.02
dmrt1	GCCAGTGTCCCAAATGTAAAC	AGAGCCACAGGACTACAAATC	118	0.91
cog5	TGGATGCTACGCTGATTTCC	CCTTGTCCAGCTGACTGATG	128	1.05

2.4. Results

2.4.2. Production of rggFSH

Recombinant ggFSH was successfully produced in *P. pastoris* using the pPIC9K expression vector utilizing the secretory pathway. Initial attempts to induce rggFSH expression in the biofermentor at the recommended culture temperature of 30°C yielded a low quantity and degraded protein products (data not shown). After optimizing the induction condition, efficient protein expression was achieved by reducing the temperature to 22°C after the yeast has adapted to methanol (~18 h). Addition of 1% casamino acid into the culture medium further enhanced the expression efficiency and at the same time reducing recombinant protein degradation. Production rate in the bioferementor ranged from 100-150 mg per litre culture media. Western blot analysis using anti-His tag antibody has shown an immuno-reactive band of about 30 kDa (Fig. 2.2., inset image).

2.4.3. *In vitro* bioactivity of rggFSH

The *in vitro* bioactivity of rggFSH was first assessed through a homologous receptor binding assay in COS-7 cells transiently expressing giant grouper FSHR and a luciferase reporter gene that responds to cAMP production. Luciferase activity displayed a dose response curve (Fig. 2.2.). Second, the potency of rggFSH to stimulate secretion of sex steroids was tested using grouper ovarian fragments. Both E_2 and T secretion increased in response to FSH-stimulation (Fig. 2.3.), following a dose-dependent manner. These results confirmed the *in vitro* bioactivity of our rggFSH.

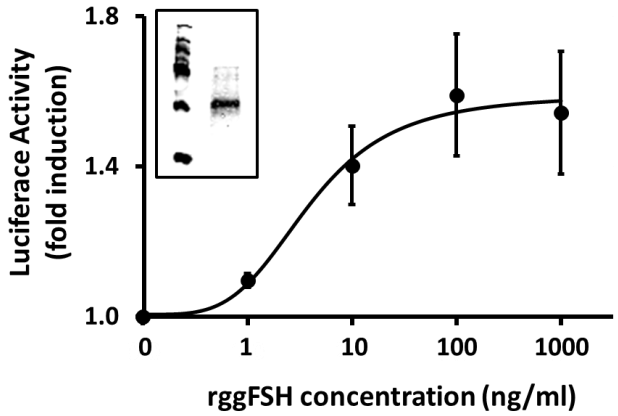

Fig. 2.2. Luciferase activity in response to rggFSH-stimulation during *in vitro* receptor binding assay. Inset: Western blot analysis of rggFSH showing an immuno-reactive band of about 30 kDa. Qiagen 6xHis ladder (laft lane) and 30 kDa rggFSH (right lane).

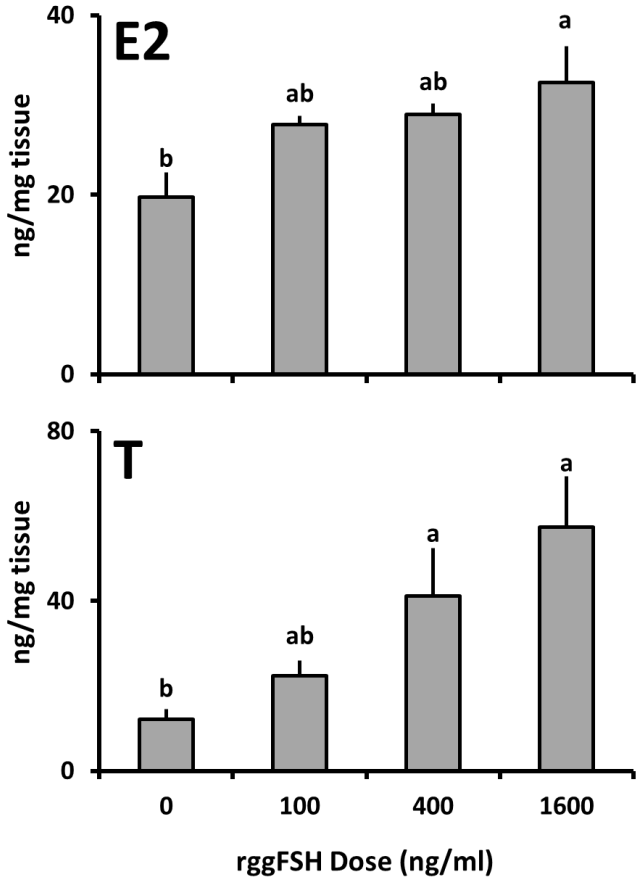

Fig. 2.3. *In vitro* sex steroid secretion of grouper ovarian fragments in response to rggFSH-stimulation. Each value is a mean of three replicates. Values with the same superscript are not significantly different.

2.4.4. Effect of rggFSH on gonadal development of immature grouper

Weekly intramuscular administration of rggFSH (100 µg/kg BW) to sexually immature tiger grouper (670-770 g BW) was conducted to assess the effect of rggFSH on reproductive development of a protogynous fish, the tiger grouper. The changes in gonad (GSI) and oocyte size (diameter) in response to rggFSH-treatment are shown in Fig. 2.4. After 8 weeks, treated fish had significantly larger and more advanced oocytes (cortical alveolar stage) compared with control, which exclusively had primary growth stage oocytes (Fig. 2.5.). Treated fish also had higher GSI although statistically significant differences were not observed. After 20 weeks of treatment, the rggFSH-treated fish exhibited significantly smaller GSI and mean oocyte diameter. Gonadal histology revealed regression of oocytes, proliferation of spermatogonial cells and occurrence of few spermatogenic cysts containing spermatocytes. In contrast, in the control group, oocytes grew larger, advancing into cortical alveolar stage. Similar histological features were observed, respectively, in the treated and control group after prolonged treatment to 38 weeks. In treated fish, regression of oocytes and proliferation of spermatogonial cells were observed. There were few spermatogenic cysts observed in the gonad containing spermatocytes, however the accumulated spermatogonial cells appeared to have remained largely undifferentiated after 38 weeks of treatment. The control group had oocytes in cortical alveolar stage similar to what was observed after 20 weeks of treatment although one out of four showed signs of spermatogonial proliferation (data not shown). This may be attributed to the grouper's natural tendency to balance the sex ratio in the population (Bright *et al*, unpublished data). Nevertheless, the consistent histological response observed among treated individuals demonstrated the biological effect of rggFSH.

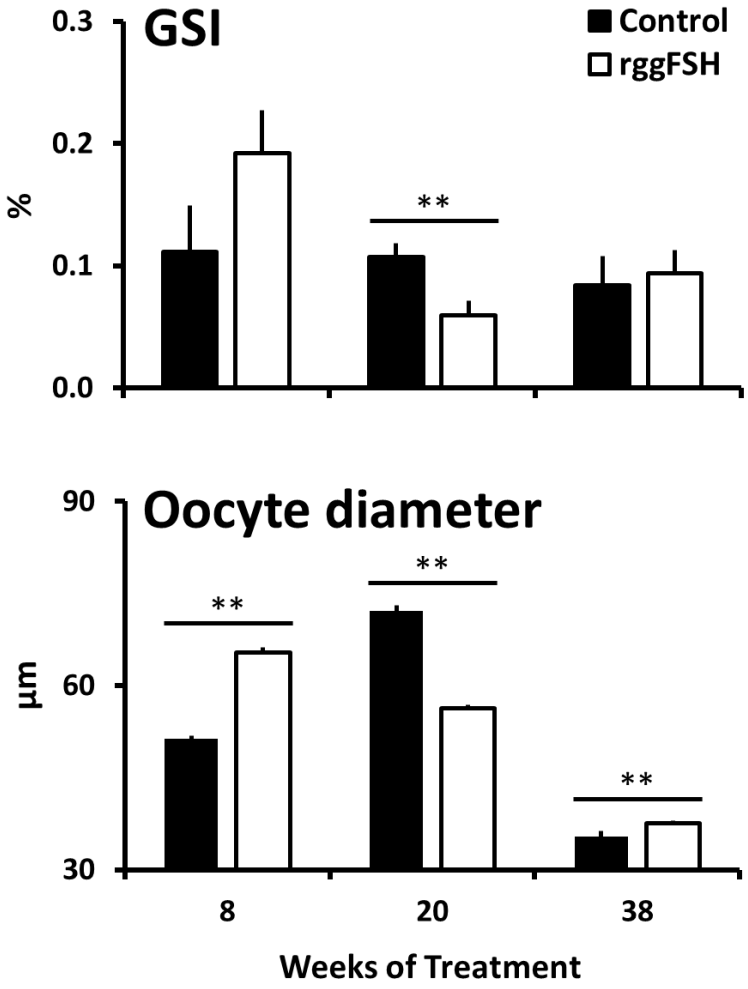

Fig. 2.4. Effect of rggFSH-treatment on GSI and oocyte diameter of immature tiger grouper at different time points. Each GSI value represents a mean of three to four fish while oocyte diameter represents mean of 150-200 measurements. Error bars denote standard error of the mean. Values marked with asterisks (**) are significantly different (P<0.05).

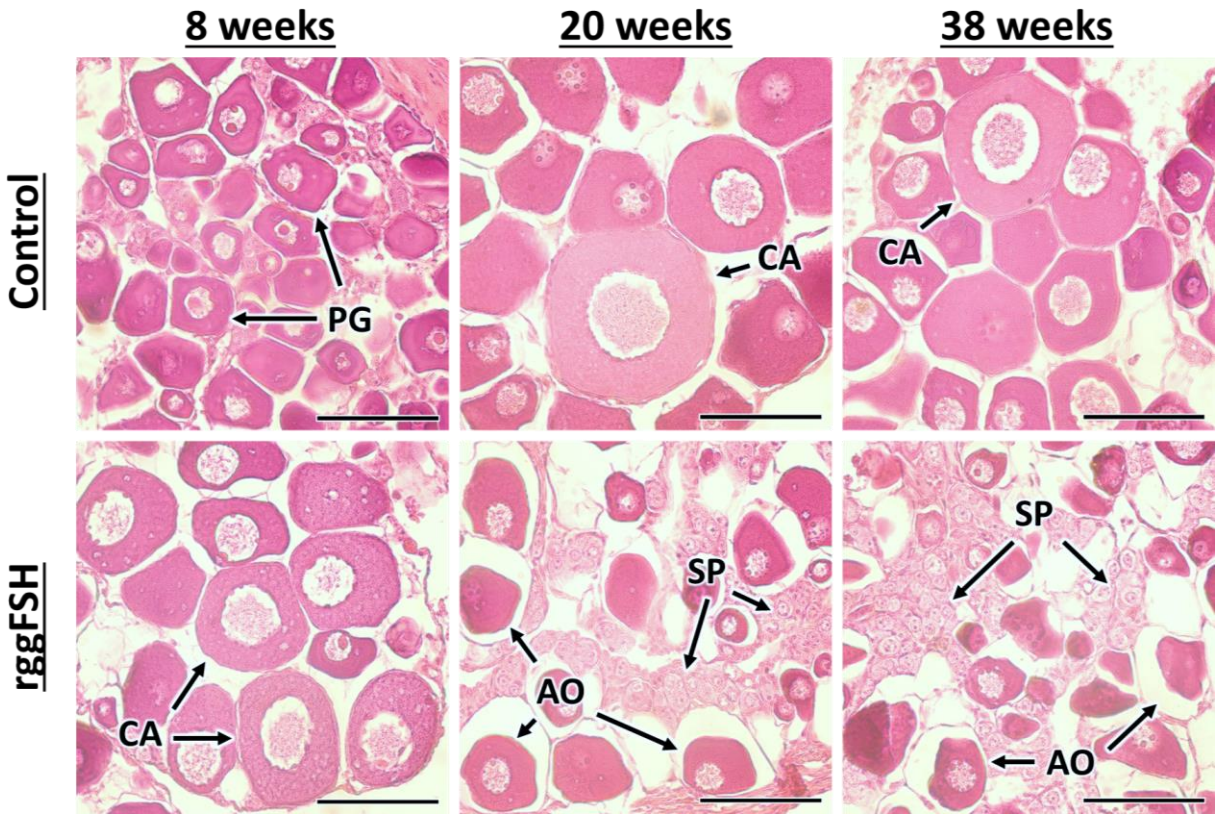

Fig. 2.5. Gonad histological changes in sexually immature tiger grouper in response to rggFSH-treatment. PG = primary growth stage oocyte, CA = cortical alveolar stage oocyte, AO = atretic oocyte, SP = spermatogonia, SC = spermatogenic cyst. Scale bar = 50 µm.

2.4.5. Effect of rggFSH on plasma sex steroids and FSH

The circulating levels of the sex steroids E_2, T and 11KT were determined at specific time points during the trial and results are presented in Fig. 2.5. After 8 weeks of treatment, there was a significantly higher level of E_2 and T in the treated group than in control. After 20 and 38 weeks of treatment, circulating E_2 in treated fish declined to control level concurrent with the observed degeneration of oocytes. On the other hand, control fish, which were advancing into cortical alveolar stage, had increased T level although these were not converted into E_2 likely due to insufficient aromatase (Fig. 2.7.: *cyp19a1a*). Levels of 11KT were notably higher in treated fish from 20 to 38 weeks of treatment, however variability was high among individuals and thus did not account for significant difference.

Throughout the trial, no significant differences were observed between treated and control groups in terms of the circulating levels of FSH. Because blood samples are collected one week after the last treatment, this suggests that the rggFSH is quickly cleared from the circulation. This result also suggests that the rggFSH did not stimulate upregulation of endogenous FSH production and that the higher steroid levels at 8 weeks did not exert a negative feedback of FSH release.

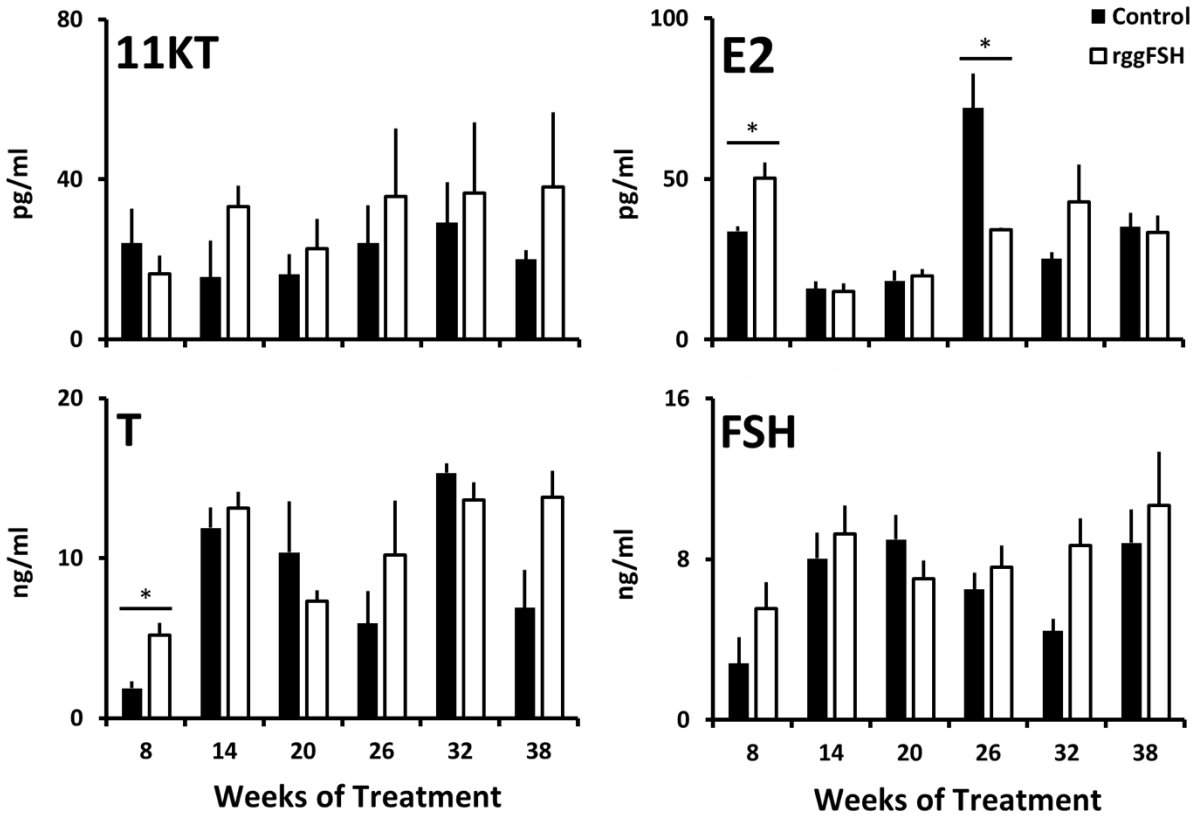

Fig. 2.6. Circulating levels of sex steroid hormones and FSH during rggFSH-treatment in sexually immature tiger grouper. Each bar represents a mean of 3-5 values ± SEM. Values marked with asterisks (*) are significantly different.

2.4.6. Effect of rggFSH on expression of reproductive genes

The mRNA expression profile of several reproduction-related genes was analysed using qPCR. Ovary-associated genes, including *cyp19a1a*, *foxl2*, and *figla*, showed increasing expression throughout the trial in the control group, suggesting progression of ovarian development. Expression of *cyp19a1a* and *foxl2* in treated fish initially increased (from 8 to 20 weeks of treatment) but was suppressed towards the end of the experiment. Interestingly, *figla* appeared to be unaffected by rggFSH treatment, showing a similar expression pattern to the control group. The level of *bmp15,* which has been shown to maintain femaleness in zebrafish (Dranow *et al*., 2016), showed progressively increasing trend in both treated and control group, similar to the *figla* expression pattern.

The testis-associated genes *cyp11b* and *sox9* both showed significantly lower expression in treated than in control after 38 weeks of treatment (Fig. 2.7.). On the other hand, *dmrt1* and *amh,* which are also testis-associated genes, displayed stable expression throughout the trial, and neither varied between the two groups (Fig. 2.8.).

Initial expression levels of *fshr* and *igf3* did not vary until 38 weeks of treatment, where expression was significantly suppressed in the treated fish (Fig. 2.7.).

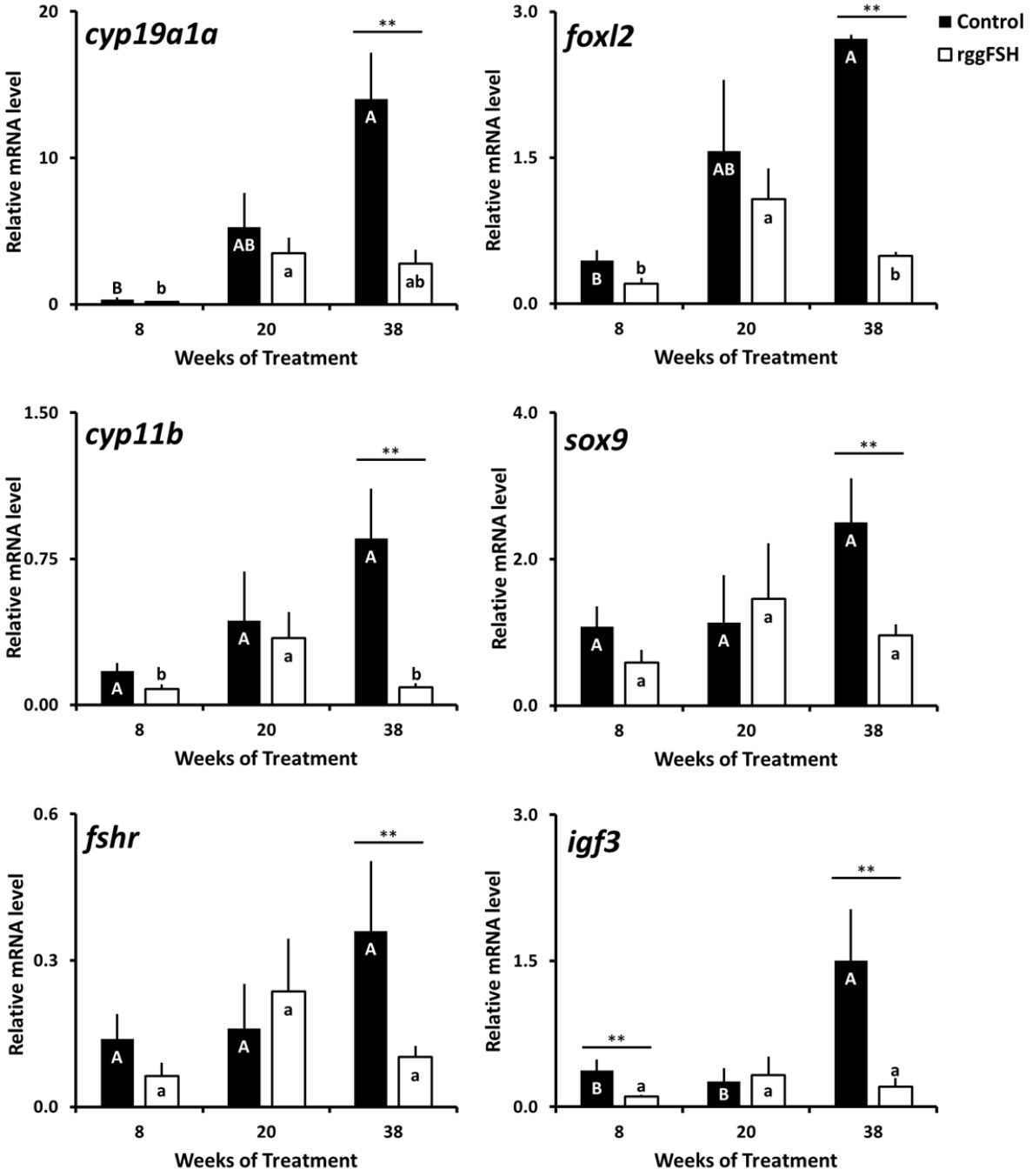

Fig. 2.7. Expression profile of reproductive genes in the gonad affected by rggFSH-treatment. Gene expression is expressed relative to *cog5* mRNA level. Each bar represents a mean of 3-4 values. In each time point, significant difference (P<0.05) between control and rggFSH values is denoted by asterisks (**). Within each treatment group, values with the same superscript are not significantly different.

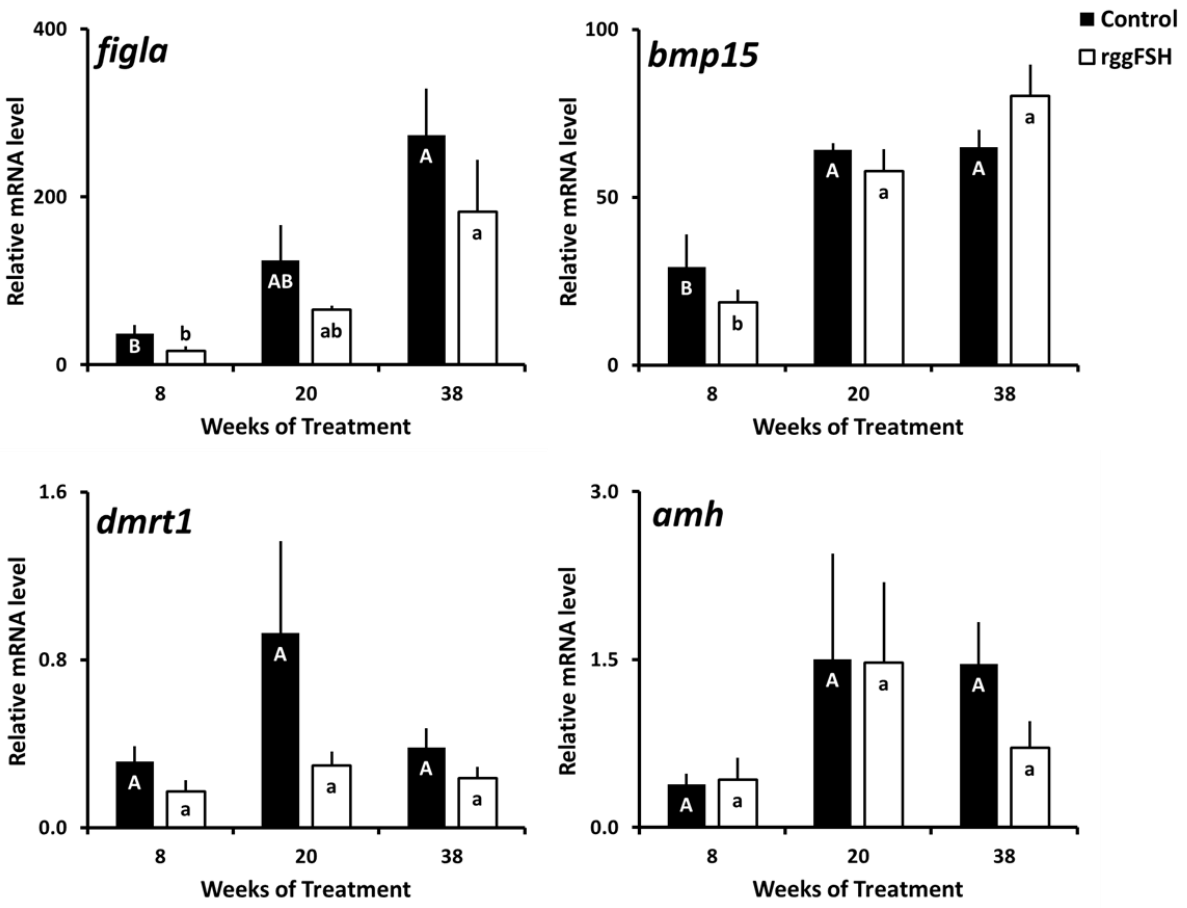

Fig. 2.8. Expression profile of reproductive genes in the gonad which were not affected by rggFSH-treatment. Gene expression is expressed relative to *cog5* mRNA level. Each bar represents a mean of 3-4 values. In each time point, no significant differences were found between control and rggFSH values. Within each treatment group, values with the same superscript are not significantly different.

2.5. Discussion

In fish, FSH is known to initiate oogenesis in females and spermatogenesis in males, however it is not clear how this model fits in the context of sex-changing species such as the protogynous groupers. A few studies have examined the effect of recombinant FSH on the reproductive development of groupers at the gonad level, describing primarily the gonad histological changes (Chen *et al*., 2012; Kline *et al*., 2008; Y. Kobayashi *et al*., 2010). Thus, only partial insight is currently available with regards to the role of FSH in grouper reproductive development. Here, we have produced a bioactive rggFSH and administered it to sexually immature tiger groupers over a period of 38 weeks. Our results show that rggFSH initially stimulated oocyte growth and development up to a certain point, and then redirects the gonad towards testicular development. We also describe the associated changes in the expression of reproduction-related genes.

The recombinant giant grouper FSH was produced using the *P. pastoris* expression system following the commonly used single-chain design (Aizen *et al*., 2007; Kasuto and Levavi-Sivan, 2005). Both the *in vitro* receptor binding assay and ovarian bioassay produced a dose-dependent response confirming the bioactivity of the rggFSH. Histidine-linked recombinant FSHs that were proven bioactive have been previously produced for Nile tilapia *Oreochromis niloticus* (Aizen *et al*., 2007), yellowtail kingfish *Seriola lalandi* (Sanchís-Benlloch *et al*., 2017), common carp *Cyprinus carpio* (Aizen *et al*., 2016), Japanese eel *Anguilla japonica* (M. Kobayashi *et al*., 2010), orange spotted grouper *E. coioides* (Chen *et al*., 2012) and Russian sturgeon *Acipenser gueldenstaedtii* (Yom-Din *et al*., 2016). Such recombinant FSHs have been shown to be potent even in inter-specific *in vitro* bioassays (Aizen *et al*., 2012b, 2012a). Their biological activity have also been demonstrated in *in vivo* trials where they consistently induced steroidogenesis and gonad development (Aizen *et al*., 2016; Chen *et al*., 2012; M. Kobayashi *et al*., 2010; Sanchís-Benlloch *et al*., 2017). Altogether, results of both previous studies and the present one demonstrate the bioactivity of single-chain recombinant FSH.

The treatment of sexually immature tiger grouper with rggFSH for eight weeks resulted in significant increase in oocyte diameter and advanced oocyte stage (cortical alveolar stage oocytes *vs* primary growth stage oocytes in control). Single injection of recombinant seabream FSH (~8.5 µg/kg BW) also stimulated oocyte development up to cortical alveolar

stage in Sevenband grouper *E. septempfasciatus* after five weeks. (Kline *et al*., 2008). In juvenile orange spotted grouper *E. coioides*, treatment with homologous recombinant FSH resulted in the occurrence of primary growth stage oocytes (Chen *et al*., 2012). Therefore results from the present and previous studies on grouper show that FSH stimulates oogenesis up to cortical alveolar stage. In female gonochoristic fish, including Yellowtail kingfish *S. lalandi* (Sanchís-Benlloch *et al*., 2017), rainbow trout *Oncorhynchus mykiss* (Ko *et al*., 2007) and Japanese eel *Anguilla japonica* (Kazeto *et al*., 2008) recombinant FSH promoted ovarian development up to vitellogenic stage. In the present study, in order to support the progression of oocytes from cortical alveolar stage into vitellogenic stage, treatment was extended and the dose was increased from 100 (first 8 weeks) to 200 µg/kg BW in the succeeding weeks of treatment.

Further treatment with rggFSH for 20 weeks resulted in a significant reduction in the size of the gonad, which contained degenerating oocytes and proliferating spermatogonial cells, indicative of sex reversal. A similar response was exhibited by mature female honeycomb grouper, where oocytes were degenerated and spermatogonial cell proliferated after single administration of purified bovine FSH (Y. Kobayashi *et al*., 2010). Although it has been discussed earlier that FSH promoted oocyte development in groupers, these findings when taken together, suggest that the effect of FSH on the reproductive development of grouper depends on their gonadal maturity. More importantly, the present study provides clear evidence that FSH shifts from promoting ovarian development to inducing sex reversal after cortical alveolar stage is reached.

During the natural process of sex reversal in honeycomb grouper, the early transition stage (initiation) is associated with regression of oocytes and proliferation of spermatogonial cells while the late stage is associated with high prevalence of differentiated spermatogenic cysts and minimal occurrence of atretic oocytes (Bhandari *et al*., 2003). On the other hand, the progression of sex reversal to the late stage is concurrent with a surge in androgen level (T and 11KT) (Alam *et al*., 2006; Bhandari *et al*., 2003). In the present study, rggFSH-injected tiger groupers appeared to have remained in the early stage of sex reversal between 20 and 38 weeks wherein spermatogonial cells were accumulated but did not differentiate and significant increase in T and 11KT was not observed. Therefore, the rggFSH-induced sex reversal observed in the present study was restricted in the early transition stage.

The expression of *dmrt1* and *amh*, which are known markers of spermatogenesis, did not show any significant changes between 20 and 38 weeks of treatment, where sex reversal was observed. Earlier studies in other grouper species (Alam *et al*., 2008; Xia *et al*., 2007) and in the protogynous rice field eel *Monopterus albus* (Huang *et al*., 2005) have shown that *dmrt1* expression peaks towards the late stage of female to male sex change. The same expression profile has been implicated for *amh* during the protogynous sex change of orange spotted grouper (Wu *et al*., 2017) and rice field eel (Hu *et al*., 2015). These observations further show that the rggFSH-treated fish in the present study did not advance to the late stage of sex reversal.

It was noted in the present study that rggFSH did not induce a significant increase in the production of endogenous T and 11KT, particularly when sex reversal was observed (20 to 38 weeks). On the contrary, our results show that rggFSH suppressed the expression of cyp11b, which encodes for the key 11KT-synthesizing enzyme. In separate *in vivo* and *in vitro* trials in other grouper species (Chen *et al*., 2012; Y. Kobayashi *et al*., 2010), a marked reduction in androgen synthesis were noted in response to high dose of FSH. *In vitro* antagonistic effects between FSH and 11KT has also been implicated in rainbow trout rainbow testis (Sambroni *et al*., 2013). Taken together, these suggest an inhibitory effect of FSH on endogenous androgen synthesis, and therefore the low level of androgen in the present study was most likely a consequence of weekly rggFSH administration. Moreover, the increased dosage of rggFSH-treatment from 20 to 38 weeks in this study may have further amplified the inhibition of androgen synthesis.

The mRNA levels of *fshr* and *sox9* are notably inhibited after 38 weeks in the present study. In an *in vitro* rainbow trout testis assay, the incubation with FSH upregulated the expression of *fshr* but not when co-incubated with androgen synthesis-inhibitor (Sambroni *et al*., 2013). On the other hand, the *in vivo* expression of *sox9* has shown a significant increase after treatment with androgen (17α-methyltestosterone, MT) in orange spotted grouper (Luo *et al*., 2010). *Sox9* is a recognized male sex determining gene whose expression is associated with the appearance of spermatocytes during sex reversal in orange spotted grouper (Luo *et al*., 2010). These observations suggest the low expression of *fshr* and *sox9* in the present study is due to the lack of androgenic stimulation. The present and previous results further reinforce

the idea that the rggFSH inhibited steroidogenic activity and thus arrested the expression of androgen-dependent genes.

It is widely recognized that sex reversal in grouper is a result of the interplay between the key sex steroid hormones, E_2 and 11KT. However, this does not reconcile with the results obtained from two separate studies that show that T and 11KT levels increases only towards the late stage of female to male sex reversal in honeycomb grouper (Alam *et al*., 2006; Bhandari *et al*., 2003). On the other hand, honeycomb grouper at the initiation of natural sex reversal exhibited a surge in *fshβ* expression, which gradually declined as the process progressed (Y. Kobayashi *et al*., 2010). Our results, in combination with the previous observations in honeycomb grouper, show that FSH has a primary role in initiating sex change in grouper. The possibility that there is an overlapping function of FSH and androgen in initiating sex reversal is ruled out by the evidence from sevenband grouper, where co-administration of FSH and androgen did not result in sex change (Kline *et al*., 2008).

The specific effect of FSH on fish gonadal development (*in vivo*) that is not linked to production of sex steroids is yet to be characterized (Schulz *et al*., 2010). However, recent *in vitro* trials in rainbow trout testis have shown that a set of genes positively respond to FSH even in the presence of trilostane, an inhibitor of androgen synthesis (Sambroni *et al*., 2013). Results from the same study indicated antagonistic effect of FSH and androgen on the expression of certain genes associated with germ cell proliferation such as *igf3*. Another *in vitro* trial in zebrafish testis has demonstrated that FSH, in the presence of trilostane, stimulated the expression of *igf3*, which promoted spermatogonial proliferation (Nóbrega *et al*., 2015). Evidence from our *in vivo* study and the *in vitro* studies above point to FSH initiating the process of sex reversal in groupers through a mechanism that involves sex steroid-independent genes or pathway.

The ovarian aromatase, which regulates estrogen production, is dependent on the expression of *cyp19a1a* gene and its transcriptional activator *foxl2*. Both of these genes were downregulated by rggFSH-treatment after 38 weeks in the present study. The *cyp19a1a* expression in the protogynous rice field eel increased during ovarian development and markedly downregulated during sex reversal (Zhang *et al*., 2008). Aromatase activity is important in maintaining female sex in groupers (Bhandari *et al*., 2005, 2004). Likewise, stable

expression of *foxl2* ensures femaleness in both hermaphroditic (Alam *et al*., 2008) and gonochoristic fish (Baron *et al*., 2004; Wang *et al*., 2007). Expression of *cyp19a1a* and *foxl2* are co-localized in the granulosa cells (Nakamoto *et al*., 2006) where FSH-R is also found (Alam *et al*., 2010). Our results, combined with the previous findings, show that FSH suppresses *cyp19a1a* and *foxl2* expression during the initiation of sex reversal.

The mRNA levels of *figla* and *bmp15*, which are known positive regulators of folliculogenesis, both displayed the same expression as the control in the present study. *In vivo* expression of *figla* during female to male sex change in wrasse was stable up to the early stage of sex change and then dropped towards the late stage (Horiguchi *et al*., 2017) concurrent with the elevation of androgen level. *In vivo* administration of androgen (MT) repressed the expression of *figla* in zebrafish (Lee *et al*., 2017). Similarly, mRNA level of *bmp15* was repressed in orange spotted grouper when treated with MT (Wu *et al*., 2015). Taken together, these observations suggest that androgen is required for the downregulation of *figla* and *bmp15*. It is interesting to note that in female zebrafish, disruption of *bmp15* gene resulted in failure of the oocytes to undergo secondary oocyte growth (cortical alveolar stage), and subsequent reversal of the fish into fully functional male (Dranow *et al*., 2016). With the negative regulatory effect of androgen on *bmp15* (Lee *et al*., 2017), inhibition of this gene could be one of the critical functions of 11KT during the advanced or final stage of sex reversal leading to the disruption of female environment (Dranow *et al*., 2016) and thus completion of the sex reversal process.

Three forms of *igf* are found in orange-spotted grouper (*igf1*, *igf2*, and *igf3*) of which *igf3* was found to be highly expressed in the gonad (Yang *et al*., 2015). We also found an *igf3* transcript in the tiger grouper gonad transcriptome. In zebrafish, *igf3* is upregulated by FSH (Morais *et al*., 2017) subsequently mediating its stimulatory effect on spermatogonial proliferation (Nóbrega *et al*., 2015). On the contrary, expression of *igf3* was constantly low in the rggFSH-treated group while it was significantly upregulated in the control group in the present study. It is therefore likely that the *igf3* is not involved in spermatogonial proliferation in tiger grouper.

In summary, the present study has produced a recombinant ggFSH using yeast and have demonstrated its *in vitro* and *in vivo* biological activities. Through long-term administration of rggFSH in sexually immature tiger grouper, we have shown that rggFSH stimulates ovarian

development but only up to cortical alveolar stage, after which rggFSH initiates sex reversal characterized by oocyte regression and spermatogonial proliferation. The initiation of sex reversal by rggFSH is mediated through a steroid-independent pathway. Prolonged administration of rggFSH during sex reversal suppresses steroidogenic activity which prevents the differentiation of accumulated spermatogonial cells thus arresting the sex reversal in the early phase.

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Chapter 3 **Oral delivery of plasmid follicle stimulating hormone for induction of gonadal development in tiger grouper *Epinephelus fuscoguttatus***

3.1. Abstract

Giant grouper is an excellent culture species due to its superior growth rate, however its large size at sexual maturity complicates physical handling and increases financial input as more food and larger facility is required. As a first step to address this issue, the present study aimed to determine whether oral delivery of FSH in the form of an expression plasmid (pFSH) can induce early sexual maturation in tiger grouper. The expression construct was designed in pcDNA3.1(-) utilizing the cytomegalovirus promoter to express a single chain follicle stimulating hormone (FSH) consisting of the complete nucleotide sequence of giant grouper FSH β subunit and mature common glycoprotein subunit α, and the carboxy-terminal peptide sequence from the human chorionic gonadotropin linking the two subunits. Next, the pFSH was encapsulated with liposome and chitosan and orally administered once to juvenile tiger grouper. The treatment prompted a significant increase in plasma level of the FSH protein. Long term trial was then conducted in sexually immature tiger grouper fed with pFSH weekly for 38 weeks. Results show that pFSH stimulated physiological effect consistent with the previously reported effect of injected FSH on groupers. Fish exhibited significantly larger oocyte diameter and cortical alveolar stage oocyte after eight weeks of pFSH feeding. Prolonged treatment (20 to 38 weeks) resulted in oocyte regression, proliferation of spermatogonial cells and appearance of spermatogenic cysts. Changes in the circulating levels of sex steroid hormones, FSH and vitellogenin as well as the expression profile of key genes in the gonad were analysed throughout the course of the trial. The present study is the first to demonstrate that an orally delivered plasmid gonadotropin impacted reproductive development in fish.

3.2. Introduction

Groupers are the primary commodity in the live reef fish trade (LRFT). The giant grouper *Epinephelus lanceolatus*, in particular, is one of the most sought-after and highly priced species in the LRFT. For fish farmers, the giant grouper is a preferred species of culture due to its remarkable growth rate, capable of attaining a commercial size of 0.6 to 1.0 kg in about six months while other grouper species take a year or more (Ho *et al*., 1997; Pierre *et al*., 2008; Sadovy *et al*., 2003).

Captive breeding of giant grouper is difficult due to its late age at maturation, taking about 4 years. Thus, broodstock development from hatchery-bred stock takes a long time during which expenses and risks (e.g. death due to diseases) are incurred. Once matured, handling of giant groupers is complicated due their large size – females reach sexual maturity at about 20 kg while males can reach up to 100 kg (unpublished data). Furthermore, their large size requires larger facility, more feed input, and additional labor hence a more expensive broodstock management operation. Their protogynous hermaphroditic nature, maturing first as female and then changing into male, is also a major challenge in obtaining breeders of both sexes.

While hormonal manipulations have been an integral part of controlling reproduction in aquaculture, techniques are primarily aimed at inducing captive broodstock to spawn (Mylonas *et al*., 2010). For instance, reproductive hormones such as the Gonadotropin-Releasing Hormone (GnRH) is extensively used for spawning induction of marine fish (Mylonas *et al*., 2010). GnRH is a hypothalamic neuropeptide that stimulates the pituitary gland to release gonadotropins into the circulation (Zohar *et al*., 2010). Gonadotropin, particularly Luteinizing Hormone (LH), prompts the gonadal synthesis of the maturation inducing hormone (17,20β-dihydroxy-4-pregnen-3-one), which in turn triggers final oocyte maturation and ovulation in females and spermiation in males, that culminates in spawning (Levavi-Sivan *et al*., 2010). The Human Chorionic Gonadotropin (HCG) is another hormone that exhibits the same effect as LH and is widely used for spawning induction (Mylonas *et al*., 2010).

Follicle-Stimulating Hormone (FSH) is another pituitary gonadotropin that is, in contrast to LH, involved during the early stages of oogenesis and spermatogenesis (Levavi-Sivan *et al*., 2010).

A number of studies have shown that administration of recombinant FSH stimulated oocyte growth in female and spermatogonial proliferation and differentiation in male in rainbow trout *Oncorhynchus mykiss* (Ko *et al*., 2007), Japanese eel *Anguilla japonica* (Hayakawa *et al*., 2008; Kazeto *et al*., 2008; M. Kobayashi *et al*., 2010), European sea bass *Dicentrarchus labrax* (Mazón *et al*., 2014), and yellowtail kingfish *Seriola lalandi* (Sanchís-Benlloch *et al*., 2017). In juvenile orange spotted grouper *E. coioides*, short-term administration of recombinant FSH stimulated the development of primary growth stage oocytes (Chen *et al*., 2012). Although these results show that FSH could be utilized to advance gonadal development, individual administration (i.e. intramuscular or intraperitoneal injection) is logistically impractical if inducing a stock of hatchery-bred groupers.

In this study, we orally delivered plasmid DNA encoding for the giant grouper FSH (pFSH) in order to induce early sexual maturation in grouper. *In vivo* expression of fed pFSH was first demonstrated followed by induction of sexual maturation in tiger grouper *E. fuscoguttatus* through long-term feeding experiment. Results show that fed pFSH successfully stimulated reproductive development in tiger grouper and particularly favored early sex reversal. To the best of our knowledge, this study is the first to demonstrate that an orally delivered plasmid gonadotropin impacted gonadal development in fish.

3.3. Materials and methods

3.3.2. Animals

Juvenile tiger groupers were obtained from the Igang Marine Station of SEAFDEC/AQD in Guimaras, Philippines. Fish were transported to SEAFDEC/AQD Tigbauan Main Station, Tigbauan, Philippines and were allowed to acclimate in the experimental set up for about a month. Two batches of juvenile tiger grouper were used in this study. The first batch consisted of 21 individuals with mean total body weight 198 ± 9 g and total length 22 ± 0.4 cm and was used for short term experiment (section 2.4). The second batch consisted of 20 individuals with mean total body weight 712 ± 37 g and total length 35 ± 0.6 cm and used for long term trial (section 2.5). Fish for each trial were separately stocked in tanks equipped with a flow-through water system. Fresh fish and squid were provided *ad libitum*. Fish were tagged with Passive Integrated Transponder (Biomark HPT9; Biomark) for subsequent identification.

This study was approved by the Animal Ethics Committee of the University of the Sunshine Coast (approval no. AN/A/16/102).

3.3.3. Plasmid FSH design and production

The complete nucleotide sequences encoding for giant grouper FSH β subunit (excluding stop codon) and mature ggCGα subunit were linked with the carboxy-terminal peptide (CTP) sequence from the human chorionic Gonadotropin (Fig 3.1.). The gene sequences were obtained from giant grouper transcriptomic data generated in-house (See Chapter 2). The whole sequence encoding for single-chain FSH was then inserted into the XhoI-EcoRI restriction site of pcDNA3.1(-) expression vector, which utilizes a cytomegalovirus (CMV) promoter. Synthesis of the expression vector was done commercially (GenScript, USA). Pure pFSH was transformed into *E. coli* which were subsequently used for production of large quantity of the plasmid. Plasmid purification was done using QIAGEN plasmid maxi kit following manufacturer's instruction.

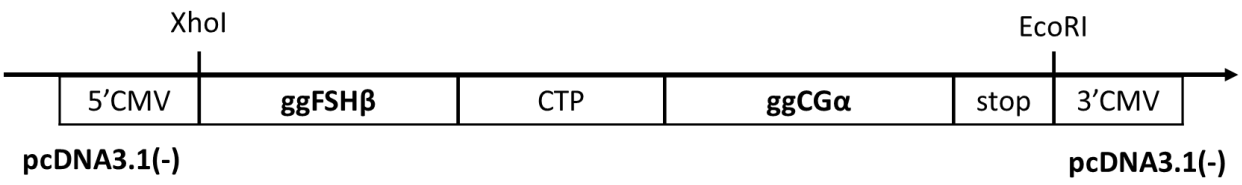

Fig. 3.1. Structure of a portion of the expression vector, pcDNA3.1, which encodes for a single-chain giant grouper FSH.

3.3.4. Encapsulation of pFSH

Liposome film was prepared by dissolving L-α-phophatidylcholine (Sigma) and cholesterol (Sigma) at 4:1 ratio in chloroform which was then aliquoted in a glass test tube and dried by heating at 65°C water bath. DNA plasmid dissolved in 1 ml water (up to 1mg/ml concentration) was then added into the liposome film followed by 6 cycles of freezing in liquid nitrogen and thawing at 55°C water bath. To break the lipoplex complex down, the solution was subjected to 10 sonication pulses and extrusion through a 100 nm membrane (10 times each side) (Avanti extruder set; Avanti Polar Lipids Inc., USA). Prior to feeding, liposome solution was mixed with 5 mg/ml chitosan solution (low molecular weight; Sigma). The procedure was done multiple times to encapsulate the needed amount of DNA plasmid for every treatment.

3.3.5. *In vivo* expression of orally delivered pFSH

To validate the encapsulation and oral delivery method, juvenile tiger groupers were fed once with either 0 (control, N=12) or 100 µg/kg BW pFSH DNA (N=12). In order to avoid unequal distribution of hormone, fish were individually fed with the exact dose of pFSH placed in a gelatin capsule. Blood samples were collected from both groups after 2, 4 and 7 days of feeding (3 fish each time point). Each fish was only used once for blood collection. Control samples were also collected prior to feeding. Blood was extracted from caudal vasculature using heparin-treated needles (23 G, 1½ in). Plasma was separated from blood by spinning at 4000 *x*g for 15 minutes at 4°C, and samples were kept at -80°C until analysis.

3.3.6. Induction of sexual maturation in tiger grouper

Fish were fed with pFSH once every week for 38 weeks at 0 (control) or 100 µg/kg BW. Oral administration was done as in section 3.3.5. Three to four individuals were then sacrificed after 8, 20 and 38 weeks of treatment. During these sampling points, gonad tissue samples

were immediately stabilised in RNALater for subsequent gene expression analysis or fixed in Boiun's solution for histological analysis. Prior to dissection, blood samples were collected from the caudal vasculature using heparin-treated needles. In addition, blood samples were collected from fish after 14, 26 and 32 weeks of feeding. Plasma were obtained and stored as in Section 3.3.5.

3.3.7. Sample analyses

Tissue processing for gonad histological analysis was done by standard histological techniques. Tissues were sectioned at 5 µm and stained with hematoxylin and eosin. Sections were then examined under Leica DM5500 B microscope and photos were taken using the built-in camera. Oocyte sizes were measured using ImageJ (version 1.51j8).

Plasma levels of E_2 and 11KT were analysed using commercially available ELISA Kit (11-KT ELISA, Cayman Chemicals). For T, ELISA was done following the previously described method (Rahman *et al*., 2000) using commercially available standard (T; Sigma), T antibody-tracer sets (CosmoBio), and anti-species antibody (mouse anti-rabbit IgG; Sigma).

FSH was quantified from plasma samples using a heterologous ELISA, which was developed for yellowtail kingfish (ytk; *Seriola lalandi*) FSH and is currently in preparation for publication. The ELISA consisted of: recombinant full length ytkFSH (standard), rabbit anti-recombinant ytkFSHβ (primary antibody), and peroxidase conjugated goat anti-rabbit antibody (secondary antibody). Serially diluted grouper FSH and recombinant ytkFSH were initially assayed and showed parallelism of reactivity.

Plasma levels of vitellogenin were analysed using grouper vitellogenin ELISA. The system utilised a sandwich design using rabbit-anti grouper vitellogenin (capture antibody), purified grouper vitellogenin (standard), and rabbit-anti grouper vitellogenin labelled with horseradish peroxidase (detection antibody). The standard and antibodies were prepared by Prof. Akihiro Takemura, University if the Ryukyus, Japan. Details of this ELISA will be published elsewhere.

3.3.8. Quantitative real-time PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen) following manufacturer's instruction. RNA quality was validated by formaldehyde denaturing gel electrophoresis and on Bioanalyzer 2100 (Agilent). RNA extracts were reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen). A 400 to 450 bp fragment of the genes of interest was amplified by PCR using primers shown in Table 2.1. The amplicons were purified using a commercial kit (QIAquick PCR Purification kit; Qiagen) and then used to construct a 7-point standard curve ranging from $1.0x10^8$ to $1.0x10^2$ copies per µl for each gene. The primers used for sample qPCR analysis and construction of standard (Table 2.2) were designed to amplify 110 to 150 bp sequence located within the span of the first primer pair.

Ten microliter qPCR reactions were prepared containing 5 µl of 2X Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 0.4 µl each of forward and reverse primers (10 µM), 3.2 µl water and 1 µl template. Each run included two standard points ($1.0x10^7$ and $1.0x10^4$ copies/µl) and a no template control. All samples and control were run in duplicate. The qPCR was done on Corbett Rotor-Gene 6000 (Qiagen) in Rotor-Disc 72. The thermal cycling conditions were: 50°C for 2 min, 95°C for 2 min, 40 cycles of 95°C and 60°C for 15 and 20 sec, respectively. To determine gene copy number, the threshold cycle (C_T) value of each sample was compared to the standard curve.

3.3.9. Statistical analysis

The data are presented as mean ± SEM. Mean values of control and pFSH were compared using independent-samples t-test and differences were considered significant at $P<0.05$. Analysis was done in SPSS Statistics 17.0.

3.4. Results

3.4.2. *In vivo* expression profile of fed pFSH

The profile of circulating FSH protein in the tiger groupers fed once with pFSH was analyzed in this study using a heterologous ELISA for FSH (Fig. 3.2.). Fish fed with pFSH exhibited a statistically significant increase in plasma FSH that peaked after four days and then declined to basal level after seven days. Variation among replicate fish was notably minimal, owing to the individual feeding with exact dose of pFSH. Control fish displayed a steady state level of FSH.

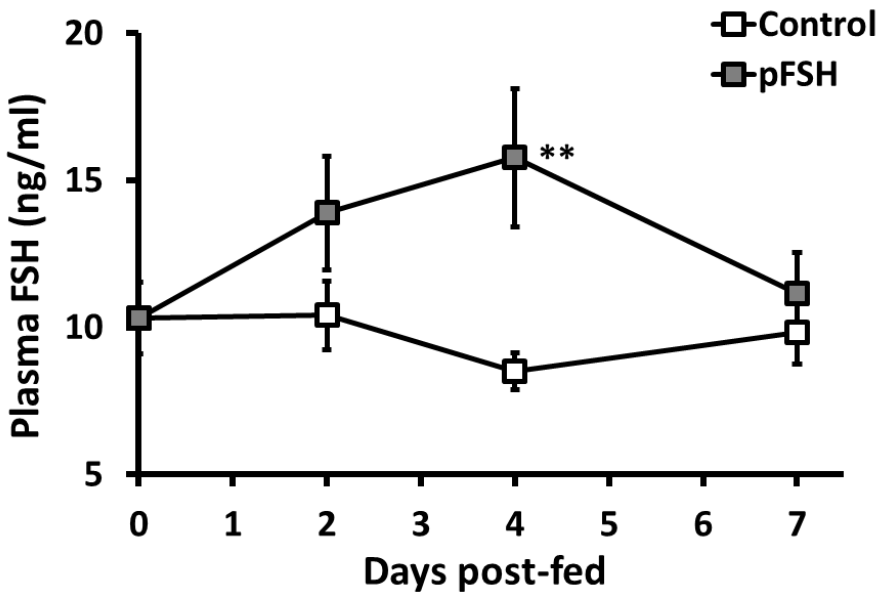

Fig. 3.2. FSH protein expression profile after feeding with pFSH (n=3). Asterisks (**) denote significant difference with control level (P<0.05).

3.4.3. Effects of pFSH on gonad and plasma hormones

Sexually immature tiger grouper (680-750 g BW) were used to demonstrate the effect of pFSH feeding on grouper reproductive development. Fish fed with pFSH for eight weeks had cortical alveolar stage oocytes, which were more advanced compared to primary growth stage oocyte found in control fish (Fig. 3.3. A, D). In addition, pFSH-fed fish had significantly larger oocyte diameter (Fig. 3.4. B) and significantly higher plasma E_2 level (Fig. 3.5. A) and plasma vitellogenin (Fig. 3.6. B). These data indicate that pFSH-feeding induced ovarian development.

Continued weekly feeding with pFSH for 20 weeks resulted in significantly lower GSI and oocyte diameter (Fig. 3.4.). The gonad histology of pFSH-fed fish was characterized by a regression of oocytes and proliferation of spermatogonial cells (Fig. 3.3. E). Plasma E_2 of pFSH-fed fish dropped to basal level (Fig. 3.5. A). These observations were indicative of sex reversal. On the other hand, control fish showed increased oocyte size and advancement of oocyte to cortical alveolar stage.

After 38 weeks, all pFSH-fed fish continued to indicate histological signs of sex reversal including regression of oocytes and accumulation of spermatogonial cells. Although few spermatogenic cysts containing spermatocytes were noted (e.g. Fig. 3.3. G), the differentiation of spermatogonial cells appeared to be minimal. Plasma 11KT level increased, although no significant difference was found. In contrast, control fish had cortical alveolar stage oocytes and significantly higher level of circulating vitellogenin (Fig. 3.6. B) indicating the progression of ovarian development except one fish which exhibited spermatogonial proliferation (data not shown).

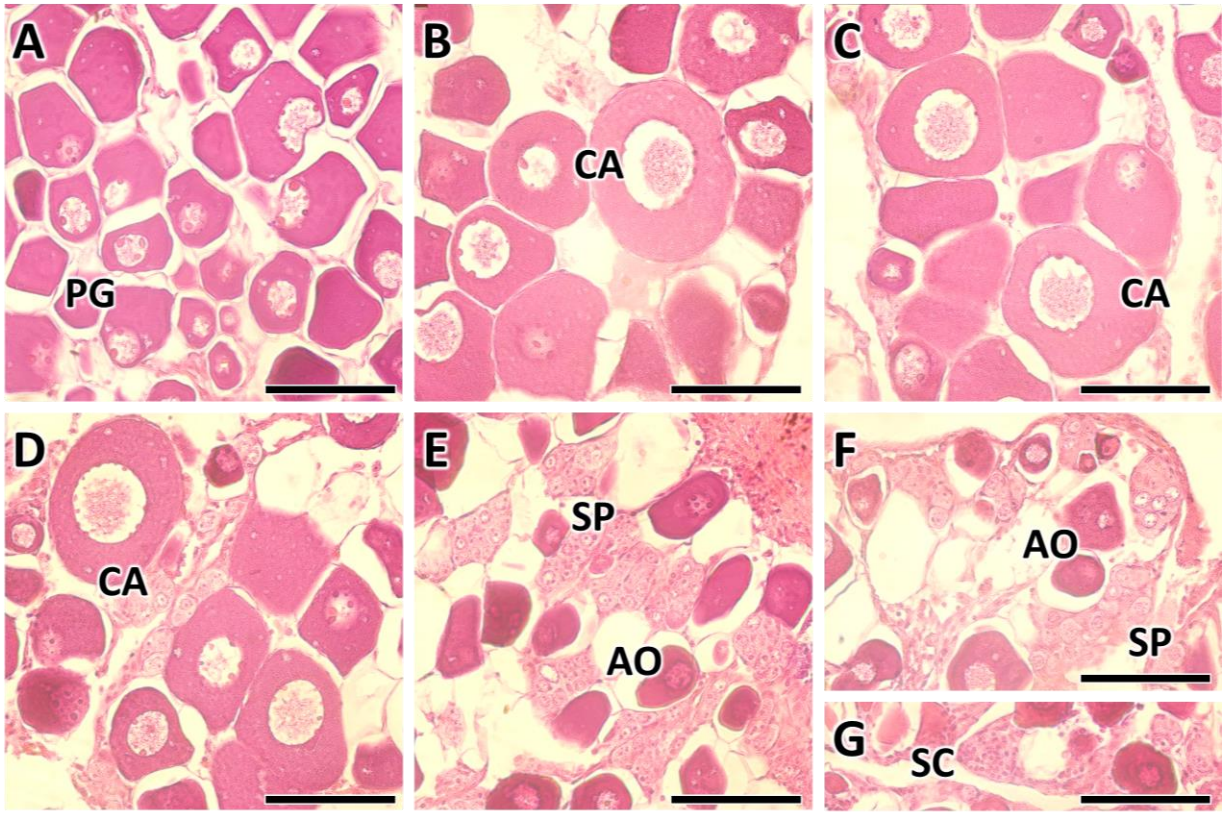

Fig. 3.3. Representative gonad histological sections of control (A-C) or pFSH-fed (D-G) tiger grouper after 8 (A, D), 20 (B, E) and 38 (C, F, G) weeks of treatment. PG = primary growth stage oocyte, CA = cortical alveolar stage oocyte, AO = atretic oocyte, SP = spermatogonial cells, SC = spermatogenic cyst. Scale bar = 50 µm.

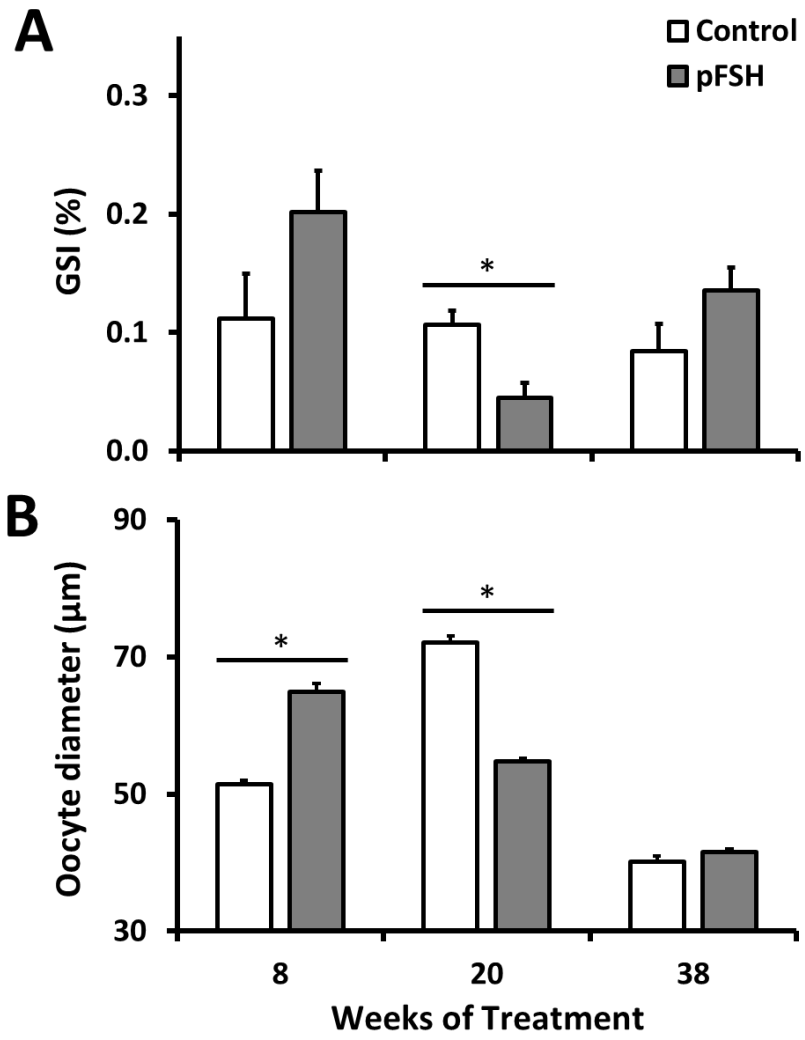

Fig. 3.4. Effect of pFSH feeding on GSI (A) and mean oocyte size (B) of sexually immature tiger grouper. Each bar represents a mean of 3-4 fish ± SEM. Values marked with asterisk (*) are significantly different at *P*<0.5.

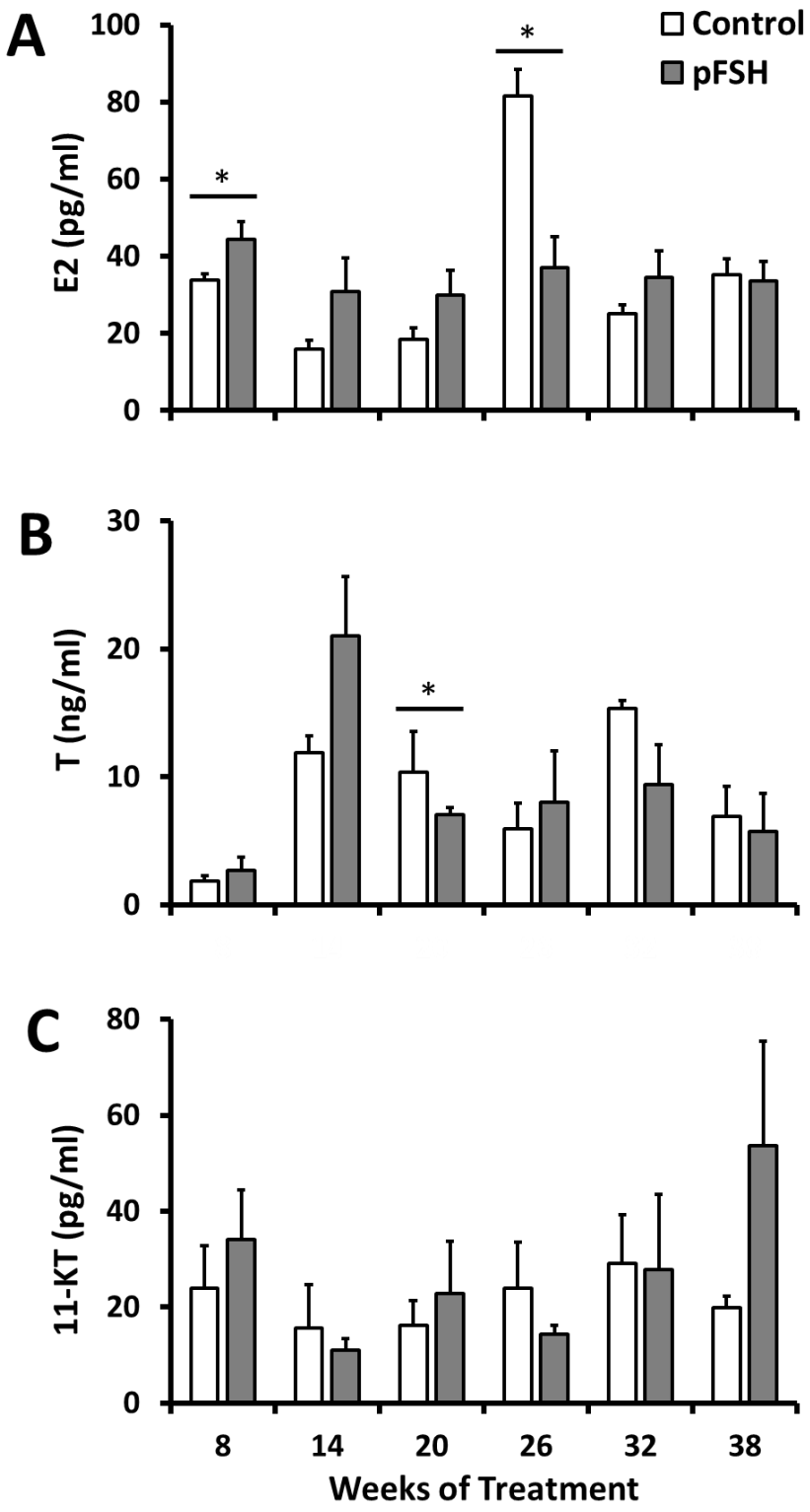

Fig. 3.5. Effect of pFSH feeding on circulating levels of sex steroid hormones: E2 (A), T (B), and 11KT (C). Each bar represents a mean of 3-5 fish ± SEM. Values marked with asterisk (*) are significantly different at *P*<0.5.

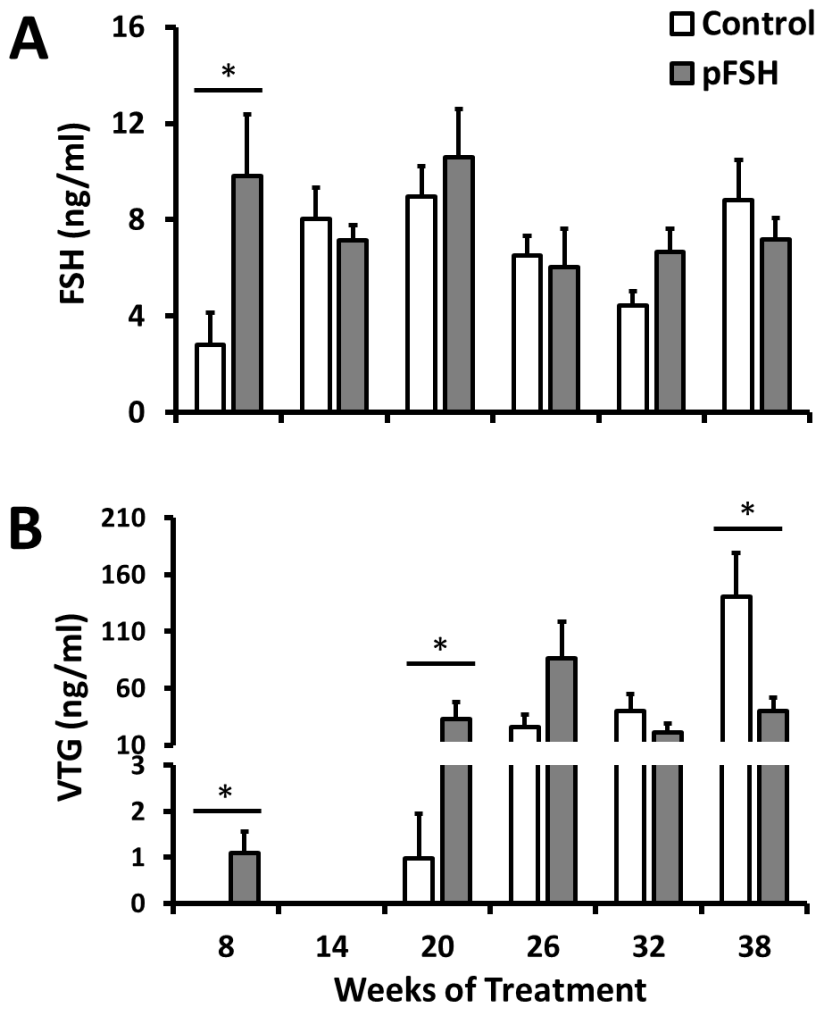

Fig. 3.6. Effect of pFSH feeding on plasma levels of FSH (A) and VTG (B). Each bar represents a mean of 3-5 fish ± SEM. Asterisk (*) indicate significant difference (P<0.05) between the two treatment groups within a time point.

3.4.4. Effects pFSH on gonadal gene expression

Quantitative PCR analysis of selected genes in the gonad showed that aromatase gene *cyp19a1a* and its transcriptional regulator *foxl2* were significantly suppressed towards the end of the trial in pFSH-fed fish. On the other hand, control fish showed a progressive increase throughout the trial supporting the histological progression of oogenesis. Expression of *figla* and *bmp15*, which are both essential in maintenance of femaleness, showed no significant difference with the control.

Gene expression of cyp11b, which codes for an enzyme that synthesizes 11KT, was significantly low after 38 weeks. Other male-related genes including *sox9*, *dmrt1*, and *amh* exhibited similar expression as in control fish.

The mRNA levels of *fshr* did not vary between control and pFSH fed fish. On the other hand, *igf3* expression was suppressed after 38 weeks of treatment.

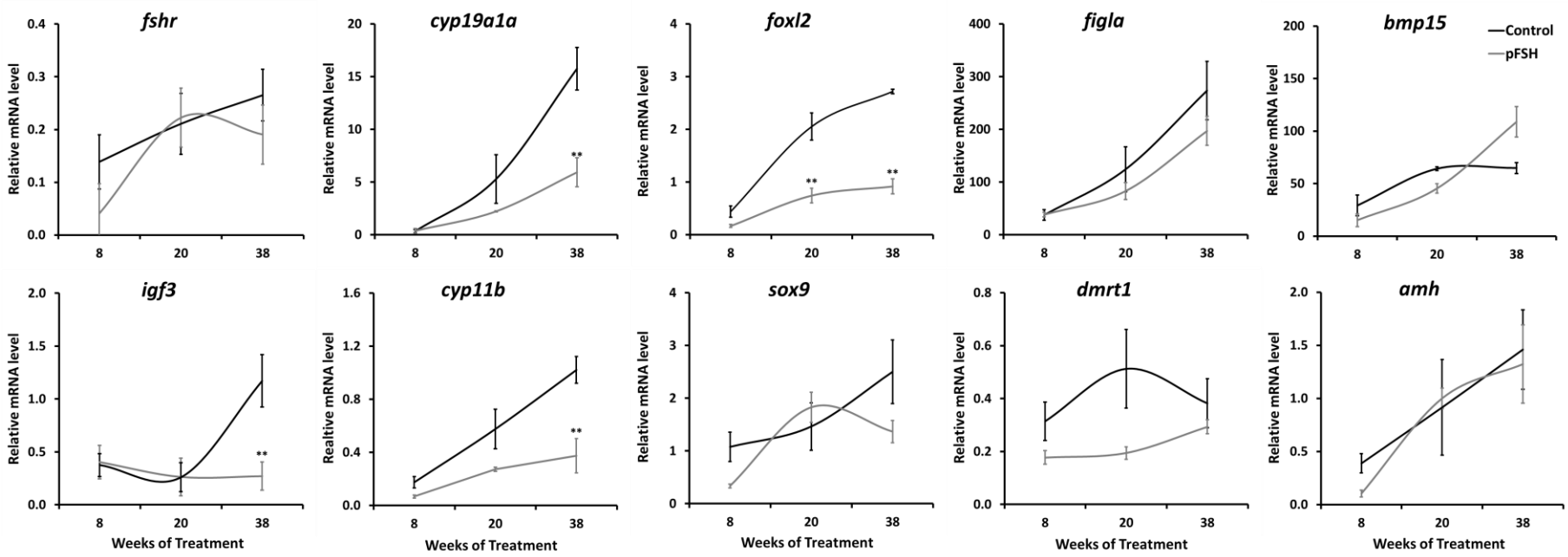

Fig. 3.7. Effect of pFSH feeding on expression of gonad genes. Error bars denote SEM. Asterisks (**) denote significant difference (P<0.05) between two treatment groups within a time point.

3.5. Discussion

This study aimed to determine whether oral delivery of FSH in the form of an expression construct can induce early sexual maturation in tiger grouper.

Gene therapy has been explored in fish, however methods employed so far still involve physical handling. Injection of plasmid DNA (pDNA) that resulted in gene expression was first reported in rainbow trout *Oncorhynchus mykiss,* wherein luciferase activity was detected in skeletal muscle following intramuscular injection of luciferase gene (Anderson *et al*., 1996). Succeeding experiments in Zebrafish *Danio rerio* (Tan and Chan, 1997), Atlantic salmon *Salmo salar* (Gómez-Chiarri and Chiaverini, 1999), Glass catfish *Kryptopterus bicirrhus* (Dijkstra *et al*., 2001), and Tilapia *Oreochromis niloticus* (Ramos *et al*., 2005) have consistently shown expression of reporter gene in the muscle following intramuscular injection. In rainbow trout fingerlings, recombinant antibody was detected in the plasma 12 days after intramuscular injection of pDNA encoding the antibody (Lorenzen *et al*., 2000) showing that the expressed protein is released by transfected muscle cells into the circulation. Recently, *in vivo* electroporation of the DNA was tested to further enhance the uptake of injected plasmid DNA (LH and FSH) in European sea bass *Dicentrarchus labrax* (Mazón *et al*., 2014, 2013). This resulted in increased plasma LH peaking after 20 days while plasma FSH was significantly higher 15 to 23 days after the treatment. More importantly, plasmid therapy of FSH and LH induced spermatogenesis and spermeiogenesis, respectively in sea bass (Mazón *et al*., 2014, 2013). Electrotransfer of plasmid LH in orange spotted grouper *E. coioides* was shown to induce ovarian primary oocyte growth (Lv *et al*., 2017). Altogether, these studies show pDNA administration could be an alternative form in providing long-lasting availability of hormones in fish. However, these studies have so far relied on individual treatment of fish through intramuscular or peritoneal injection. Hormonal administration that involves physical handling is time consuming and stressful for fish especially when dealing with a population of cultured fish or large-sized broodstock such as giant grouper.

In the present study, we have tested an oral route of administering plasmid DNA that encodes for giant grouper FSH. Encapsulation with liposomes was first developed to deliver pharmacological agents, which are vulnerable to degradation in the digestive tract. Among the first evidences of the efficacy of this method was a study which demonstrated in rats that

oral administration of liposome-encapsulated insulin moderated the blood glucose to a level comparable to that of insulin administered intramuscularly (Patel and Ryman, 1976). The encapsulation method was later modified to coating the liposome with chitosan, a mucoadhesive substance, which improved the *in vivo* physiological effect of insulin (Takeuchi *et al*., 1996). The use of low molecular weight chitosan in preparing the modified mucoadhesive liposomes was shown to prolong the physiological effect (i.e. lowering the blood calcium level) of calcitonin in rat compared with using high molecular weight chitosan (Thongborisute *et al*., 2006). Recently, liposomes were also used to encapsulate plasmid DNA containing influenza M1 protein gene which was expressed *in vivo*, stimulating immune response after feeding to mice (Liu *et al*., 2014). In the present study, the liposome and chitosan encapsulated pFSH significantly increased the plasma level of FSH protein and induced physiological effects after long term administration. Altogether, these findings show the efficacy of liposome encapsulation in stabilizing bioactive compounds including protein and DNA during passage through digestive tract.

In fish, studies on oral delivery of plasmid DNA has been undertaken for its application in DNA vaccines. Feeding of chitosan-encapsulated plasmid DNA containing β-galactosidase reporter gene resulted in positive enzyme activity in spleen, stomach and gill tissues of Tilapia *Oreochromis niloticus* (Ramos *et al*., 2005), and in gut, liver and muscle tissues in gilthead sea bream *Sparus aurata* (Sáez *et al*., 2017). In two separate studies in Asian sea bass *Lates calcarifer*, chitosan facilitated intestinal uptake of an encapsulated DNA vaccine which resulted in immune resistance when challenged with pathogen (Rajesh Kumar *et al*., 2008; Vimal *et al*., 2014). Similarly, liposome encapsulation stabilized and promoted uptake of antigens (e.g. *Aeromonas salmonicida* antigen) that are able to generate immune response in Common carp *Cyprinus carpio* (Irie *et al*., 2005, 2003). In the present study, plasma FSH significantly increased in fish fed with pFSH encapsulated with liposome and chitosan. Taken together, our results and those from previous studies confirm that DNA can be orally administered and subsequently expressed when encapsulated with liposome and chitosan.

The pFSH fed to tiger groupers in the present study was presumably transfected and expressed in the digestive, gill and muscle tissues similar to that reported in Tilapia (Ramos *et al*., 2005) and gilthead sea bream (Sáez *et al*., 2017). However, previous studies used only chitosan to encapsulate plasmid DNA while the present study used both liposome and

chitosan. Thus, further studies are required to compare *in vivo* transfection sites of orally delivered plasmid DNA encapsulated using different protocols.

Previous studies validated *in vivo* expression of fed pDNA, however data were limited to either qualitative (or descriptive) observations regarding the activity of reporter gene in various tissues or the immune response elicited by antigen gene expression. In the present study, we have quantified the plasma levels of FSH protein following a single administration of the DNA construct thus providing clear information as when and how long is pFSH expressed and translated into protein.

Tiger grouper fed with pFSH for eight weeks developed a more advanced oocyte stage (cortical alveolar stage) and significantly larger oocyte diameter, which was associated with a marked increase in plasma estrogen level. These responses are identical to that described in tiger grouper (see chapter 2) and in sevenband grouper *E. fasciatus* (Kline *et al*., 2008), which exhibited cortical alveolar stage oocytes after receiving recombinant FSH injection. Previous trials in female gonochoristic fish have shown that oogenesis is hastened upon treatment with FSH protein as described in rainbow trout *Oncorhynchus mykiss* (Ko *et al*., 2007), Japanese eel *Anguilla japonica* (Kazeto *et al*., 2008) and yellowtail kingfish *Seriola lalandi* (Sanchís-Benlloch *et al*., 2017). Finally, the effect of pFSH after eight weeks of feeding coincides with the effect of intramuscular injection of rggFSH in tiger grouper (refer to Chapter 2). Our results show that orally delivered pFSH prompted a physiological response that is comparable to injected protein FSH.

After 20 and 38 weeks, a significant reduction in GSI and oocyte diameter was observed. Gonad histology was characterized by regression of oocytes, proliferation of spermatogonial cells and occurrence of spermatogenic cysts containing spermatocytes. These observations indicate that prolonged feeding of pFSH after cortical alveolar stage induces sex reversal. Again, this is identical to the response exhibited by tiger grouper after 20 to 38 weeks of rggFSH treatment (refer to Chapter 2). FSH-induced sex reversal was described in mature female honeycomb grouper *E. merra* after single injection of purified bovine FSH at 50 or 500 ng/fish (Kobayashi *et al*., 2010). In the bidirectional sex changing gobiid fish *Trimma okinawae*, the direction of sex reversal is dependent on which gonadal tissue (ovary or testis) possess higher receptor numbers for FSH (Kobayashi *et al*., 2009). In the case of female zebrafish

Danio rerio (Zhang *et al*., 2015) and medaka *Oryzias latipes* (Murozumi *et al*., 2014), genetic disruption of FSH or its receptor causes sex reversal into males. Taken together, these findings show that FSH plays a role in sex reversal of grouper. Our results suggest that the regulatory role of FSH on sex reversal is acquired at cortical alveolar oocyte stage.

In the present study, the mRNA expression of key male-related genes such as *dmrt1* and *amh* showed no significant variation between control and pFSH fed fish. In comparison, in orange spotted grouper at the late transition stage of female to male sex reversal, where the gonad is predominated by differentiated spermatogonial cells, expression of *dmrt1* and *amh* was significantly elevated (Alam *et al*., 2008; Wu *et al*., 2017; Xia *et al*., 2007). In protogynous rice field eel, a significant increase in gonadal expression of *dmrt1* and *amh* was exclusively observed in the late transition stage of sex reversing individuals (Hu *et al*., 2015; Huang *et al*., 2005). The expression of *sox9* gene, which is a marker of spermatocytes and spermatids in orange spotted grouper (Luo *et al*., 2010), showed no significant variation between control and pFSH-fed fish in the present study. Moreover, female associate gene including *figla* and *bmp15* did not significantly vary between treatment groups throughout the trial. Both *figla* and *bmp15* are known to play vital role in folliculogenesis in fish. These observations suggest that the sex reversal observed in the present study between 20 and 38 weeks has remained at the initiation or early stage. The present study shows that pFSH treatment only acted to initiate sex reversal.

Between 20 and 38 weeks, when sex reversal was observed in the present study, no significant increase in the endogenous levels of 11KT was noted while the expression of *cyp11b* was significantly low than control fish. *In vitro* incubation of orange spotted grouper ovarian tissue with homologous recombinant FSH protein resulted in release of T and E2 however steroid secretion was significantly reduced at high FSH dose (Chen *et al*., 2012). Similarly, honeycomb grouper injected with high dose of FSH (500 ng/fish) had significantly lower plasma 11KT than those injected with low dose (50 ng/fish) (Kobayashi *et al*., 2010). The present and previous results show that FSH inhibits 11KT production, which could explain why only limited differentiation of spermatogonial cells were observed in the present study. In honeycomb grouper, pituitary expression of *fshβ* displayed a surge at the initiation of natural female to male sex change but subsequently dropped as the process progress (Y. Kobayashi *et al*., 2010). Taken together, a withdrawal of pFSH treatment after the initial treatment may be necessary

to release the androgen inhibition which will then allow for the differentiation of spermatogonial cells thus the completion of sex reversal.

In summary, this study is the first to demonstrate that plasmid FSH can be orally administered and induce gonadal development in tiger grouper. We have shown that oral delivery of liposome- and chitosan-encapsulated pFSH results in subsequent *in vivo* expression of FSH protein. The effect of pFSH feeding on gonadal development of sexually immature tiger grouper is stage specific, such that it promotes oocyte development in immature individuals but initiates sex reversal in individuals that have attained cortical alveolar stage.

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Chapter 4 **Final conclusions**

Captive breeding of giant grouper is difficult due to their large size and late age at sexual maturation. In order to address this issue, the present study was undertaken to develop a method to induce early sexual maturation so that breeding could be done using smaller and younger broodstock. Follicle stimulating hormone, a pituitary gonadotropic hormone that regulates the early stages of oogenesis and spermatogenesis in fish, was utilized in this study. Using sexually immature tiger grouper as a model species, induction of gonadal development was done through intramuscular administration of recombinant giant grouper FSH (rggFSH) and oral administration of expression plasmid that codes for giant grouper FSH (pFSH).

Recombinant ggFSH was produced using the yeast *P. pastoris* expression system. Through *in vitro* bioassays, the rggFSH was shown to be capable of activating the FSH-Receptor and stimulating ovarian synthesis of Estardiol (E_2) and Testosterone (T). The use of recombinant protein technology enabled this study to obtain sufficient quantity of pure FSH to demonstrate the *in vivo* function of FSH in grouper reproductive development. The use of rggFSH also presented an advantage of not possessing biosafety risk (e.g. transmission of disease) which is associated with the use of pituitary extract.

Weekly intramuscular injection of rggFSH for 8 weeks in sexually immature tiger grouper promoted oocyte growth and development to cortical alveolar stage. This observation was concurrent with a significant increase in circulating E_2 level. Further treatment to 20 weeks resulted in the degeneration of oocytes, proliferation of spermatogonial cells and significant decrease in plasma E_2, which are all indicative of female to male sex change. These observations clearly show that the function of FSH in grouper reproductive development varies depending on the developmental stage of the gonad. Based on these results, we conclude that FSH initially simulates ovarian development but only up to cortical alveolar stage, after which it initiates sex reversal.

It was noted in the present study that the prolonged treatment with rggFSH to 38 weeks, after the initiation of sex reversal was observed (at 20 weeks), did not advance the transition of gonad to functional testis, as the accumulated spermatogonial cells remained largely

undifferentiated. During this period, circulating 11KT, T and E_2 were restricted at basal level. Gene expression analysis showed that the rggFSH treatment significantly suppressed steroidogenic genes including *cyp11b* gene (involved in 11KT synthesis) as well as *cyp19a1a* and *foxl2* genes (involved in E_2 synthesis). These results show that the rggFSH actively inhibited steroidogenic activity while initiating female to male sex reversal. Therefore, we conclude that in the grouper, FSH-induced sex reversal involves a pathway that is independent of sex steroids. This also suggests that withdrawal of rggFSH-treatment might be needed in order to release the inhibition of sex steroid production which, in turn, would have advanced the female to male transition. Since the specific effect of FSH on fish gonadal development that is not mediated through steroid hormones is primarily unknown, this area merits deeper investigation.

Results from the present study show that individuals that exhibit cortical alveolar stage oocyte were prompted to initiate sex reversal when treated with rggFSH. This raises the question on how, and what, induces vitellogenesis in grouper. The involvement of luteinizing hormone during vitellogenesis is highly possible as it is capable of stimulating ovarian secretion of E_2, the known enhancer of vitellogenin synthesis. For FSH, regulation of vitellogenesis may require critical maintenance of low circulating level. The distinct or complementary roles of FSH and LH during this stage of gonadal development in grouper needs to be further evaluated.

The findings from the present study challenge the widely-accepted model of grouper sex reversal as a result of an interplay between androgen and estrogen. Results from this study suggest that the FSH actively inhibited androgen synthesis while successfully initiating sex reversal. On the other hand, it is clear that androgen's role in sex reversal is primarily focused on the differentiation of spermatogonial cells, which takes place at the late transition stage. This study recognizes that the complementary effect of FSH and androgens is fundamental to the completion of sex reversal. The interplay between FSH and androgens needs in-depth study in order to better understand the molecular basis of sex change in grouper.

An alternative administration method of FSH through oral delivery in plasmid form was assessed in the present study. In a short-term trial, single oral administration of liposome and chitosan-encapsulated pFSH resulted in the increase of circulating FSH protein level that

peaked after 4 days and declined to basal level after 7 days. This confirmed that liposome and chitosan facilitated pFSH passage through digestive tract and *in vivo* expression. Results from the short-term trial provided the basis for further studies on optimizing treatment dose so as to manipulate the onset and duration of fed-pFSH *in vivo* expression for application in research or in the industry.

Long term weekly oral administration yielded physiological responses that were consistent with the effects of injected rggFSH. Initially, fed pFSH stimulated oocyte growth and development up to cortical alveolar stage and then initiated female to male sex reversal. Extended treatment with pFSH, however, did not advance the process of sex reversal but rather inhibited the expression of steroidogenic genes (i.e. *cyp11b*, *cyp19a1a* and *foxl2*), which resulted in the lack of sufficient level of androgen to stimulate differentiation of spermatogonial cells. These results reinforce the idea that FSH promotes either ovarian or testicular development, depending on the developmental stage of the gonad and recognises the cortical alveolar stage as a critical point where there is a shift in the effect of FSH. Results from this experiment also support the idea that the initiation of sex reversal by FSH is based on a steroid-independent mechanism. The present study is the first to demonstrate successful oral delivery of plasmid gonadotropin which impacted reproductive development in fish.

The successful oral delivery of pFSH provides an alternative hormonal administration method in aquaculture. Hormones are generally administered through injection which requires a labor intensive and stressful process of capturing, crowding, and exposing the fish to anesthetic agent. Oral delivery is an excellent method for inducing early maturation in a large stock of cultured fish or in large-sized fish such as giant groupers. With further optimization, hormones can be administered to obtain higher level and prolonged presence in the circulation. The method can also be used to administer LH which will significantly cut the time, labor and stress during routine induced-spawning operation.

Overall, this study has shown that sexual maturation in grouper can be successfully induced using FSH manipulation either through intramuscular injection of rggFSH or feeding of pFSH. The FSH treatment can be strategically employed to obtain either female or male broodstock. This study provides a valuable tool that could significantly shorten the period for broodstock development and will allow captive breeding using more manageable sized fish. By shortening

the life cycle in captivity, this will also hasten the progress in marker-assisted broodstock management programs. This study successfully addressed one of the major gaps in giant grouper captive breeding and can also be extended to other late-maturing and large-sized culture species.