Application of aquaculture biotechnology to fish reproductive endocrinology

Thesis by

Pablo J. Sanchis-Benlloch

BSc: Marine Science, MSc: Sustainable aquaculture

Submitted in partial fulfilment for the degree of

Doctor of Philosophy (PhD)

Faculty of Science, Health, and Education

University of the Sunshine Coast

Sippy Downs, QLD, Australia

December 5, 2016 (Defended September 6, 2016)
Dedicated to all my family, specially to my parents Jose Manuel & Maria Jose

“If I have seen further than others, it is by standing upon the shoulders of giants”

(Isaac Newton)
Abstract

This project is an industrial PhD research, which means that it is a university research planned to have a direct application for the industry collaborator of the project, CleanSeas Tuna Ltd (CST, Arno Bay, South Australia). Relevant biotechnological methods were applied to manipulate the endocrine regulation of reproduction of the main farmed species at CST. Each of the species cultured presents a number of bottlenecks that affect directly or indirectly the reproductive performance of the broodstock. This PhD reports on the investigations aimed in the long term to enhance the aquaculture of Yellowtail kingfish (*Seriola lalandi*, YTK) and Southern Bluefin tuna (*Thunnus maccoyii*, SBT).

Currently, a genetic breeding program is taking place at CST and there is interest in shortening the breeding period to increase genetic gain. The first step towards shortening the breeding period is the advancement of sexual maturation or puberty. We generated slow release EVAc implants for chronic peripheral administration of kisspeptin peptides (Kiss1-15 and Kiss2-12) to immature YTK female. The estradiol (E2) and Vitellogenin (Vtg) plasma levels increased during the course of the trial, and by the end of the trial, fish from the Kiss1-15 treatment group showed the highest GSI, E2 and Vtg levels, although differences between the groups were not statistically significant. Plasma E2 levels from the Kiss1-15 treatment group at the start and end of the trial were significantly different. Results provide evidence that Kiss1-15 treatment has a stimulatory effect in female YTK and set up the basis for further studies in order to induce early maturation in this species. We also engineered a Fsh recombinant hormone to induce reproductive development in immature female and early maturing male YTK. The recombinant hormone was produced in *Pichia pastoris* and its biological activity was confirmed by *in-vitro* and *in-vivo* bioassays. Incubation of ovarian and testicular fragments with the recombinant hormone stimulated E2 and 11 Testosterone (11KT) secretion, respectively. *In-vivo* trial in immature female YTK resulted in a significant increase of plasma E2 levels and development of oocytes. In males at the early stages of puberty, advancement of spermatogenesis was observed, however plasma 11KT levels were reduced when administered with rytkFsh. Results suggest that optimization of dosage, frequency and duration of administration could facilitate achieving
commercially applicable protocol for inducing early maturation in farmed female YTK and other late maturing species such as SBT. In this project, a hybrid competitive enzyme linked immunosorbent assay (ELISA) for YTK Vtg was developed. The developed ELISA would be useful for the YTK aquaculture industry as it can be used to assess maturation stage and effectiveness of hormonal therapies. This ELISA could also be used in SBT due to the similarity of the VTG nucleotide sequences of the two species.

The current method of hormone administration in large bodied fish, such as spawning induction in SBT, is a very labor-intensive method for the workers and stressful method for the fish. In this context, methods using oral delivery would create a breakthrough in the capacity to control spawning and reproductive development. As part of this PhD research, diets were developed containing GnRH encapsulated either in alginate-chitosan particles (containing GnRHa) or in yeast (expressing SBT GnRH1) and tested in tilapia as model animal for SBT. These encapsulation methods are intended to protect the hormone from degradation while in the digestive tract until its release to the bloodstream. Results reveal that both encapsulation methods appeared to hold the desired hormones. HPLC analysis revealed that alginate-chitosan particles incorporate 1% (w/w) GnRHa and that all the encapsulated GnRHa was released after 1hr. The expression of recombinant SBT GnRH1 was confirmed by PCR, HPLC and mass spec. Yeast particles where found to contain 0.03% of rsbtGnRH1 (w/w). In-vivo experiments in tilapia showed that doses of 0.6µg GnRHa/g did not stimulate spawning at temperatures under 23°C. Results reveal that both of the encapsulation methods appeared to hold successfully the desired hormones setting up the basis for further studies in exploring these techniques as potential effective vehicle for the oral delivery of GnRHs and other relevant related peptides.
Declaration of originality

This thesis is formed of my original work, and consists of no material previously published or written by another person except where due references has been made in the text.

I have stated the contribution of the others to my thesis as a whole, including statistical assistance, data analysis and significant technical procedures and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my higher degree by research candidature. None of the results presented in this thesis were submitted to qualify for another degree.

I hereby declare that the content of the above statement is completely true.

Signature: Date: December 5, 2016
Statement of contributions to jointly authored works contained in the thesis:

**Chapter 3 (Published):** Sanchis-Benlloch, P J., Nocillado, J., Ladisa, C., Aizen, J., Miller, A., Shpilman, M., Levavi-Sivan, B., Ventura, T., Elizur, A. *In-vitro and in-vivo biological activity of recombinant yellowtail kingfish* (*Seriola lalandi*) *follicle stimulating hormone.* doi: [http://dx.doi.org/10.1016/j.ygcen.2016.03.001](http://dx.doi.org/10.1016/j.ygcen.2016.03.001).

PS; Wrote the manuscript, contributed to the design of the experiments, and carried out the recombinant hormone expression and production in yeast, *in-vivo* and *in-vitro* assays, histology and the analysis of the results. JN; Conceived and contributed to the design of the experiments, performed the cloning and identification of ytkFSH, obtained the funding, contributed to all the experimental work, and the preparation of the manuscript. CL; Assisted during the hormonal production and sampling of the *in-vivo* assay. AM; Carried out all fish work husbandry; MS and BL; Carried out the *in-vitro* heterologous receptor assay. JA and TV; Contributed to the yeast work. AE; Conceived and designed the experiments, obtained the funding and contributed to the preparation of the manuscript. All authors read and approved the final manuscript.

**Statement of contributions by others to the thesis of a whole**

This project benefited from support from other people including, Y. Zohar, provided protocol for the EVAc implants (Chapter 2); A. Miller and M. Harrison, carried on fish husbandry at CleanSeas Tuna Ldt. facilities (Chapters 2 and 3). N. H. Nguyen, offered statistical assistance (Chapters 3 and 4); D. Powel, assembled transcriptome and identified Vtg sequence (Chapter 4); N. Cheetham, performed the Vtg purification (Chapter 4); T. Wang, carried out mass spectrometry analysis (Chapters 4 and 5) and T. Correa offered his assistance on developing the alginate-chitosan particles (Chapter 5).
Acknowledgments

I would like to thank every single person that has been part of my life during this incredible journey. Thank you all for helping me to make it happen.

Particularly, I am deeply thankful to my supervisor, Prof. Abigail Elizur for providing me with the opportunity, tools, strength and motivation to develop my carrier at PhD level. I am very grateful to her for sharing her extensive knowledge and wisdom in the research field and beyond it, for her personal and professional advice to help me growing not only as a scientist but as a person. Thank you for being a model of commitment, courage and passion to work. I am greatly thankful to my co-supervisor, Dr. Josephine for her generosity and patience in sharing her expert knowledge and laboratory techniques through her constant availability and never ending encouragement. I am very thankful to her for teaching me how the complicated can be turn into easy if properly explained, and for helping me to see a big picture of what PhD means. I can confirm that both of my supervisors are the best example that a student can have. You have been and you will always be an example of good scientists, hard workers and good hearted people.

I would like to thank Clean Seas Pty Ltd (CST), the research partner of this PhD project, for providing me with the facilities and animals to carry on this research. I am very thankful to all staff members, specially to Michael Harrison for making me fell at home by giving me such a good time every time that I was visited CST facilities in Arno Bay.

I would like to thank the University of the Sunshine coast staff and all my Australian, Spanish and international friends, mates and collaborators who helped and assisted to accomplish this stage of my life and career.

I pleased to thank all my family, including my brother Alejandro Sanchis, my sister Gemma Sanchis, and especially to my partner, best friend and magnificent scientist Nikoleta Ntalamagka. Thank you for all your support, love and for always encouraging me to develop a better version of myself. Finally, I am delighted to thank my parents, Jose Manuel Sanchis and Maria Jose Benlloch without whom none of my success would be possible.
**Table of Contents**

Abstract ........................................................................................................................................... 4

Declaration of originality ................................................................................................................ 6

Statement of contributions to jointly authored works contained in the thesis: ........ 7

Acknowledgments .......................................................................................................................... 8

List of Figures ............................................................................................................................... 13

List of Tables ................................................................................................................................. 14

List of published papers and works to be submitted by the author, incorporated into the thesis ................................................................................................................................. 15

List of common abbreviations .................................................................................................... 16

Chapter 1: Introduction and literature review .............................................................................. 18

1.1 Introduction ............................................................................................................................. 18

1.2. Endocrinology of fish reproduction ..................................................................................... 19

1.2.1 RFamide peptides .............................................................................................................. 20

1.2.2 Gonadotropin-releasing hormone ..................................................................................... 21

1.2.3 Gonadotropins .................................................................................................................. 22

1.2.4 Sex steroids ....................................................................................................................... 23

1.3. Yeast, *Pichia pastoris*, expression system ......................................................................... 24

1.4. Yellowtail Kingfish aquaculture ......................................................................................... 25

1.5. Southern Bluefin Tuna aquaculture .................................................................................... 26

1.6. Tilapia as a model species ..................................................................................................... 26

1.7 References .............................................................................................................................. 27

Chapter 2: Effect of kisspeptin administration in immature female yellowtail kingfish (*Seriola lalandi*) during the breeding season .................................................................................. 32

2. 1. Introduction .......................................................................................................................... 33

2.2. Materials and Methods ....................................................................................................... 35

2.2.2. In-vivo administration of Kiss1–15 and Kiss2–10 on immature female YTK 35

2.2.3. E<sub>2</sub> and Vtg analysis ................................................................................................. 37

2.2.4. Statistical analysis ............................................................................................................ 37

2.3. Results ................................................................................................................................... 37

2.3.1 E<sub>2</sub> analysis .................................................................................................................. 38

2.3.2 Vtg analysis ....................................................................................................................... 39

3.4. Discussion ............................................................................................................................... 40
Chapter 3: *In-vitro* and *in-vivo* biological activity of recombinant Yellowtail kingfish (*Seriola lalandi*) follicle stimulating hormone

3.1. Introduction

3.2. Materials and methods

3.2.1. Isolation of full-length *fshb* and *fsha* cDNA sequences

3.2.2. Synthesis of a single chain *fshba* and ligation into the pPIC9K expression vector

3.2.3. Selection of high expressing clones and induction of protein expression

3.2.4. Purification of rytkFsh by dialysis and Western blot analysis

3.2.5. Prediction of rytkFsh 3D structure

3.2.6. *In-vitro* heterologous receptor binding assay

3.2.7. *In-vitro* bioactivity assay using YTK gonads

3.2.8. *In-vivo* bioassay in immature female YTK and maturing male YTK

3.2.9. Sex steroids analysis

3.2.10. Histological analysis

3.2.11. Statistical analysis

3.3. Results

3.3.1. Yellowtail Kingfish Fsh and predicted structure of single chain rytkFsh

3.3.2. Recombinant single chain ytkFsh (rytkFsh)

3.3.3. *In-vitro* bioactivity of rytkFsh

3.3.4. *In-vivo* bioactivity of rytkFsh

3.4. Discussion

3.5. References

Chapter 4: Development and Validation of a Competitive Hybrid ELISA for *Seriola lalandi* Vitellogenin

4.1. Introduction

4.2. Materials and methods

4.2.1. Generation of *S. lalandi* liver transcriptome and identification of Vtg sequences

4.2.2. Generation of *S. lalandi* Vtg antiserum

4.2.3. Induction of Vtg synthesis in *S. lalandi* males

4.2.4. Vtg purification

4.2.5. Protein in-solution digestion and LC-MS/MS analysis
4.2.6. Protein gel electrophoresis and Western blot analysis................................. 75
4.2.7. ELISA development .................................................................................. 76
4.2.8. Generation of standard curve .................................................................... 77
4.2.9. Validation of the ELISA ........................................................................... 78
4.2.10. Assay precision and antibody specificity .................................................. 78
4.3. Results ........................................................................................................... 79
  4.3.1. *Seriola lalandi* Vtg sequences ................................................................. 79
  4.3.2. Purification and identification of *S. lalandi* Vtg ......................................... 81
  4.3.3. Development of the hybrid competitive ELISA ........................................ 83
  4.3.4. Hybrid competitive ELISA validation. ..................................................... 86
4.4. Discussion ....................................................................................................... 86
4.5. References ...................................................................................................... 89
4.6. Supplementary figures .................................................................................. 93

Chapter 5: Development of oral delivery technologies to induce spawning in Southern Bluefin tuna using Tilapia as a model animal ................................................. 98
5.1. Introduction ..................................................................................................... 99
5.2. Material and methods .................................................................................... 101
  5.2.1. Encapsulation of GnRHa in alginate-chitosan based particles ................. 101
  5.2.2. Intracellular expression of recombinant sbtGnRH1 in yeast ..................... 101
  5.2.3. Recombinant sbtGnRH1 verification by PCR and Mass spectrophotometry.
       .................................................................................................................... 102
  5.2.4. *Pichia pastoris* cell lysis for peptide extraction ....................................... 104
  5.2.5. Quantification of GnRH using high-performance liquid chromatography
         (HPLC). ......................................................................................................... 104
  5.2.6. Formulation of *on-site* produced tilapia pellets .................................... 104
  5.2.7. *On-site* tilapia pellets containing GnRHa particles and recombinant yeast105
  5.2.8. *In-vivo* experiments ............................................................................... 106
  5.2.9. Statistical analysis .................................................................................... 107
5.3. Results ............................................................................................................ 107
  5.3.1 *On-site* tilapia pellets formulation ........................................................... 107
  5.3.2 GnRHa alginate-chitosan particles and rsbtGnRH1 yeast ........................ 108
  5.3.3. *In-vivo* tilapia feeding experiment of alginate-chitosan encapsulated GnRHa
         and rsbtGnRH1 in yeast .............................................................................. 111
5.4. Discussion ...................................................................................................... 113
List of Figures

Fig 1.1. Simplified representation of the BPG axis in teleosts. ......................... 20
Fig 2.1. In-vivo experiment of Kiss1-15 and Kiss2-12 on immature female YTK. ..... 36
Fig 2.2. GSI vs body weight at the end of the experiment. ................................ 38
Fig 2.3. Mean GSI values................................................................. 38
Fig 2.4. Mean plasma E2 levels. .................................................................. 39
Fig 2.5. E2 levels vs body weight at the end of the experiment. ......................... 39
Fig 2.6. Mean Vtg values over the time per treatment. ................................... 40
Fig 2.7. Vtg levels vs body weight at the end of the experiment. ....................... 40
Fig 3.1. Multiple comparative sequence alignment of ytkFshb (A) and ytkFsha (B) with other vertebrate Fsh sequences......................................................... 56
Fig 3.2. Structure of the single chain rytkFsh construct .................................. 57
Fig 3.3. Western blot analysis of deglycosylated single chain rytkFsh. .......... 57
Fig 3.4. In-vitro binding assay of rytkFsh and recombinant tilapia Fsh (rtiFsh) to the tilapia Fsh receptor................................................................. 58
Fig 3.5. In-vitro bioassay of rytkFsh on gonadal segments. ....................... 58
Fig 3.6. rytkFsh in-vivo trials on YTK. .................................................... 59
Fig 3.7. Histological sections of YTK ovary and testis from the in-vivo trial. .... 60
Fig 4.1. Multiple comparative sequence alignment of S. lalandi Vtg......................... 79
Fig 4.2. Phylogenetic tress of the S. lalandi Vtg ........................................ 81
Fig 4.3. HPLC of the purified S. lalandi Vtg from plasma of E2-induced males ........ 82
Fig 4.4. SDS-PAGE and Western blot analysis of purified Vtg and plasma samples... 83
Fig 4.5. Checkerboard titration panel showing the optimal S. lalandi Vtg ELISA concentration and antiserum dilution. ........................................ 83
Fig 4.6. Validation of anti-M. cephalus Vtg for the hybrid competitive ELISA. .... 84
Fig 4.7. Hybrid competitive ELISA binding curves. .................................. 85
S4.1 Fig. Multiple comparisons of the peptides raised based on suspected epitopes and S. lalandi VtgAa and VtgAb sequences. ........................................ 93
S4.2 Fig. The S. lalandi Vtg amino acid sequence alignment. .......................... 93
S4.3 Fig. Sequence coverage of *S. lalandi* VtgAa using LC-MS. ........................................ 94
S4.4 Fig. Sequence coverage of *S. lalandi* VtgAb using LC-MS........................................ 95
S4.5 Fig. Checkerboard titration using anti-*S. lalandi* Vtg. ............................................. 96
S4.6 Fig. Vtg levels in plasma of *S. lalandi* males prior to (Time 0) and after treatment with E\(_2\). ......................................................................................................................... 96

Fig 5.1. The sbtGnRH1 expression construct. ............................................................................. 102
Fig 5.2. Verification of most resistant yeast transformants by PCR. ....................................... 108
Fig 5.3. MALDI TOF mass spectrum of yeast (*P. pastoris*) expressing rsbtGnRH1. .. 108
Fig 5.4. GnRH HPLC profiles. .................................................................................................... 109
Fig 5.5. GnRHa standard curve. ................................................................................................. 110
Fig 5.6. GnRH quantification of encapsulated GnRH............................................................... 110
Fig 5.7. GnRHa release from alginate-chitosan particles over time........................................ 111
Fig 5.8. E\(_2\) levels in Tilapia after GnRH oral treatment (Experiment 3). ............................. 113

List of Tables

Table 2.1. Weight at the start and end of the treatment and GSI % of the identified females. ........................................................................................................................................ 37
Table 3.1. Gene-specific primers used for the isolation of *fhsa* and *fshb* cDNA sequences in yellowtail kingfish ......................................................................................................................... 50
Table 4.1. Identity percentage of the predicted Vtg amino acid sequences in *S. lalandi* .................................................................................................................................................. 80
Table 5.1. sbtGnRH1-specific primers. ...................................................................................... 103
Table 5.2. *In-vivo* experiments from GnRH oral administration at 0.6µg/g. ..................... 111
List of published papers and works to be submitted by the author, incorporated into the thesis

2. Sanchis-Benlloch, P J., Nocillado, J., Miller, A., Zohar, Y., Elizur, A Effect of kisspeptin administration in immature female yellowtail kingfish (*Seriola lalandi*) during the breeding season
To be submitted to Aquaculture
Incorporated as Chapter 2

Published in General and Comparative Endocrinology,
doi: [http://dx.doi.org/10.1016/j.ygcen.2016.03.001](http://dx.doi.org/10.1016/j.ygcen.2016.03.001)
Incorporated as Chapter 3 and attached in the published form at the end of the thesis.

Submitted to PLoS ONE
Incorporated as Chapter 4

5. Sanchis-Benlloch, P J., Nocillado, J., Elizur, A Development of oral delivery technologies to induce spawning in Southern Bluefin tuna using Tilapia as a model animal
To be submitted to Aquaculture research
Incorporated as Chapter 5
### List of common abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>11KT</td>
<td>11 Ketotestosterone</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BPG</td>
<td>Brain-pituitary-gonadal axis</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CST</td>
<td>CleanSeas tuna Ltd.</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>E₂</td>
<td>17-β estradiol</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin releasing hormone</td>
</tr>
<tr>
<td>GnRHa</td>
<td>Gonadotropin-releasing hormone analogue</td>
</tr>
<tr>
<td>GSI</td>
<td>Gonadosomatic index</td>
</tr>
<tr>
<td>GV</td>
<td>Germinal vesicle</td>
</tr>
<tr>
<td>HPLC</td>
<td>High liquid chromatography</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography–mass spectrometry</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>rsbtGnRH1</td>
<td>Recombinant Southern Bluefin tuna Gonadotrophin releasing hormone</td>
</tr>
<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rytkFsh</td>
<td>Recombinant yellowtail kingfish follicle stimulating hormone</td>
</tr>
<tr>
<td>SBT</td>
<td>Southern Bluefin tuna (<em>Thunnus maccoyii</em>)</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the main</td>
</tr>
<tr>
<td>Vtg</td>
<td>Vitellogenin</td>
</tr>
<tr>
<td>YTK</td>
<td>Yellowtail kingfish (<em>Seriola lalandi</em>)</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction and literature review

1.1 Introduction

The world’s human population is expected to exceed 9 billion by 2050, which means that to feed all these people, at least 70 percent more protein would be needed than what is available to humankind today. Limited attention has been given so far to fish (fisheries and aquaculture) as a key element in food security and nutrition (Béné et al., 2015). While the capture fisheries has remained almost constant, aquaculture has doubled its production during the last 20 years, and just in recent years has increased from 55.7 (2009) to 73.8 million tonnes (2014) (FAO, 2016a). The Food and Agriculture Organization (FAO) of the United Nations recognizes the fast-growing contribution that aquaculture is making to fish production and indeed to food security. The farming of about 550 aquatic species represents nearly 50 percent of the world’s food fish (FAO, 2015). However, the aquaculture industry is still far away from the sophistication of other industries such as poultry and cattle livestock production (Zohar et al., 2001). Applying biotechnology to aquaculture can be one of the means to increase the intensity, capacity and sophistication that the industry needs in order to meet the rising demand for food security in the coming years (Hew et al., 2001). The selection of the biotechnological tools to apply is dependent on the biology and physiology of the desired species and the farming challenges that need to be overcome.

The aquaculture of large-body fishes, such as the yellowtail kingfish (Seriola lalandi, YTK), is an actual priority, considering the high growth rate of these fishes and the high economic value and quality of the flesh produced. Nevertheless, in many cases, as is the case of YTK, the development of the aquaculture of this species is blocked by critical reproductive problems, mostly related to puberty and fecundity. Thus, the objectives of this PhD thesis were to investigate the reproductive endocrinology of YTK and to develop techniques to address the reproductive problems of this species in captivity, with the long term view of applying those techniques to other large-bodied cultured fishes, such as Southern Bluefin Tuna (Thunnus maccoyii, SBT).

The main hypothesis of this PhD project is that the use of appropriate biotechnological tools a deep scientific knowledge of the mechanisms of action of the
reproductive genes and hormones can help develop effective therapies applicable to the different processes of reproduction. Based on this hypothesis, a recombinant YTK follicle stimulating hormone and Kisspeptin slow release implants were generated to advance sexual maturation in YTK (Chapter 2 and 3). An enzyme-linked immunosorbent assay (ELISA) for YTK vitellogenin (Vtg) detection was developed to assess the effectiveness of the above hormonal manipulations (Chapter 4). Based on the results obtained, a final hypothesis was formulated on how to improve the biological activity and efficacy of the kisspeptin and Fsh treatments using oral administration protocols. Thus, two different methodologies for oral delivery were explored using tilapia (Oreochromis mossambicus) as a model animal aiming to create a breakthrough in the capacity to control spawning and reproductive function of captive broodstock (Chapter 5). These results overall can support the commercial growth of the YTK aquaculture industry (Chapter 6).

1.2. Endocrinology of fish reproduction

In vertebrates, including fish, the reproductive cycle is centrally controlled by the brain-pituitary-gonadal (BPG) axis (Fig 1.1). The axis can be subdivided into four main components, namely: (I) RFamide peptides (RFa) Kisspeptin and gonadotropin-inhibiting hormone (GnIH), (II) gonadotropin-releasing hormones (GnRH), (III) gonadotropic hormones; follicle stimulating hormone (Fsh) and luteinizing hormone (Lh), and (IV) sex steroids; in males (Testosterone (T) and 11 Ketotestosterone (11KT)) and in females estradiol (E2) (I. Parhar et al., 2012; Zohar et al., 2010).
Fig 1.1. Simplified representation of the BPG axis in teleosts. Grey arrows on the left represent the negative (-) or positive (+) feedback caused by the gonadal stage to the BPG axis. Orange colour represents hypothalamus, where RFa neurons interact with the GnRH neurons. In the pituitary (yellow) GnRH stimulates the secretion of the Fsh and Lh gonadotropins, which act at the gonadal level (blue) stimulating the production of sex steroids 11KT and E2 (A). E2 in the liver stimulates Vtg production, followed by uptake (green dotted line) by the oocytes in the gonads (B).

1.2.1 RFamide peptides

RFamide (RFa) are neuropeptides with an arginine and an amidated phenylalanine-motif at its C-end which in vertebrates are involved in neuroendocrine, behavioural and sensory functions (Findeisen et al., 2011; I. Parhar et al., 2012; Sandvik et al., 2014). The Kisspeptin peptides and the gonadotropin-inhibitory hormone (GnIH) are RFa that are considered one of the key important elements of the BPG axis regulation (Biran et al., 2014; I. Parhar et al., 2012).

Molecular studies across vertebrates have demonstrated that the highest expression sites of all Kiss and Kissr isoforms is the hypothalamus, confirming that the Kisspeptin system has conserved neuroendocrine and neuromodulatory roles in the control of reproduction (Lee et al., 2009). Although it may play other putative roles not related with reproduction, such as fear response in fish (Ogawa, Nathan, et al., 2014), there is
strong evidence that kisspeptin mainly regulates the vertebrate reproductive system via GnRH (Oakley et al., 2009; Zmora et al., 2014). In fact, the kisspeptin system is considered as the “gatekeeper” of puberty and reproduction in most vertebrates including fish (Elizur, 2009; Zohar et al., 2010). It activates the reproductive hormone-signaling cascade of the BPG axis by stimulating the GnRH neurons in the hypothalamus (Kauffman, 2010; I. Parhar et al., 2012).

Whereas the role of kisspeptin across vertebrates is commonly accepted, the role of the GnIH is inconclusive and varies among vertebrate species (Biran et al., 2014). In vertebrates, including teleosts, GnIH or LPXRFa (piscine ortholog of GnIH) neurons are in close association with cells expressing other reproductive neuropeptides such as GnRH and kisspeptin neurons, indicating a general reproductive role in vertebrates (Ogawa & Parhar, 2014). More specifically, in fish, recent studies confirm its general reproductive role. In Sea bass, there is evidence that LPXRFa suppresses reproduction in males by acting at both brain and pituitary levels, regulating steroidogenesis and gametogenesis (Paullada-Salmerón et al., 2016a) as well as potentially mediating rhythmic processes associated with photoperiod (Paullada-Salmerón et al., 2016b). In orange-spotted grouper (Epinephelus coioides), molecular identification of GnIH/GnIHR pointed to the reproductive role of this system including sex differentiation, gonadal development and sex reversal via GnRH and gonadotropin regulation (Wang et al., 2015).

1.2.2 Gonadotropin-releasing hormone

Hypothalamic gonadotropin-releasing hormone (GnRH) is the pivotal hypothalamic hormone regulating the BPG axis in all vertebrates (Millar, 2005). The mature peptide is composed of 10 amino acids and it contains an N-terminal pyroglutamate and C-terminal glycinamide, which are important for its activation and receptor binding (Millar, 2005). It is regulated by steroids, neurotransmitters, neuropeptides and RFamide peptides (I. Parhar et al., 2012). Teleosts can express up to three GnRH isoforms (GnRH1, GnRH2 and GnRH3) (Powell et al., 1994). GnRH1 is the most species-specific isoform, expressed mainly in the ventral telencephalon and preoptic area of the basal hypothalamus and in less number of neurons in the anterior olfactory bulb (OB) region (Zohar et al., 2010). It is the most relevant isoform for reproductive function, specifically being involved in gonadal maturation and gonadotropin regulation, and in some cases
it has been considered as the gatekeeper of BPG axis activation (I. Parhar et al., 2012; I. S. Parhar et al., 2003). GnRH2 is the most evolutionarily conserved isoform across teleost species (Lethimonier et al., 2004). It is expressed in the midbrain tegmentum (Zohar et al., 2010), suggesting that it may play supplementary roles in reproductive behaviours (I. Parhar et al., 2012). While GnRH1 and GnRH2 are found in fish through to terrestrial vertebrates, GnRH3 includes only salmon GnRH (fish), and is therefore considered as a teleost-specific isoform (Zohar et al., 2010). If present, it is expressed in neurons localized in the olfactory nerve (ON), ventral OB, and transitional area between OB and ON, suggesting its possible involvement in reproductive behaviours such as male aggression, nest-building and homing migration (I. Parhar et al., 2012). In species were GnRH1 gene is lost, GnRH3 neurons are localized in the preoptic area of the hypothalamus covering the function of GnRH1 (Adams et al., 2002).

Dopamine (D) is a neurotransmitter secreted also by the hypothalamus that in some, but not all, teleost fish species exerts inhibitory effect on the GnRH system and gonadotropin release, at the hypothalamic and pituitary levels, respectively (Zohar et al., 2010). Interestingly, recent studies demonstrated a dopaminergic control of GnRH1 neurons via D2 receptor interactions in the hypothalamus across vertebrates (Bryant et al., 2016). In general terms, some species have been reported to have strong dopaminergic inhibition such as in cyprinids, therefore spawning induction would require the use of dopamine antagonists, while in marine species a lack of dopamine inhibition has been reported (Levavi-Sivan et al., 2010).

1.2.3 Gonadotropins

When stimulated by GnRH, the anterior pituitary area produces and releases into the bloodstream two gonadotropins: Fsh and Lh, which act at the gonad by binding to their cognate receptors (Fshr and Lhr), inducing steroidogenesis and gametogenesis (Levavi-Sivan et al., 2010). It is well established that Fsh plays a key role at the onset of puberty (Taranger et al., 2010) and a dominant role during early phases of gametogenesis including oocyte growth, vitellogenic processes in females, and spermatogenesis in males (Yaron et al., 2003). On the other hand, Lh plays a key role at the advanced stages of maturation and is considered to be responsible for the final
maturational processes including oocyte maturation, ovulation in females and spermiation in males (Yaron et al., 2003).

Fsh and Lh are glycoproteins composed of two subunits. The α subunit is well conserved among fish species at the amino acid level while the β subunit is more species-specific. The β subunit is strongly linked to the α subunit but not covalently associated which determines the biological activity and specificity of the hormone (Yaron et al., 2003). The most notable characteristics in the primary structure of the gonadotropin subunits is the presence of the “cysteine knot motif” which consists of a series of 6 cysteines linked in a specific way by molecular disulfide bonds and the existence of 3 loops (L1 and L3) on one side and L2 loop the other (Levavi-Sivan et al., 2010).

1.2.4 Sex steroids

Sex steroids are the final effectors of reproductive function as they are released into circulation at different stages of gametogenesis to induce the process of oocyte maturation and spermiation. They exert positive or negative feedback into the hypothalamus and pituitary to control GnRH and gonadotropin release depending on the maturational stage (Diotel et al., 2011).

Three main types of sex steroids derived from the cholesterol molecule can be found in fish. These are: (I) progestogens (17α, 20β Dihidroxy-4-pregnen-3-one), which are involved in oocyte and sperm maturation (in males and females, respectively) as well as inhibiting the secretion of gonadotropins as a form of negative feedback on the BPG axis, preventing sex hormone levels from getting too high; (II) androgens; testosterone (in both male and female) and the main androgen 11-Ketosterone (in both sexes but in females is present at low concentrations with a minor biological function) which are involved in the initiation of spermatogenesis (Ikeuchi et al., 2001) and (III) oestrogen-17β (E₂), which stimulates oogenesis and the hepatic synthesis of Vtg in female fish (Hara et al., 2016). Testosterone is the precursor of E₂; it is converted into oestrogen by cytochrome P450 aromatase, which is more highly expressed in females than in males (Piferrer et al., 2005).

In the testis of teleost males, Leydig cells stimulated by gonadotropins produce 11KT, which activates spermatogenesis, composed of three phases: mitotic proliferation of spermatogonia, meiosis of spermatocytes, and spermiogenesis (Schulz et al., 2002). In
female fish, oogenesis is also composed of three main stages: follicle growth, maturation and ovulation (Patiño et al., 2002). Follicle growth can be divided in two stages, previtellogenic growth and vitellogenic growth which are marked by Vtg synthesis in the liver in response to $E_2$ entering the liver and binding to its specific $E_2$ receptor (Nagahama et al., 2008). Vtg is a female specific serum protein which contains phosphorus, lipids, carbohydrates, calcium and iron and has been identified as the egg yolk precursor in most oviparous vertebrates. It also marks gonadal maturation (Hara et al., 2016).

1.3. Yeast, *Pichia pastoris*, expression system

The methylotrophic yeast *Pichia pastoris* is a powerful biotechnological tool that was developed as an expression system for high-level production of recombinant proteins (Daly et al., 2005). The main advantage of recombinant hormones over the purified hormones is that hormones produced in yeast and other host organisms can be continually produced without relying on the main species as starting source (Levavi-Sivan et al., 2010). These proteins are analogous to purifications from naturally expressing tissue sites and can produce a biological response. Recombinant proteins do not cross-react with other related hormones, which are often co-purified (Levavi-Sivan et al., 2008).

The yeast expression system has many advantageous features over a prokaryotic expression system. It has the additional benefits of an eukaryote expression system, including improved folding and the ability to carry out post-translational modifications, such as glycosylation, which is required for the bioactivity of gonadotropins (Cereghino, 2000). Additionally, the yeast expression system has been improved by the incorporation of the tightly regulated promoter of the alcohol oxidase I (AOX1) gene for the expression of recombinant proteins (Daly et al., 2005). Depending on the secretion signal on the expression vector, heterologous expression in *Pichia pastoris* can be either intracellular or extracellular. Extracellular or secreted expression is when the recombinant protein is secreted to the culture media. On the other hand, intracellular expression is when the recombinant protein is expressed within the yeast cells and not secreted to the culture media. This is especially useful when the hormone is intended to be orally administrated as yeast can act as a delivery system, which facilitates the
transport through the cells of the digestive tract while protecting the recombinant hormone from degradation during digestion in the stomach (Kim et al., 2014).

1.4. Yellowtail Kingfish aquaculture

The genus Seriola includes highly active pelagic fish belonging to the Carangidae family and Perciform order. The culture of Seriola began in Japan over 70 years ago (Nakada, 2008). Three Seriola species are the main farmed species; S. quinqueradiata, S. dumerili and S. lalandi (Kolkovski et al., 2004).

S. lalandi is one of the larger members of the genus. It is found in the Atlantic, Indian and Pacific Oceans (Fishbase.org, 2016). Its importance for the aquaculture industry is growing worldwide due to its fast growth, high flesh quality and suitability for farming in both cage and recirculating aquaculture systems (Chen et al., 2006; Moran et al., 2007; Orellana et al., 2014; Poortenaar et al., 2001). Its culture is well established in Australia and New Zealand. In Australia, Clean Seas Tuna Ltd. (Arno Bay, Australia, CST) is the main commercial producer, with over 2000 tonnes produced per year (CST, 2016), exporting to European, Asian and American countries.

YTK is a gonochoristic species with an asynchronous oocyte development, which provides the capacity for multiple spawning within a reproductive season, between spring and summer (Moran et al., 2007). Age at puberty can vary between male and female fish, and between geographical locations. One of the commercial limitations of this species is that females take 4–5 years to reach sexual maturity while precocious males can start to spermiate from 12 months old onwards (Poortenaar et al., 2001). Currently, a genetic selection program is being undertaken for YTK (Knibb et al., 2016; Whatmore et al., 2013) and there is interest to shorten its generation time to increase genetic gain (Sanchis-Benlloch et al., 2016). Advancing pubertal development in females is the first step towards shortening the breeding period (Taranger et al., 2010). The application of biotechnological techniques such as exogenous hormonal treatment to advance puberty and molecular tools to assess maturational stages and effectiveness of hormonal therapies could help to further understand S. lalandi reproductive biology, overcome the commercial limitations and support the commercial growth of this important aquaculture species.
1.5. Southern Bluefin Tuna aquaculture

The genus *Thunnus* holds eight species of bony fishes belonging to the Scombridae family and Perciformes order (Chow et al., 1995). Currently, three species of tunas have been the focus of the aquaculture industry due to its high market value. These are the Atlantic (*Thunnus thynnus*), Pacific (*Thunnus orientalis*), and Southern bluefin tuna and the effort of farming them started in Spain Japan, Australia, respectively (Shamshak, 2011). Japan was the first country to close the life cycle of Pacific bluefin tuna over 10 years ago (Sawada et al., 2005). Recently, Spain reported closing the life cycle of Atlantic Bluefin tuna (IEO, 2016). Australia was the first country to establish an onshore facility capable of producing juveniles of Southern bluefin tuna. However, bottlenecks in closing its life cycle, such as long maturation time or inconsistent spawning in captivity, remain major challenges in SBT farming (Bain et al., 2013). Currently, the aquaculture of this species is “capture-based”, which involves capturing wild fish and fattening them in sea cages until the desired market weight is reached. This method of farming imposes a threat for this species due to its dependence on the already heavily overfished wild SBT stock. The high demand and the high market value of this species are causing risk through overfishing. In fact, SBT was listed as a threatened species by the Commonwealth threatened species scientific committee from 2010 (TSSC, 2010).

When tunas are reared in captivity, GnRH therapies are necessary to induce spawning (Zohar et al., 2001). The current method of hormonal administration in large tunas is very stressful and dangerous for fish and handlers alike. Therefore, there is an interest to control the reproductive development and spawning of SBT using non-invasive methodologies. The combination of oral delivery technologies and hormonal reproductive therapies is an approach towards achieving this aim. In the long term, closing the cycle of this species would not only alleviate the overfishing issue, but would also provide Australia with a new economically viable and sustainable aquaculture industry.

1.6. Tilapia as a model species

Although more than 70 species of fish are referred by the common name “tilapia” only 8-9 species are considered as aquaculture species. The two most important species
in terms of aquaculture are *Oreochromis niloticus* and *Oreochromis mossambicus* (Coward et al., 2000). *O. mossambicus* is originally a native species of the Southern African rivers. It was introduced in Australia during the 1970s for aquaculture and biological control purposes, however it escaped and established itself in the wild and nowadays it is considered as a pest (Russell et al., 2012), however *O. mossambicus* aquaculture is well established around the world. In 2014 alone, over 42000 tonnes were produced (FAO, 2016b).

The robustness of tilapia to adapt to a wide range of salinity and temperature, its disease resistance, the relatively short reproductive cycles and the fact that they breed productively under culture conditions makes this species easy to manipulate in the laboratory (Russell et al., 2012). The well-studied reproductive biology of this species, and its similarity to the available tilapia genome model *Oreochromis niloticus* (Guyon et al., 2012) made this species an excellent model animal and a foundation for comparative studies with other perciforms, such as Yellowtail kingfish or Southern bluefin tuna.

1.7 References


Chapter 2: Effect of kisspeptin administration in immature female yellowtail kingfish (*Seriola lalandi*) during the breeding season

Pablo J. Sanchis¹, Josephine Nocillado¹, Adam Miller² Yonatan Zohar³ and Abigail Elizur¹

¹Genecology Research Centre, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Queensland, Australia. ²Clean Seas Tuna Ltd, Port Lincoln, SA, Australia, ³Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland, USA.

Abstract

Kisspeptins are considered potent key regulators of vertebrate reproductive function, particularly the onset of puberty. We utilized slow-release implants to chronically administer kisspeptins (Kiss1–15 and Kiss2–12) to immature female (1-2 years old) yellowtail kingfish (*Seriola lalandi*, YTK). The trial was conducted for 12 weeks coinciding with the reproductive season. The effect of the treatment was assessed by measuring plasma estradiol-17β (E₂) and vitellogenin (Vtg) levels as well as determining gonadosomatic index (GSI). The E₂ and Vtg plasma levels increased during the course of the trial, and by the end of the trial, fish from the Kiss1-15 treatment group showed the highest GSI, E₂ and Vtg levels, although differences between the groups were not statistically significant. Plasma E₂ levels from the Kiss1-15 treatment group at the start and end of the trial were significantly different.

Key words: Kiss1–15, Kiss2–10, *Seriola lalandi*, puberty, aquaculture
2. 1. Introduction

Kisspeptins, members of the RFamide peptide family, were first discovered in 1996 as a metastasis suppressor of malignant melanoma in human cells (J. Lee et al., 1996). There is now strong evidence that the kisspeptins are potent central regulators of the mammalian and non-mammalian vertebrate reproductive system (Oakley et al., 2009). Kisspeptins activate the reproductive hormone signaling cascade of the Brain-pituitary-gonadal (BPG) axis by controlling the release of gonadotrophin-releasing hormone (GnRH) in the hypothalamus (Parhar et al., 2012; Um et al., 2010). Therefore, it is known that in vertebrates kisspeptins play a key role in adult regulation of gonadotropin secretion, puberty onset and sexual differentiation (Kauffman, 2010; Pinilla et al., 2012).

The number of Kiss genes in vertebrates can vary from none to three and the number of Kiss receptors (Kissr or GPR54) genes can vary from none to four depending on the species (Pasquier et al., 2012). In mammals and reptiles, a single Kiss ligand binds to one Kissr (Pasquier, Kamech, et al., 2014). Exceptions have been found in the platypus (Ornithorhynchus anatinus), which was found to possess 2 Kiss receptor genes and in amphibians such as Xenopus species that has up to three Kiss and Kissr genes (Kitahashi et al., 2009; Y. Lee et al., 2009). The kisspeptin system has not been confirmed in birds, although a kiss2-like gene was found, however its function is still unknown (Pasquier, Lafont, et al., 2014). In teleost fish, Biran et al. (2008) described for first time the co-existence of two paralogous Kiss genes (Kiss1 and Kiss2) originating from the duplication of an ancestral gene was reported in sea bass (Dicentrarchus labrax) (Felip et al., 2009). At the same time, the coexistence of these two paralogous genes was reported for two different species zebrafish (Danio rerio) and medaka (Oryzias latipes) (Kitahashi et al., 2009). This dual kisspeptin system comprised of two different genes (Kiss1 and Kiss2) and two different receptors named Kissr1 (also called GPR54-1) and Kissr2 (also called GPR54-2) have been reported for the majority of fish species (Carrillo et al., 2009; Tena-Sempere et al., 2012). Interestingly, a recent study has reported a third Kiss receptor (Kissr3) in the brain and gonads of sea bass, medaka and zebrafish (Felip et al., 2015).

The highest expression sites of all Kiss and Kissr isoforms in fish, amphibians and mammals are in the brain, notably in the hypothalamus, suggesting a conserved neuroendocrine and neuromodulatory roles in the control of puberty and reproduction.
(Y. Lee et al., 2009; Zmora et al., 2014). As in mammalian vertebrates, the kisspeptin system has been considered as the “gatekeeper” of puberty and reproduction in teleosts (Elizur, 2009; Zohar et al., 2010). Such is supported by studies in pre-pubertal *Seriola lalandi* males where kisspeptin administration stimulated gonadal development (Nocillado et al., 2013) and in juvenile *Morone* species where Kisspeptin mediated pubertal initiation (Beck et al., 2012). Furthermore, it was also shown that Kisspeptin can stimulate vitellogenesis in sexually immature chub mackerel (Selvaraj et al., 2013).

Although the importance of kisspeptins in regulating fish reproduction, including puberty, is well established (Kauffman, 2010; Zmora et al., 2014), fish kisspeptins are considered the most poorly documented among all vertebrate kisspeptins and thus their modulatory roles are not well understood (Escobar et al., 2013). Some authors reported a reproductive role, such as gonadotropins activity modulation, but only for Kiss2 but not for kiss1 (Espigares et al., 2015). Other authors reported a reproductive role, like estrogen feedback, but only for Kiss1 and not for Kiss2 (Mitani et al., 2010). Moreover, other non-reproductive roles have been pointed out (Akazome et al., 2010). In zebrafish, only Kiss2 was reported as being responsible for reproductive events while Kiss1 may play other functions (Servili et al., 2011). Other work in zebrafish suggested that both kisspeptins may play roles other than in reproduction (Ogawa et al., 2014). Tang et al., (2014) demonstrated that kisspeptin system also modulates fear regulation in the same species. All in all, this situation demonstrates a complex functional scenario for the kisspeptin system in fish, which merits investigation.

Recent studies in sexually immature chub mackerel (*Scomber japonicus*) treated with exogenous synthetic Kiss1–15 and Kiss2–12 peptides showed that Kiss1-15 but not Kiss2–12 induced gonadal development (Selvaraj, Ohga et al. 2013). Results obtained from a previous work in our lab revealed that administration of kisspeptin decapeptides can induce earlier gonadal development in pre-pubertal YTK males (Nocillado et al., 2013). Considering these findings, the hypothesis of this study was that administration of synthetic YTK Kiss1-15 and Kiss2-12 to immature YTK females could induce earlier maturation. The effect of the exogenous administration of synthetic YTK Kiss1-15 and Kiss2-12 peptides to immature YTK females was evaluated through the GSI, and plasma estradiol-17β (*E₂*) and Vtg levels as they are considered as key indicators of fish maturation (Hara et al., 2016).
YTK is rapidly growing in importance worldwide as an aquaculture species due to its fast growth, high flesh quality and suitability for farming in both cage and recirculating aquaculture systems (RAS) (Chen et al., 2006; Orellana et al., 2014; Poortenaar et al., 2001). In Australia, a genetic selection program is being undertaken for this species (Knibb et al., 2016; Whatmore et al., 2013) and there is interest from the aquaculture industry to shorten its generation time in order to increase genetic gain. Advancing pubertal development is the first step towards shortening the breeding period, and as such has been the focus of many studies in fish (Carrillo et al., 2009; Taranger et al., 2010) as it is in this study.

2.2. Materials and Methods

All experiments were conducted with approval from the Animal Ethics Committee of the University of the Sunshine Coast (Queensland) (approval number AN/A/12/67). In vivo experiments at CleanSeas Tuna Ltd (CST, Arno Bay, South Australia) commenced on the 17th of October 2013 and terminated on the 9th of January 2014 (12 weeks in total) coinciding with the reproductive season, between spring and summer (Poortenaar et al., 2001).

2.2.2. In-vivo administration of Kiss1–15 and Kiss2–12 on immature female YTK

Fish husbandry: YTK were kindly provided by CST. Experimental animals (n=36) were weighed, tagged and randomly distributed into 4 experimental tanks of 6m³ each equipped with a flow-through water supply system (Fig 2.1A and B). Each tank had one confirmed mature 3-year old male and an equal number of 1 to 2 year-old fish (n=8). One and 2 year-old fish were combined due to the restricted number of available immature individuals at that time. Tanks were aerated with a combination of compressed air and pure oxygen. Water temperature and dissolved oxygen were monitored daily. Fish were acclimatized 2 weeks prior the start of the experiment. Sex of the 1 to 2 year-old fish could not be determined at the beginning of the experiment although gonadal biopsy was attempted. It was technically difficult to obtain a gonadal biopsy of the very immature gonad without damaging the fish. During the experiment, fish were exposed to ambient water temperatures (15–20°C) and to a natural
photoperiod which ranged from 12L:12D (October) to 14L:10D (January). The fish were fed with a commercially formulated diet (CST).

**Fig 2.1. In-vivo experiment of Kiss1-15 and Kiss2-12 on immature female YTK.** Experimental Tanks (A). Red arrowheads indicate each one of the tanks used. One to two-year-old YTK (B). Red circle indicates the area where the implantation was performed.

*Kiss implants:* The YTK-specific 15 amino acid kisspeptin1 (Kiss1-15) and 12 amino acid kisspeptin2 (Kiss2-12) peptides (Nocillado et al., 2013) and Gonadotropin-releasing hormone analogue (GnRHa) (D-Ala$^6$,Pro$^9$,NEt) were commercially synthesized (China Peptides, Shanghai). Slow-release poly [ethylene-vinyl acetate] (EVAc) implants were prepared according to the protocol described by Mylonas et al., (2007). Implants were prepared so that a dose of 50µg/kg was contained in one or two pellets. The four experimental groups were: (1) Fish implanted with GnRHa, served as a positive control due to the routine use of GnRHa to induce final oocyte maturation and ovulation in fish when reared in captivity (Zohar and Mylonas, 2001); (2) Fish given blank implants and served as negative control; (3) Fish implanted with Kiss1-15; and (4) Fish implanted with Kiss2-12. Five implantations were conducted at an interval of 15-17 days. At the termination of the experiment, fish were sacrificed and sex was determined by visual identification. Total body weight and gonad weight data were collected from those identified as females. Gonadosomatic index (GSI) values (%) were calculated by following the formula; GSI = gonad weight / body weight without gonads × 100 (Selvaraj et al., 2013). Blood samples were collected at initial, middle part (third implantation) and at the end of the experiment. Fish were bled by caudal puncture using heparinized
syringes (heparin, 30 iu/ml Sigma). Plasma was separated from blood by centrifugation at 4000 xg at 4°C for 15 minutes and then stored at -80°C for E₂ and Vtg analysis.

2.2.3. E₂ and Vtg analysis

Levels of E₂ were determined by a commercially available enzyme immunoassay (EIA) kits (Cayman Chemical Company, Ann Arbor, MI, USA). Vtg levels in the plasma were determined by a competitive hybrid ELISA (Chapter 4).

2.2.4. Statistical analysis

Body weights (BW), GSI, E₂ and Vtg values were expressed as mean±SEM. Values from these data were analyzed with one-way analysis of variance (ANOVA) followed by a multiple comparison test (Tukey). Differences were reported as statistically significant when p < 0.05.

2.3. Results

At the end of the trial, only 13 of the 32 fish were identified as females, 3 females in each of the treatment groups and 4 in the control. The weights at the start and end of the trail as well as GSI from all female fish are in Table 2.1.

Table 2.1. Weight at the start and end of the treatment and GSI % of the identified females.

<table>
<thead>
<tr>
<th></th>
<th>Weight start (kg)</th>
<th>Weight end (kg)</th>
<th>Weight gain (%)</th>
<th>GSI %</th>
<th>Weight start (kg)</th>
<th>Weight end (kg)</th>
<th>Weight gain (%)</th>
<th>GSI %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>3.75</td>
<td>4.94</td>
<td>24.1</td>
<td>0.55</td>
<td>3.3</td>
<td>4.38</td>
<td>24.7</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>5.38</td>
<td>25.7</td>
<td>0.55</td>
<td>4.55</td>
<td>5.49</td>
<td>17.1</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>1.85</td>
<td>3.61</td>
<td>48.8</td>
<td>0.27</td>
<td>1.75</td>
<td>3.14</td>
<td>44.3</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>1.75</td>
<td>3.55</td>
<td>50.7</td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GnrHa</td>
<td>4.1</td>
<td>5.75</td>
<td>28.7</td>
<td>0.51</td>
<td>2.05</td>
<td>3.7</td>
<td>44.7</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>1.65</td>
<td>3.05</td>
<td>45.9</td>
<td>0.24</td>
<td>1.65</td>
<td>3.25</td>
<td>49.2</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>1.75</td>
<td>1.99</td>
<td>12.3</td>
<td>0.4</td>
<td>2.75</td>
<td>4.24</td>
<td>35.1</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Overall there was a clear growth in all the fish, with fish adding between 12.3% and 50.7 % body weight. As there was size and age variations between the fish, the relationship between GSI and body weight at the end of the trial was calculated and found to be linear in the control fish, making it possible to see increases in GSI in response to treatment in individual fish (Fig 2.2). While fish receiving Kiss 1-15
treatments showed slightly elevated GSI, nonetheless, no statistically significant differences were found between the GSI from the different treatment groups (Fig 2.3).

**Fig 2.2. GSI vs body weight at the end of the experiment.** Regression line represents the linear relationship between GSI and body weight in the control fish (●) after 12-week chronic treatment with GnRHa (x), Kiss1-15 (■) and Kiss2-12 (▲).

**Fig 2.3. Mean GSI values.** Mean GSI index of YTK females implanted with blank implants (control), GnRHa at 50µg/Kg (GnRH), Kiss1-15 at 50µg/Kg (Kiss1) and Kiss2-12 at 50µg/Kg (Kiss2). error bars represent mean and SEM, respectively.

### 2.3.1 E$_2$ analysis

Measurement of plasma E$_2$ levels detected significant differences only when comparing the Kiss1-15 treatment at the start and end of the trial. However, a trend can be observed in the other groups showing higher values at the end of the experiment when compared with the time zero values (Fig 2.4). At the end of the experiment, the highest E$_2$ value was 0.76±0.05 pg/ml in the Kiss1-15 treatment and the lowest E$_2$ (0.47
±0.12 pg/ml) was observed in the Kiss2-12 treatment. When E$_2$ levels were examined after adjusting to body weight, there was a linear relationship in the control, and all of the Kiss 1-15 fish showed a higher level of E$_2$ (Fig 2.5).

**Fig 2.4. Mean plasma E$_2$ levels.** YTK females implanted with blank implants (control), GnRH at 50µg/Kg (GnRH), Kiss1-15 at 50µg/Kg (Kiss1) and Kiss2-12 at 50µg/Kg (Kiss2). Columns and error bars represent mean and SEM, respectively. Asterisk (*) indicates significant difference (p < 0.05).

**Fig 2.5. E$_2$ levels vs body weight at the end of the experiment.** Regression line represents the linear relationship between E$_2$ and body weight in the control fish (●) after 12-week chronic treatment with GnRH (×), Kiss1-15 (■) and Kiss2-12 (▲).

### 2.3.2 Vtg analysis

Plasma Vtg also increased in all groups at the end of the experiment when compared with the time zero values, however the results appear much more variable. At the end of the experiment, the highest Vtg value was 22.43 ±16.58 µg/ml in the Kiss1-15 treatment, which was also the largest fish. The lowest Vtg value was 11.87±13.78 in the control treatment. No significant differences were found between the treatment groups.
or during the course of the trial (Fig 2.6). Looking at the results after adjusting to body weight showed a trend indicating bigger fish showed higher Vtg levels (Fig 2.7).

**Fig 2.6. Mean Vtg values over the time per treatment.** YTK females implanted with blank implants (control), GnRHα at 50µg/Kg (GnRH), Kiss1-15 at 50µg/Kg (Kiss1) and Kiss2-15 at 50µg/Kg (Kiss2). Columns and error bars represent main and SEM, respectively.

**Fig 2.7. Vtg levels vs body weight at the end of the experiment.** Regression line represents the linear relationship between Vtg and body weight in the control fish ( ) after 12-week chronic treatment with GnRHα (×), Kiss1-15 (■) and Kiss2-12 (▲).

### 3.4. Discussion

Our previous work showed that Kiss1-10 and Kiss2-10 treatments can stimulate gonadal development in pre-pubertal YTK males (Nocillado et al., 2013). In zebrafish, ligand receptor binding analysis indicated that Kiss1 pentadecapeptide (Kiss1-15) and Kiss2 dodecapeptide (Kiss2-12) are much more potent in receptor activation than Kiss1-10 or Kiss2-10 (Y. Lee et al., 2009). Recently, it was also shown that Kiss1 pentadecapeptide (Kiss1-15) can stimulate vitellogenic onset in sexually immature chub.
mackerel (Selvaraj et al., 2013). In the light of these findings, and in the interest of further investigating the kisspeptin system in fish, the present study examined the effects of exogenous administration of synthetic YTK kiss1-15 and kiss2-12 on immature YTK females. Administration was performed using slow release EVAc implants, which allow the hormone to be released in a diffusion-controlled process for a period of at least 7 days (Mylonas et al., 2007). As in this study, the effectiveness of these implants on the delivery of Kisspeptins in YTK has been already reported (Nocillado et al., 2013).

The results of this study did not show a parallel increase of the plasma E₂ levels and Vtg levels over the course of the trial, indicating that while a direct relationship between Vtg production and circulating E₂ levels has been reported in other teleosts (Nagahama et al., 2008), fish that are immature might be responding in a differential way to the stimulation by E₂. Despite the fact that the YTK fish were very immature, the trend of rising plasma E₂ levels and Vtg levels in control group over the time indicates some reproductive development during the 12 weeks of the trial. According to observations in farm conditions in South Australia, YTK females take 4-5 years to reach sexual maturity (Sanchis-Benlloch et al., 2016). Thus, the experimental female YTK were 2-4 years away from sexual maturity, hence only a minimal seasonal response was expected while the weather was warming up in South Australia. When the effectiveness of the hormonal treatment was adjusted to body weight, all the fish that responded better to the treatment were those biggest in size and more advanced in age, indicating higher efficiency of hormonal treatment as fish were closer to sexual maturity. The Vtg plasma levels found in this study in all treatments and control ranged between 1 to 45 µg/ml, this levels are within the levels reported for female fish approaching to sexual maturity, which can fluctuate from 1 µg/ml to several tens of mg/ml (Hara et al., 2016). The levels of E₂ found in the control, GnRHa and Kiss2-12 treatments during the course of the trial were within the range of that found in 18-month old farmed YTK females (control and FSH-treated) during the reproductive season in South Australia (Sanchis-Benlloch et al., 2016). Although no significance in the Vtg or E₂ levels was observed among treatment groups, significant difference was found in the E₂ levels within the Kiss1-15 group, at the start and end of the trial. Moreover, when the E₂ levels where adjusted to body weight a higher level of E₂ in all of the Kiss 1-15 fish was observed indicating a stimulatory effect of the treatment. Our previous work in male YTK showed also stimulatory effect of the
Kiss1 decapeptide on pubertal development on male YTK, both during the breeding and non-breeding season, while the Kiss2 decapeptide was more effective during the non-breeding season (Nocillado, Zohar et al. 2013). Results in the present study are consistent with our previous observations although it remains to be determined whether Kiss2 would have the same effect in female YTK as in males. Studies in medaka using in situ hybridization revealed that Kiss1 but not kiss2 is involved in estrogen feedback (Mitani, Kanda et al. 2010), consistent with the results obtained here. Recent studies in immature chub mackerel reported that single subcutaneous implantation of mini-osmotic pumps loaded with Kiss1-15 induced vitellogenic onset, however, no GSI differences were found during the 6 weeks experimental period (Selvaraj et al., 2013). As in Chub mackerel, only Kiss1-15 treatment appeared to be an effective treatment in this study. This result suggests a role of Kiss1 in gonadal maturation in female YTK, as reported in juvenile European sea bass (Escobar et al., 2013). Studies in striped bass suggest a stimulatory effect of Kiss1 on Fshβ expression and gonadal development, pointing to a role of Kiss1 in early stages of reproduction (Zmora et al., 2014) as in our previous work in male YTK (Nocillado et al., 2013). However, the dose used in this study was half (50µg/kg BW) of that tested in our previous work (100 µg/kg BW) due to the fact that the exposure time in this study was longer (12 weeks) compared to that used in our previous Kiss study on YTK (4-8 weeks), and might have limited the response. This study was compromised by the difficulty of sexing the experimental animals at the beginning of the trial, as we have experienced in another study in YTK (Sanchis-Benlloch et al., 2016), however due to the size of the animals and limited experimental facilities, we were not able to start with a larger cohort. Nevertheless, results of the present study provide evidence that Kiss1-15 treatment has a stimulatory effect in female YTK. Moreover, for further studies on inducing earlier maturation of late maturing species as YTK using kisspeptins treatments authors suggest to use higher kisspeptin doses and animals closer to sexual maturity as there was a higher response to the hormonal treatment as fish were closer to sexual maturity. Hence, this study set up and justified the bases for further studies into evaluating the effect of kisspeptins and determining its effect on the brain-pituitary-gonadal axis in more detail.
2.5. References


Chapter 3: *In-vitro* and *in-vivo* biological activity of recombinant Yellowtail kingfish (*Seriola lalandi*) follicle stimulating hormone*<sup>*</sup>

Pablo J. Sanchis-Benlloch 1, Josephine Nocillado 2, Claudia Ladisa 3, Joseph Aizen 1, Adam Miller 2, Michal Shpilman 3, Berta Levavi-Sivan 3, Tomer Ventura 1, Abigail Elizur 1

1 Genecology Research Centre, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Queensland, Australia; 2Clean Seas Tuna Ltd, Port Lincoln, SA, Australia; 3Department of Animal Sciences, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot 76100, Israel.

**Abstract**

Biologically active recombinant yellowtail kingfish follicle stimulating hormone (rytkFsh) was produced in yeast *Pichia pastoris* and its biological activity was demonstrated by both *in vitro* and *in-vivo* bioassays. Incubation of ovarian and testicular fragments with the recombinant hormone stimulated 17β-estradiol (*E*₂) and 11 ketotestosterone (11KT) secretion, respectively. *In-vivo* trial in immature female YTK resulted in a significant increase of plasma *E*₂ levels and development of oocytes. In males at the early stages of puberty, advancement of spermatogenesis was observed, however plasma 11KT levels were reduced when administered with rytkFsh.

**Keywords:** Recombinant protein, FSH, Teleost puberty, Yeast, *Pichia pastoris, Seriola lalandi*.

* This Chapter was published in General and comparative endocrinology (Sanchis-Benlloch et al., 2016; http://dx.doi.org/10.1016/j.ygcen.2016.03.001). It is attached in the published form at the end of the thesis.
3.1. Introduction

The genus Seriola includes highly active pelagic fish belonging to the Carangidae family and are found in the Atlantic, Indian and Pacific Oceans. The culture of Seriola began in Japan over 70 years ago (Nakada, 2008). Yellowtail kingfish (*Seriola lalandi*, YTK) is one of the largest members of the genus and its importance for the aquaculture industry is growing worldwide due to its fast growth, high flesh quality and suitability for farming in both cage and recirculating aquaculture systems (B. Chen et al., 2006; Orellana et al., 2014; Poortenaar et al., 2001). YTK is a gonochoristic species with an asynchronous oocyte development, which provides the capacity for multiple spawning within a reproductive season, between spring and summer (Poortenaar et al., 2001). YTK puberty age can vary between male and female fish, and between geographical locations. According to observations in farm conditions in South Australia, precocious males can start to spermiate from 12 months old onwards, however females take 4-5 years to reach sexual maturity. Currently, a genetic selection program is being undertaken for YTK (Whatmore et al., 2013), and there is interest to shorten its generation time to increase genetic gain. Advancing pubertal development is the first step towards shortening the breeding period, and as such has been the focus of many studies (Carrillo et al., 2009; Taranger et al., 2010). Strategies to advance the onset of puberty range from the control of environmental parameters, as reported for Atlantic salmon (*Salmo salar*) where the control of salinity and photoperiod modulates pubertal development (Melo et al., 2014), to hormonal therapies such as in female red sea bream (*Pagrus major*), where continuous administration of GnRHα resulted in precocious induction of puberty (Kumakura et al., 2003).

The development of the gonads in fish is mainly controlled by two pituitary gonadotropins: follicle stimulating hormone (Fsh) and luteinizing hormone (Lh) (Levavi-Sivan et al., 2010). Evidence suggests that Fsh plays a key role in the onset of puberty and early stages of reproductive development while Lh plays a role at the advanced stages of maturation (Yaron et al., 2003). However, studies in Japanese eel and goldfish reported recombinant Fsh activates the larger stages of maturation (Hayakawa et al., 2008; Kobayashi et al., 2010). Gene knockout studies of *fsh* and *lh* in zebrafish have confirmed that the *fsh-fshr* signalling pathway is essential in puberty onset and gonadal
growth in male and female fish (Zhang et al., 2015; Zhang et al., 2014). Until recently, knowledge gaps still exist in relation to the exact actions of Fsh and Lh (Mazón et al., 2014) in most perciforms. This is partly due to the lack of biologically active pure forms of these hormones (Zhang et al., 2014). This problem has recently been circumvented with the production of recombinant gonadotropins in heterologous expression systems resulting in pure forms of the hormones with biological activity (Levavi-Sivan et al., 2010).

To date, piscine recombinant Fsh have been produced successfully for 12 species using different heterologous expression systems (Chen et al., 2012; Hayakawa et al., 2008; Kobayashi et al., 2010; Levavi-Sivan et al., 2010; Mazón et al., 2014; Yu et al., 2010). However, recombinant Fsh has been tested in vivo in six species only. Recombinant goldfish (Carassius auratus) Fsh (Hayakawa et al., 2008; Kobayashi et al., 2006) and recombinant Manchurian trout (Brachymystax lenok) Fsh (Ko et al., 2007) successfully induced milt production in goldfish. Also, recombinant goldfish Fsh induced ovulation in bitterling (Rhodeus ocellatus ocellatus) and promoted the initiation of spermatogenesis in sexually immature male Japanese eel (Anguilla japonica) (Hayakawa et al., 2008; Hayakawa et al., 2009). Recombinant Japanese eel Fsh induced spermatogenesis and oocyte maturation in sexually immature Japanese eel (Kobayashi et al., 2010). Recombinant orange-spotted grouper (Epinephelus coioides) Fsh administrated to juvenile grouper increased serum sex steroid levels and induced early ovarian development (Chen et al., 2012). In zebrafish (Danio rerio) it was fond that the native form of the recombinant Fsh had higher activity when compared to its 6His-tagged form (Yu et al., 2010). In prepubertal European sea bass male (Dicentrarchus labrax), recombinant Fsh (rFsh) was able to trigger the process of spermatogenesis (Mazón et al., 2014). In the same species rFsh was tested for its in-vivo stability, allowing the comparison of the recombinant hormones produced in two different expression systems (Molés et al., 2011).

In the present study, we have generated a single chain recombinant yellowtail kingfish Fsh (rytkFsh) using the yeast (Pichia pastoris) expression system (Hollenberg et al., 1997). The recombinant hormone was tested both in-vitro and in-vivo for its efficacy in inducing sex steroid secretion and promoting gonadal development in immature YTK females and males at the onset of sexual maturation.
3.2. Materials and methods

All experiments were conducted with approval from the Animal Ethics Committee of the University of the Sunshine Coast (Queensland, Australia) under approval number AN/A/12/69.

3.2.1. Isolation of full-length fshb and fsha cDNA sequences

Total RNA was extracted from frozen YTK pituitaries previously stabilised in RNALater (Ambion Life Technologies, Carlsbad, CA, USA) with Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA quality and quantity was established using Agilent’s 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). First-strand cDNA was generated from 1 µg total RNA using 5’- and 3’- SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). Synthesised 5’ and 3’ cDNAs were tested by PCR amplification of the reference gene acidic ribosomal phosphoprotein P0 (ytkARP) (Nocillado et al., 2012).

Gene-specific RACE PCR primers (Table 3.1) for fshb were designed from the previously isolated partial YTK fshb sequence (GenBank Accession No. HQ449731). The primers used for the first and semi-nested 3’ RACE PCR were Primer 1 and Primer 3, respectively. The first and semi-nested 5’ RACE PCR reverse primers were Primer 2 and Primer 4. In both reactions, the fshb primers were paired with Nested universal primer (NUP). The first round PCR reaction mix contained 1.5 µl 10x PCR buffer, 1.2 µl MgSO4 (50 mM), 0.3 µl dNTPs (10 mM each), 0.3 µl of NUP (10 µM), 0.3 µl of each forward and reverse primer (10 µM), 0.06 µl Platinum high fidelity Taq DNA polymerase (Invitrogen Life Technologies) and 1 µl of the first-strand cDNA. The semi-nested PCR amplification reaction mix contained 2.5 µl 10x PCR buffer, 2 µl MgSO4 (50 mM), 0.5 µl dNTPs (10 mM each), 0.5 µl of NUP (10 µM), 0.3 µl of each forward and reverse primer, 0.1 µl Platinum high fidelity Taq DNA polymerase and 1 µl of the first round PCR product (diluted 20-fold). No template control reactions were performed in order to rule out contamination. The thermal cycling parameters for the first and semi-nested reactions were: initial denaturation at 94°C (3 min); followed by 35 cycles of denaturation at 94°C (30 s), annealing at 53°C (30 s) and extension at 72°C (1 min); and final extension at 72°C (5 min). PCR product purification and cloning were as previously described (Nocillado et al.,
2012). Plasmid DNA from positive colonies were sequenced by the Australian Genome Research Facility (Brisbane, Australia). Sequences were analysed using NCBI’s Basic Local Alignment Search Tool (Altschul et al., 1990) and Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA).

Table 3.1. Gene-specific primers used for the isolation of fhsa and fshb cDNA sequences in yellowtail kingfish

<table>
<thead>
<tr>
<th>Primer</th>
<th>5’ to 3’ sequence</th>
<th>Direction</th>
<th>Sequence Amplified</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1</td>
<td>CCATATGTCAGTAGGCTTGTTAACCC</td>
<td>Forward</td>
<td>fshb</td>
<td>53°C</td>
</tr>
<tr>
<td>Primer 2</td>
<td>ACATCCAGGAGTGTTCTGACACTC</td>
<td>Reverse</td>
<td>fshb</td>
<td>53°C</td>
</tr>
<tr>
<td>Primer 3</td>
<td>GAGGTGAAACACACTGCGGATGTT</td>
<td>Forward</td>
<td>fshb</td>
<td>53°C</td>
</tr>
<tr>
<td>Primer 4</td>
<td>GGTACACTGCTCTGAGCATATGG</td>
<td>Reverse</td>
<td>fshb</td>
<td>53°C</td>
</tr>
<tr>
<td>NUP</td>
<td>AACGAGTTGATCAACGCGAGT</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer 6</td>
<td>GGAACTTTCTCTCAATGGGTTGAC</td>
<td>Forward</td>
<td>fsha</td>
<td>53°C</td>
</tr>
<tr>
<td>Primer 7</td>
<td>GCTGCAACCAGATGGGCTCAGTG</td>
<td>Forward</td>
<td>fsha</td>
<td>53°C</td>
</tr>
</tbody>
</table>

*NUP; Nested Universal primer

The full length fsha cDNA sequence was isolated by 3’RACE PCR. Primers 6 and 7 (Table 1) were designed according to the conserved regions of Dicentrarchus labrax (GenBank Accession No. AF269157) and Scomber japonicus (GenBank Accession No. JF495131) glycoprotein alpha sequences. The first and semi-nested PCR amplification was performed as for fshb. The thermal cycling parameters for both reactions were: initial denaturation at 94°C (2 min 30 s); followed by 30 cycles of denaturation at 94°C (30 s), annealing at 60°C (30 s) and extension at 72°C (45 s); and final extension at 72°C (7 min). PCR products were purified, cloned, sequenced and sequence analysed as described for fshb. The signal sequences of fshb and fsha were identified using SignalP 4.1 software (Petersen et al., 2011).

3.2.2. Synthesis of a single chain fshba and ligation into the pPIC9K expression vector

The rytkFshba was designed according to Kasuto et al. (2005) and Aizen et al. (2007). The cDNA sequences encoding for YTK fshb and YTK fsha (excluding the signal sequence) were linked with the nucleotide sequence encoding for GSGSHHHHHHGSGS and were then codon-optimised according to the yeast (Pichia pastoris) coding preference. The codon-optimised sequence was synthesised and ligated into the EcoRI-NotI sites of
pPIC9K (GenScript, Piscataway, NJ, USA). Sufficient quantity of the construct plasmid DNA was generated in JM109 *E. coli*. Plasmid DNA was purified with QIAprep spin midiprep columns (Qiagen, Hilden, Germany). The purified plasmid DNA was linearized with *SalI* (New England Biolabs, Ipswich, MA, USA). Nine µg of the linearized construct were transformed into SuperMan5-His- strain of the yeast (*Pichia pastoris*) cells (Biogrammatics, Carlsbad, CA, USA) by electroporation (BioRad GenePulser, Hercules, CA, USA).

### 3.2.3. Selection of high expressing clones and induction of protein expression

Positive yeast transformants were selected in histidine-deficient media. High copy number colonies were screened based on the resistance to the antibiotic Geneticin (G418 sulfate, Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 0.5-2 mg/ml. Using this protocol, 10 most resistant clones were selected from 800 colonies. From these 10 clones, the highest expressing clones were identified by methanol induction in 125 ml cultures. Protein expression induction by methanol in 1 Litre (L) volume was carried out in 5 L shaker flask at 30°C. Molecular grade methanol (Sigma-Aldrich) was added every 24 hr at 1% final concentration. Yeast was harvested 72 h after induction. The rytkFsh was purified from the culture media using nickel nitrilotriacetic acid-agarose (Ni-NTA, Qiagen) that binds His-tagged proteins. Media from yeast without recombinant DNA served as negative control. All the mentioned steps including electroporation protocol, selection in histidine-deficient media, antibiotic resistance, and protein expression induction by methanol were carried out according to Invitrogen’s *P. pastoris* expression manual (Invitrogen Life Technologies).

### 3.2.4. Purification of rytkFsh by dialysis and Western blot analysis

The recombinant protein was dialyzed against 0.1 PBS in Slide-A-Lyzer G2 Dialysis Cassettes that had a molecular weight cut-off of 20 kDa (Thermo Fisher Scientific, Pittsburgh, PA, USA), following the manufacturer’s protocol. The expected size of the deglycosylated rytkFsh was 23 to 35kDa. For Western blot analysis, 25 µl of the purified recombinant hormone was deglycosylated at 37°C overnight with *N*-glycosidase F (PNGase F, New England Biolabs) following the supplier recommendations. SDS-PAGE was performed using 20 µl of the deglycosylated protein on a 12% Mini protean gel (BioRad) at 160 V for 1 h. 6XHis protein ladder (Qiagen) was used as a marker (4 µl). The
recombinant protein was mixed with loading buffer composed of Tris-Cl, glycerol, 20 % SDS, Bromophenol blued and DTT at final concentration of 0.045M, 10%, 1%, 0.01 and 0.05 M, respectively (Sigma-Aldrich). Then the sample and the protein ladder were heated to 98°C (7 min) prior to loading into the gel. Proteins were transferred onto a nitrocellulose membrane using Trans-blot turbo transfer system transfer pack (BioRad). The membrane was blocked (1h at room temperature) with 25 ml of Tris-buffered saline (TBS), pH 7.5, containing BSA and Tween-20 at 3% and 0.1%, respectively (Sigma-Aldrich). Membrane was then washed twice with TBS-T (TBS supplemented with 0.1% Triton) (Sigma-Aldrich), followed by incubation with primary antibody (mouse Penta-His antibody, Qiagen) at 1:2000 dilution for 1h at room temperature in an orbital shaker, followed by two consecutive washes as described above, then incubation with secondary antibody diluted 1:5000 (Goat anti mouse-IR Dye 800CW; Li-cor Bioscience, Lincoln, USA). Bands were visualized at 800 channel using the Li-cor Odyssey CLx Infrared Imaging System (Li-cor Bioscience).

3.2.5. Prediction of rytkFsh 3D structure

To predict the folding of rytkFsh, a 3D structure of was generated using the iterative online software I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). Visualization and labelling of the model were performed using Discovery Studio 4 and Adobe Photoshop CC2014.

3.2.6. In-vitro heterologous receptor binding assay

To study the receptor binding of the novel rytkFsh, we utilised the tilapia Fsh receptor (tiFshr) as heterologous receptor. The assay used a light sensitive luciferase (LUC) reporter, with cAMP response element (CRE-LUC; Invitrogen) that was previously demonstrated as a useful tool for discriminating cAMP signalling pathway (Biran et al., 2008). Fsh is known to exert its effect via cAMP upon binding to its cognate receptor (Levavi-Sivan et al., 2010). The entire coding region of tiFshr was inserted into pcDNA3.1 (Invitrogen). Three microgram of tiFSHR-pcDNA3.1 construct with three microgram of luciferase reporter plasmid were transiently transfected into COS-7 cells (American Type Culture Collection). Forty-eight hours after transfection, cells were treated with increasing doses of rytkFsh (16, 80, 400, 2000 and 5000 ng/ml) or rtiFsh (0.8, 4, 20, 100 and 275 ng/ml). The hormone treatment and the subsequent measurement of luciferase
activities were carried out as previously described (Biran et al., 2008). The EC\textsubscript{50} values were calculated from concentration response curves by means of computerized nonlinear curve fitting with Prism version 6 software (GraphPad).

**3.2.7. In-vitro bioactivity assay using YTK gonads**

The bioassay procedure was performed according to (Aizen et al., 2007) with the following modifications. Briefly, testes from male YTK at the start of spermatogenesis/onset of puberty (3.2 kg body weight (BW), 59 cm fork length (FL) and 0.07 gonadosomatic index (GSI) or immature ovaries from immature YTK female (2.4 kg BW, 49 cm FL and 0.23 GSI) were divided into uniformly sized fragments (20 mg each). The fragments were pre-incubated at 21°C with gentle shaking in a 24-well culture plate that had 1ml/well of basal medium eagle (BME) containing NaHCO\textsubscript{3} (2.5 g/L), penicillin (50 iu/ml), streptomycin (0.05 mg/ml), and nystatin (1.25 iu/ml) and 0.05% BSA buffered to pH 7.4 with 2.1 mM of Hepes (all from Sigma-Aldrich). The culture media was replaced every hour during pre-incubation. After the 3 hr pre-incubation, the fragments were incubated in the same medium containing recombinant rytkFsh at doses of 800, 400, 200 and 100 ng/ml. As a positive control, gonadal fragments were incubated in the same medium containing YTK pituitary extracts. Freshly dissected pituitaries from YTK male at the start of the spermatogenesis (3.12 kg BW, 59 cm FL and 0.08 GSI) and immature YTK female (3.5 kg BW, 60.5 cm FL and 0.29 GSI) were homogenized in 0.1% PBS and stirred for 30 min at 4°C. The mixture was centrifuged at 3000g for 4 min and 50 µl from the top layer were used as a positive control. Negative control wells contained culture medium only. Incubation with rytkFsh, YTK pituitary homogenate and media alone was conducted for 18 h at 21°C. These incubations were performed in triplicate wells per treatment. The incubation medium was collected and stored at -80°C until assayed for 11KT or E\textsubscript{2} concentrations.

**3.2.8. In-vivo bioassay in immature female YTK and maturing male YTK**

Eighteen months old YTK were used as experimental animals for the in-vivo experiments at CleanSeas Tuna Ltd (Arno Bay, South Australia). At the start of the trial, average BW and FL were 2.79±0.10 kg and 52.26±0.72 cm (mean ± SD), respectively. Fish were reared in a 6000 L tank using ambient sea water. In order to determine whether there were spermiating males in the group, gentle pressure was applied on the abdomen,
however no fish released sperm. Gonadal biopsy was not conducted as it is technically difficult to obtain a gonadal biopsy of very immature gonad without damaging the fish. At the end of the trial, fish BW and FL were 3.2±0.14 kg and 58.36±0.80 cm for females (n=17) and 2.88±0.08 kg and 56.86±0.52 cm for males (n=24), respectively.

The experiment commenced on October 2014 and was terminated on December 2014, which coincides with the spawning season for YTK in South Australia. The experimental animals were tagged and randomly assigned to 2 experimental groups. During the experiment, the fish were under natural photoperiod which ranged from 12L:12D (October) to 14L:10D (December). Water temperature ranged between 15°C and 20°C. Experimental animals were injected as described by Mazón et al. (2014) with the following modifications. YTK was injected intramuscularly in the right epaxial muscle anterior to the dorsal fin. Animals belonging to control group (n=22) were injected saline only. Animals belonging to treated group (n=19) were injected at doses of 10-20 µg/kg with lyophilised rytkFsh resuspended in sterile saline (0.9% NaCl). Injections were repeated 6 times with 10 day’s interval. At the end of the trial, fish were terminally anesthetized with Aqui-S (Aquatic anaesthetic) at a dose of 1 ppt. Then, BW and FL were recorded. Fish were bled by caudal puncture using heparinised syringes (heparin 30 iu/mL, Sigma). Two ml of blood per fish were collected. All fish gonads were then dissected out and weighed. In addition, male gonads were inspected for the presence of sperm when processed for gonadal fragment collection. Both; males and females gonad fragments corresponding to the medial area were fixed in 4% paraformaldehyde for histological analysis. Plasma from the experimental animals was extracted from blood by centrifugation at 3000g at 4°C for 20 minutes and then stored at -80°C until 11KT and E2 analysis.

3.2.9. Sex steroids analysis

Levels of 11KT and E2 were determined by commercially available enzyme immunoassay (EIA) kits (Cayman Chemical Company, Ann Arbor, MI, USA), which are known to be highly specific for these steroids, as detailed in their specifications. For the in-vitro assay, the intra-assay coefficient of variation (CV) was 7.3% and 9.8%, while the inter-assay CV was 10.7% and 13.9% for E2 and 11KT, respectively. For the in-vivo assay,
the intra-assay CV was 9.8% and 9.6% while the inter-assay CV was 13% and 11.8% for E₂ and 11KT, respectively.

Steroids were extracted from 200 µl of plasma with 5 ml of diethyl ether. Solvent was evaporated and extracts were resuspended in the EIA assay buffer. Plasma levels of 11KT and E₂ were measured for males and females, respectively. For E₂ assays, the plasma was diluted 2 times while for 11KT, 10 times dilution was required to optimise the sensibility of the assay defined as 90% binding.

3.2.10. Histological analysis

Right and left gonads were removed and weighed from each male (n=24) and female (n=17) fish. Cross section samples were taken from the medial area of both gonads as being representative for the whole lobe and fixed in 4% paraformaldehyde for 24h and then transferred to 70% ethanol until tissue processing for histology. All samples were processed for histological investigation. The gonad samples were embedded in paraffin and sectioned transversely at 6 µm width. Sections were stained with haematoxylin and eosin. The classification of developmental stage of oocytes was according to (Gillanders et al., 1999). Histological analysis was performed under a DM5500B Microscope equipped with a DFC550 camera system (Leica Microsystems, Wetzler, Germany). Measurements (n=600 oocytes/group) of the main oocyte diameter were taken from each sample using an eye piece micrometre mounted on a light microscope (BS51, Olympus, Tokyo, Japan).

3.2.11. Statistical analysis

Body weights (BW), fork length (FL) and GSI were expressed as mean±SD. Levels of E₂ and 11KT from the in-vitro and in-vivo experiments were expressed as mean±SEM. Values from these data were analysed with one-way analysis of variance (ANOVA) followed by a multiple comparison test (Tukey). Differences were reported as statistically significant when p<0.05.
3.3. Results

3.3.1. Yellowtail Kingfish Fsh and predicted structure of single chain rytkFsh

Multiple sequence alignment showed 97% homology of ytkFshb (GenBank accession no. HQ449731) with those of amino acid sequences from the same genus (S. dumerili and S. quinqueradiata) (Fig 3.1A). Homology dropped to 57% when compared to other perciforms, such as O. niloticus, and to 37% when compared to mammals, such as H. sapiens. However, ytkFsha (GenBank accession no. KT364712), the gonadotropin alpha subunit, was 100% identical to that of S. dumerili and 99% with S. quinqueradiata (Fig 3.1B).

Fig 3.1. Multiple comparative sequence alignment of ytkFshb (A) and ytkFsha (B) with other vertebrate Fsh sequences. Sequence logo indicates homology conservation with bigger letters indicating higher conservation.

The percentage homology dropped to 85% when compared to other perciform fish, such as O. niloticus, and to 53% when compared to mammals, such as H. sapiens. The construct of the ytkfsh is presented in Fig 3.2A. Modelling of the single chain recombinant ytkFsh predicted a properly folded 3D structure (Fig 3.2B). The model showed 3 loops in each subunit, the expected binding sites and the linker sequence does not obstruct the alpha and beta subunits.
3.3.2. Recombinant single chain ytkFsh (rytkFsh)

The single chain ytkFsh construct was successfully electroporated into the yeast (*P. pastoris*) genome and recombinant Fsh was produced. Yield ranged from 400-900 µg/L, depending on the specific yeast clone used (n=6). Western blot analysis of deglycosolated rytkFsh using the His tag antibody showed 3 bands: the expected 23 kDa and >30 kDa bands representing rytkFsh and the enzyme PNGase F, respectively, and a third band below 15 kDa, that might be due to partial degradation of the recombinant protein (Fig 3.3).
3.3.3. *In-vitro* bioactivity of rytkFsh

The tilapia Fsh receptor was activated by both rytkFsh and recombinant tilapia FSH (rtiFsh) in a dose-dependent manner using the cAMP signal transduction system as a reporter. The rtiFsh was 7-fold more effective than rytkFsh in inducing tiFshr activity (EC\text{50} 73.78 ng/ml for rtiFsh and 530.5 ng/ml for rytkFsh) (Fig 3.4).

*In-vitro* bioassay using YTK gonadal fragments assessed the efficiency of rytkFsh in inducing E\text{2} secretion from immature female gonadal fragment or 11KT secretion from testicular fragment taken from males at the onset puberty. In females, the rytkFsh stimulated a dose response in terms of E\text{2} secretion (Fig 3.5A). However, only the highest dose (800 ng/ml) was significant when compared with the negative control. In males, 11KT secretion from YTK testicular tissues treated with increasing doses of rytkFsh also showed a response in a dose-dependent manner (Fig 3.5B).

**Fig 3.4.** *In-vitro* binding assay of rytkFsh and recombinant tilapia Fsh (rtiFsh) to the tilapia Fsh receptor. rytkFsh and rtiFsh-induced CRE-derived transcriptional activity. Data are expressed as the change in luciferase activity over basal activity. The experiment was repeated 3 times and each point was determined in quadruplicate. Data are presented as mean ± SEM.

**Fig 3.5.** *In-vitro* bioassay of rytkFsh on gonadal segments. E\text{2} secretion from YTK ovarian fragments (A) and 11KT release from YTK testicular fragments (B) incubated with increasing doses of rytkFsh. The doses in ng/ml are shown on the X-axis. YTK pituitary extract was used as positive control. Column and bar
represent the mean and the SEM, respectively. Different letters indicate significant difference (p<0.05, N=3).

As in the females, only the highest dose (800 ng/ml) showed significant difference when compared with the control. The level of E₂ and 11KT secretion in gonads incubated with the highest rytkFsh dose was comparable to that obtained from the pituitary extracts (positive control).

3.3.4. In-vivo bioactivity of rytkFsh

In-vivo assays in sexually immature female YTK resulted in a significant increase in plasma E₂ in treated fish when compared with the control (Fig 3.6A). In males, plasma levels of 11KT showed significantly lower levels of 11KT compared with control group (Fig 3.6B). Although the GSI of females was slightly higher in the treated group (0.2±0.03 vs 0.23±0.05), the difference was not significant. In males, the GSI in control group was slightly higher than in the treated group (0.07±0.02 vs. 0.054±0.03), however the difference was not also significant.

Histological analysis of the cross section from the middle area of the right and left gonads showed no developmental differences between the two lobes, either in males or in females. Oocyte diameter did not significantly differ between the two groups, with means ± SEM of 40.55 ± 0.59 vs 41.41± 0.83 µm for control and treated females, respectively. Oocytes at the chromatin nucleolar and perinucleolar stages of development (Stages 1 and 2, respectively) were present in both rytkFsh-treated and untreated fish (Fig 3.7A and 3.7B). However, oocytes at early cortical alveolar stage
(Stage 3) were present only in the rytkFsh treated group, suggesting the onset of gonadal development.

YTK males presented an asynchronous pattern of spermatogenesis, where all germ cell stages were present at the same time. However, within each spermatocyst, germ cell development was synchronous. Stages observed were mature (stage 1), developing (stage 2) and mature (stage 3). Stages 1 and 2 were present in both control and treated groups (Fig 3.7C). Stage 3, which is characterised by spermatozoa in lumen of the lobules, was found only in the treated group (Fig 3.7D).

**Fig 3.7.** Histological sections of YTK ovary and testis from the in-vivo trial. Ovary (A) and testis (C) from YTK injected with saline only (control) while B and D are ovary and testis, respectively, from YTK treated with rytkFsh. Oocytes at chromatin nucleolar stage of development (Stage 1) and perinucleolar stage (Stage 2) were present in both rytkFsh treated and untreated groups. However, oocytes at cortical alveolar stage (Stage 3) (B) were only present in the rytkFsh treated group. Mature testes (stage 3) were observed only in rytkFsh-treated fish (D). (SG= spermatogonia; SD= spermatids; SZ= spermatozoa; CNS= chromatin nucleolar stage; PS= perinucleolar stage; CAS= cortical alveolar stage. Scale bar represents 50 µm.
3.4. Discussion

Yellowtail kingfish is of growing importance in aquaculture around the world, and as such there is interest in having better control over its reproductive development, particularly the onset of sexual maturation in female broodstock. We have explored the use of recombinant ytkFsh in manipulating reproduction in 18 month old YTK in South Australia. Age at sexual maturation varies between male and female YTK. It also varies between geographical locations as well as within populations. In South Australia, males may start to spermiate from 12 month old onwards based on observations under farm conditions. In contrast, females take 4-5 years to reach sexual maturity. In New South Wales (Australia), where temperatures are higher, males can reach sexual maturity at the age of one year and females at 3+ years (Gillanders et al., 1999). In New Zealand, where it is colder, it can take longer than in either South Australia and New South Wales (Poortenaar et al., 2001). Earlier sexual maturation has been reported for YTK raised in Western Australia under commercial conditions when compared to wild YTK in this area (Kolkovski et al., 2004). These differences suggest that YTK age of puberty can be influenced by environmental parameters such temperature and photoperiod as reported in other fish species (Taranger et al., 2010).

As a first step towards developing the tools to control reproductive function in farmed YTK, we cloned the alpha and beta subunits of YTK fsh. Alignment of the alpha and beta ytkfsh sequences showed that, as in other fish species, fshb subunit is less conserved than the fsha subunit, indicating species specificity of the beta subunit and a high conservation of the alpha subunit over vertebrate evolution from fish to mammals, as reviewed by (Yaron et al., 2003). We used the alpha and beta ytkFsh sequences to design a single chain construct, with a linker composed of 2x (GSGS) and 6His in between, which is slightly modified from that designed in tilapia (Aizen et al., 2007). Although other literature demonstrated that the native form of Fsh has higher activity when compared to 6His-tagged recombinant hormone (Yu et al., 2010), we have utilised 6His tagging to facilitate the purification and detection of the recombinant hormone as there are no specific antibodies produced yet for the YTK Fsh. The prediction of the three-dimensional structure of the single chain protein confirmed that the linker sequence did not disturb the proper folding of the recombinant hormone, and therefore was not
expected to interfere with its biological function, as reported in other recombinant
glycoproteins (Xing et al., 2004). We generated a single chain rytkFsh hormone in the
yeast (Pichia pastoris), which is capable of post-translational modifications typically
associated with higher eukaryotes, such as the addition of N-linked high mannose
moiety (Cereghino et al., 2000). The consensus sequence for N-linked glycans in yeast is
Asn-Xaa-Ser/Thr, which is critical for bioactivity of the recombinant hormone (Cereghino
et al., 2000). The deglycosolation of rytkFsh with PNGase F to the nascent translated
protein implies that the carbohydrate modification of the mature rytkFsh protein occur
exclusively through the N-linked glycosylation as suggested for tilapia and Japanese eel
Fsh (Aizen et al., 2007).

Western blot analysis of the deglycosylated rytkFsh, using antibodies raised against
the histidine tag, revealed the expected 23 kDa band indicating that the hormone was
successfully synthesized, secreted into the media and harvested using the Ni-NTA beads.
The sizes of other reported deglycosylated recombinant Fsh range from 23 kDa in tilapia
(Aizen et al., 2007) to 35 kDa in grouper (Chen et al., 2012) and Manchurian trout (Ko
et al., 2007). Western blot analysis also revealed a >30 kDa band representing the
deglycosylation reagent PNGase F, and an additional band which might represent partial
degradation of the recombinant protein, as described by (Kasuto et al., 2005).

In-vitro receptor assay and gonadal bioassay confirmed that the recombinant single
chain ytkFsh is biologically active. The receptor assay confirmed bioactivity of the
rytkFsh showing that tilapia Fsh receptor was activated by both rytkFsh and rtiFsh dose-
dependently in the cAMP signal transduction system. As expected, the efficacy of
rytkFsh activation was not as potent as the rtiFsh, as the two Fshb subunits only share
57% homology at the amino acid level, however confirming that the rytkFsh is
biologically active. When using the rytkFsh to activate YTK testicular and ovarian sections
examined through 11KT and E2 secretion, a dose response to the hormone was detected,
significantly for the highest dose (800 ng/ml), which was as potent as the YTK pituitary
extract. These results further confirm the biological activity of rytkFsh. In a study in
tilapia, where the sex steroid levels were assayed using an enzyme-linked immunoassay,
it was found that the rtiFsh stimulated the secretion of E2 and 11KT in mature gonads at
the same level as that of the tilapia pituitary extract (Aizen et al., 2007). In the present
study, the levels of E2 and 11KT secreted were found to be lower when compared with
other *in-vitro* studies using recombinant Fsh in tilapia (Aizen et al., 2007) and European sea bass (Mazón et al., 2014), probably due to the difference in the maturational stage of the tested gonads.

The effect of the rytkFsh was examined *in-vivo* by administering 6 times, at 10 day’s intervals, rytkFsh to 18-month old YTK and following the changes in circulating sex steroid levels and changes in gonadal development at the end of the trial. Augmenting E$_2$ plasma levels is a prerequisite for advancing reproductive development and vitellogenin synthesis (Pankhurst, 2008). In a short term study, the E$_2$ levels found in immature rainbow trout and mature goldfish were significantly elevated 24 hours after a single injection of recombinant Fsh (Ko et al., 2007; Kobayashi et al., 2006). The E$_2$ levels found in mature goldfish were 2 times higher while the levels reported for immature rainbow trout were 2 times lower compared to the levels observed in the present study. However in juvenile grouper treated five times with recombinant Fsh at a dose of 200 µg/kg at 48 h intervals (Chen et al., 2012), the results are within the range of those found in this study for immature YTK. Although, some differences can be observed between the present study and the grouper one, such as the doses used (10 fold higher for the grouper), and particularly the duration of the trial and the stage of the gonads, the results of the present study are consistent in showing a clear E$_2$ response when a female fish is treated with recombinant Fsh, further confirming the biological activity of the recombinant hormone.

Histological analysis of the female gonads revealed that the oocytes at the chromatin nucleolar and perinucleolar stages (Stages 1 and 2 of development) were present in both ryrkFsh-treated and untreated fish. However, ovaries from the treated group exhibited onset of development as indicated by the presence of oocytes at early Stage 3 or cortical alveolar stage. Although differences in mean oocyte diameter were not statistically significant between the treated and the control groups, there was a trend showing higher oocyte diameter in rytkFsh-treated fish compared with the control fish. The plasma E$_2$ levels together with the ovarian histology suggest that the administration of rytkFsh to immature female YTK promoted gonadal development although the response was still at the early stages of manifestation and would have benefited from a longer duration of the treatment.
In male teleosts, 11KT is recognised as the major androgen responsible for testicular development (Dahle et al., 2003). In this study, the effect of rytkFsh administration at 10 day’s intervals for 6 times was assayed in 18-month old males through measurement of 11KT levels in plasma and by histological analysis of the testes. Histological examination revealed that, Stage 3, characterised by spermatozoa in lumen of the lobules was found only in the treated group. In accordance, sperm was found within the gonads of the majority of the rytkFsh-treated males indicating the activation of a late stage of spermatogenesis. Plasma testosterone levels showed a lower trend in the rytkFsh-treated males compared with the control (data not shown). Taken together with the fact that levels of plasma 11KT were significantly lower in the rytkFsh-treated fish when compared with those from the control group suggest that rytkFsh-treated males experimented an overall androgen reduction due to the fact that fish were already towards a late developmental stages as described by Gillanders et al. (1999) that correspond to a decreasing plasma 11KT levels (Poortenaar et al., 2001). Activation of later stages of maturation by Fsh has been reported already in goldfish (Hayakawa et al., 2008; Kobayashi et al., 2006). Such a reduction in 11KT was also observed exactly seven days after spermiation in three-spined stickleback (Gasterosteus aculeatus) (Páll et al., 2002). Juvenile European sea bass treated with recombinant Fsh resulted to increase 11KT plasma levels (Mazón et al., 2014). This results differ to the results of this study due to the maturational stage of the fish. Other long term studies in rFhs administration showed that recombinant goldfish Fsh (5–6 administrations at 7 day intervals) induced milt production in male goldfish and initiation of spermatogenesis in sexually immature male Japanese eel although no differences were found in size of the eel testicular lobes (Hayakawa et al., 2008). When recombinant Fsh was administrated in the same species at a higher dosage and frequency (8 times with 2-5 days interval), the treatment was more effective in promoting the size of testicular lobes (Hayakawa et al., 2009). Using the same frequency of recombinant Fsh treatment at a dose of 2 µg/g, spermatogenesis was also induced in-vivo in sexually immature Japanese eel (Kobayashi et al., 2010).

In summary, this study demonstrated that biologically active recombinant yellowtail kingfish Fsh can be produced in yeast P. pastoris and its biological activity was confirmed by both in-vitro and in-vivo bioassays. In future studies, further research on administration at different periods of gonadal development could help define the most
appropriate time frame of administration. Also, optimisation of dosage, and frequency and duration of administration could facilitate achieving commercially applicable protocol for inducing early maturation particularly in farmed female YTK.

3.5. References


Chapter 4: Development and Validation of a Competitive Hybrid ELISA for *Seriola lalandi* Vitellogenin

Pablo J. Sanchis-Benlloch, Josephine Nocillado, Norm Cheetham, Adam Miller, Daniel Powell, Tianfang Wang, Abigail Elizur

1Genecology Research Centre, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Queensland, Australia. 2Clean Seas Tuna Ltd, Port Lincoln, SA, Australia.

Abstract

The main objective of this study was to develop an enzyme-linked immunosorbent assay (ELISA) for *Seriola lalandi* vitellogenin (Vtg). Plasma from male *S. lalandi* treated with 17β-estradiol (E₂) was fractionated to produce the Vtg standard. LC-MS/MS analysis showed that the fraction corresponding to the highest peak when the NaCl gradient was at 90-100% consisted mainly of VtgAa and VtgAb. Nucleotide sequences corresponding to VtgAa, VtgAb and VtgC were obtained from a *Seriola lalandi* liver transcriptomic data. Two peptides (12 amino acids) were synthesized according to the predicted conserved regions of VtgAa and VtgAb, and used to generate two polyclonal antibodies in rabbits. Two homologous antibodies (anti-*S. lalandi* VtgA and anti-*S. lalandi* VtgB) and one heterologous (anti-*Mugil cephalus* Vtg) were tested in order to develop the *S. lalandi* ELISA. SDS-PAGE and western blot analyses of the ELISA standard, using both *S. lalandi* antibodies and the *M. cephalus* Vtg antibody, revealed a main band of 195 KDa. This band was present in plasma from E₂ treated males but not in males prior to E₂ treatment. Both homologous antibodies (anti-*S. lalandi* VtgA and anti-*S. lalandi* VtgB) did not result in acceptable absorbance levels during ELISA development. In contrast, the heterologous *M. cephalus* Vtg antibody resulted in acceptable absorbance values. Parallelism was confirmed between the *M. cephalus* Vtg standard and the *S. lalandi* Vtg standard. Serial dilutions of plasma from E₂-treated *S. lalandi* males and maturing females but not from males prior to E₂-treatment paralleled the standard curve. The ELISA developed is highly specific (no cross-reaction with other proteins present in plasma when samples were diluted over 1:20), precise (the inter-assay coefficient of variation at 50% binding was 13%) and sensitive (19ng/ml). The assay was validated by
quantifying the plasma Vtg levels in females at the start of the reproductive season and in E2-treated males.

Key words: Seriola lalandi, Vitellogenin, ELISA
4.1. Introduction

Vtg is the major egg yolk precursor synthesised by the liver of non-mammalian oviparous vertebrates in response to circulating estrogens (Hara et al., 2016). Vtg is transported through the bloodstream to the ovaries where it is incorporated by the developing oocytes via receptor-mediated endocytosis and processed into egg yolk proteins (YPs) (Mommsen et al., 1988). In teleosts, the three YPs derived from Vtg (lipovitellin, phosvitin and β’-component) play essential roles in embryonic and larval fish nutrition (Hiramatsu et al., 2002). The most evolved groups of fish (Paracanthopterygii and Acanthopterygii) possess three Vtg orthologues, namely: Vtg type A (VtgA or VtgAa), Vtg type B (VtgB or VtgAb) and Vtg type C (VtgC) (Rawat et al., 2013). VtgAa and VtgAb are known as “complete” Vtg forms because they contain the three main YPs while VtgC is known as an “incomplete” form due to the presence of lipovitellin only (Rawat et al., 2013). The complete form of Vtg gives undergo proteolytic cleavage during oocyte maturation yielding yolk proteins that are needed for oocyte hydration and embryonic and larval development, while the incomplete form gives rise to nutrients that are necessary at the later stages of embryonic development (Amano et al., 2007; Carnevali et al., 1999; Hara et al., 2016; Hiramatsu et al., 2002; Pousis et al., 2011).

In teleosts, Vtg levels in the plasma directly indicates the females’ reproductive stage (Mañanós et al., 1994; Wallace, 1985). Therefore, Vtg levels in plasma can be used to determine sex and maturational stages in females (Heppell et al., 1999; Maltais et al., 2014). Males are also capable of producing Vtg if exposed to estrogenic compounds (Mañanós et al., 1994). Among the estrogens, E2 is the most potent in inducting Vtg synthesis (Van Bohemen et al., 1981).

The first Vtg ELISA was developed in 1984 as a method for sexing salmonids (Gordon et al., 1984). Since then, Vtg ELISA has been widely used for assessing the maturational stages in different fish species (Heppell et al., 1999; Maltais et al., 2014; Mañanós et al., 1994; Mosconi et al., 1998; Susca et al., 2001). Numerous studies have developed ELISAs using Vtg as a biomarker for environmental endocrine disruptors (Hansen et al., 1998; Heppell et al., 1995; Holbech et al., 2001; Ndiaye et al., 2006; Parks et al., 1999; Swart et al., 2009; Tyler et al., 2002).
The cross-reactivity of Vtg in different species of fish has been used to develop heterologous Vtg ELISAs (Palumbo et al., 2009; Tyler et al., 2002). For instance, fathead minnow (*Pimephales promelas*) Vtg was quantified based on carp (*Cyprinus carpio*) Vtg (Tyler et al., 1999). Using *O. niloticus* Vtg as a standard, the levels of circulating Vtg in seven different cichlid species were determined (Ndiaye et al., 2006). The cross-reactivity between Vtg from different species has also been used to develop hybrid ELISAs, where a heterologous antibody is used against a homologous Vtg standard (Carnevali and Belvedere, 1991; Maltais et al., 2014; Maltais et al., 2010; Mylchreest et al., 2003; Vega-López et al., 2006). In the present study, we evaluated the use of homologous antibodies developed against synthetic peptides (anti-*S. lalandi* Vtg antiserum) compared with heterologous antibodies developed against the whole Vtg molecule (anti-*M. cephalus* Vtg antiserum) in order to develop a competitive ELISA for *S. lalandi* Vtg.

Seriola species, and in particular *S. lalandi*, are important aquaculture species and their importance is growing worldwide due to their fast growth, high flesh quality and suitability for farming in both cage and recirculating aquaculture systems (Chen et al., 2006; Mandich et al., 2004; Orellana et al., 2014; Poortenaar et al., 2001). The hybrid Vtg ELISA we developed can be used to further understand *S. lalandi* reproductive biology and to support the development and commercial growth of this important aquaculture species.

4.2. Materials and methods

All experiments were conducted with approval from the University of the Sunshine Coast Animal Ethics Committee (Queensland, Australia) under approval number AN/A/15/95.

4.2.1. Generation of *S. lalandi* liver transcriptome and identification of Vtg sequences

Total RNA was extracted from liver of 3 females *S. lalandi* (6-8 kg, 2-3% GSI; provided by CleanSeas Tuna Ltd, South Australia) using Direct-zol RNA miniprep kit (Zymo Research) that includes a genomic DNA elimination step. The total RNA concentration was measured by a fluorometer (Quantus, Promega). RNA integrity was verified on a
BioAnalyzer (Agilent). One microgram of total RNA was used to construct an RNA-Seq library according to Illumina’s TruSeq low RNA sample preparation v2 guide.

The library was sequenced using the Illumina MiSeq benchtop sequencer at the University of the Sunshine Coast, producing over 20 million 250 bp paired-end (PE) reads. Fastq files were recovered from the sequencer and quality of the raw sequencing reads were assessed using FastQC (http://www.bioinformatics.babraham.ac.uk). Trimming of adaptors, filtering of low quality reads and removal of contaminants was performed using Trimmomatic (Bolger et al., 2014) with a custom screening database. Clean PE reads from the MiSeq platform were assembled using Trinity software (release r20140413) (Haas et al., 2013), using the default settings for paired-end reads except for -min contig length 100 and -normalize reads. The assembled transcripts were screened for putative protein-coding regions using the program TransDecoder (release r20140704) (http://transdecoder.github.io/) with the option of including matches to the Pfam domain database (Finn et al., 2013) and with a minimum size of 30 amino acids (aa), then clustered using CD HIT with a sequence identity threshold of 0.9 (Fu et al., 2012).

Transcript sequences for Vtg were identified by PFam protein domain searches using the HMMER3 suite and BLASTp alignment to publically available Perciforms Vtg nucleotide sequences (Eddy, 2011). Multiple sequence alignments were performed using ClustIO (Sievers et al., 2011). Phylogenetic trees were constructed from amino acid sequences with 500 bootstrap replicates using the maximum likelihood algorithm from MEGA6 software (Tamura et al., 2013).

4.2.2. Generation of S. lalandi Vtg antiserum

Two peptides, C-NIKKTQNVYELQ (S4.1 Fig A) and C-NHLVTHNMVRH (S4.1 Fig B), which are conserved within the S. lalandi VtgAa and VtgAb sequences, were synthesized. Two polyclonal antibodies, anti-S. lalandi VtgA and anti-S. lalandi VtgB were raised against these peptides in rabbits by a commercial provider (Abmart, Shanghai, China). The peptides were injected in 4 rabbits 8 times with a general immunization schedule. The three firsts injections were provided every 14 days. Then, the rabbits were injected every 7 days until blood collection (Abmart, Shanghai, China). The M. cephalus Vtg antibody generated in rabbits was kindly provided by Professor Akihiko Hara from Hokkaido University, Japan (Amano et al., 2007).
4.2.3. Induction of Vtg synthesis in *S. lalandi* males

Vtg induction in *S. lalandi* males was performed at CleanSeas Tuna Ltd (Arno Bay, South Australia). Three *S. lalandi* males (28 months-old; around 10 Kg in body weight) were injected with E₂ (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 5 mg/kg. E₂ was first dissolved in 99.45 % molecular grade ethanol (EtOH, Sigma-Aldrich) at a ratio of 1:2 weight/volume. The E₂/EtOH dilution was then mixed with melted edible cocoa butter at ratio of 1:9 (v/v). *S. lalandi* were bled for time zero controls. The fish were intraperitoneally injected as previously reported (Sun et al., 2003). Injections were repeated 3 times at 7 days interval.

Seven days after the last injection, fish were terminally anaesthetised with Aqui-S (1 ppt; AQUI-S, New Zealand). Ten millilitres of blood were collected from the caudal vasculature from each fish using heparin-treated needle (19-gauge) and syringe. Immediately after collection, blood was transferred into heparin-treated microcentrifuge tubes and placed on ice. Plasma was separated from blood by centrifugation at 3000 x g at 4°C for 20 minutes and then stored at -80°C in tubes containing 10mM protease inhibitor (PMSF, Sigma-Aldrich) until further analysis.

Plasma from female *S. lalandi* (during the breeding and non-breeding season) was obtained from commercial fish production at CleanSeas Tuna.

4.2.4. Vtg purification

*S. lalandi* Vtg was purified using anion-exchange chromatography as previously described (Amano et al., 2007) with the following modifications. Six hundred microliters *S. lalandi* plasma from E₂-induced males were lyophilized using a ModulyoD-230 (Thermo Fisher Scientific; Waltham, MA, USA), resuspended in 6 ml molecular grade water. A volume of 600 µl from the resuspended plasma was injected into a column (GoPure™ POROS™ HQ50, Life Technologies, Bedford, MA, USA), which was equilibrated with a starting buffer of 0.02M Tris-HCl (pH 9.0). Fractions were eluted by step-wise addition of Tris-HCl buffer containing a gradient (from 0 to 100%) of 0.25M NaCl at a flow rate of 4ml/min and purified using an ÄKTA purifier system (GE Healthcare, Uppsala, Sweden). The whole procedure was performed at 4°C in order to prevent proteolytic breakdown. Eluted fractions of 5 ml were collected using a Frac-920 (GE Healthcare) fraction collector from a total volume of 300ml and stored at -80°C. The system was
controlled by a personal computer running Unicorn software. The protein concentration of the purified fractions was determined with a Quantus\textsuperscript{TM} Fluorometer (Promega, USA) following the manufacturer’s instructions (Promega, Madison, WI, USA).

**4.2.5. Protein in-solution digestion and LC-MS/MS analysis**

The fraction corresponding to the highest peak when the NaCl gradient was at 90-100% was lyophilised and reconstituted in 100 µl 8 M urea buffer, followed by reduction with 5 µL of 200mM dithiothreitol (DTT) for 60 min at 37°C to break the disulphide bands. Akylation was carried out in 20 µl of 200 mM of iodoacetamide (IAA) in 25 mM of ammonium bicarbonate for 60 min at room temperature, then 20 µl of 200 mM DTT was added and the mixture was incubated at room temperature for 30-60 min. The urea concentration was reduced with 775 µl double distilled water and trypsin in a 1:50 ratio. The digestion was performed overnight at 37°C. The reaction was stopped by adjusting the pH of the solution to <3 by adding 10% formic acid, and the samples were subjected to LC-MS/MS.

The tryptic peptides were analysed by LC-MS/MS on a Shimadzu Prominance Nano HPLC (Japan) coupled to a Triple Tof 5600 mass spectrometer (AB SCIEX, Canada) equipped with a nano electrospray ion source as previously described (Adamson et al., 2016). Briefly, approximately 6 µl of each extract were injected and de-salted on the trap column using solvent A [0.1% formic acid (aqueous)] before entering a nano HPLC column (Agilent Technologies, Australia) for mass spectrometry analysis. Peptide elution used a linear gradient of 1-60% solvent B [90:10 acetonitrile: 0.1% formic acid (aqueous)] over 30 min at 300 nl/min flow rate, and then solvent B was increased to 80% over 5 min and held at 80% for 5 min to wash the column and then returned to 1% solvent B. The mass spectrometer acquired 500 ms full scan TOF-MS data followed by 20 by 50 ms full scan product ion data. Full scan TOFMS data were obtained over the mass range 350-1800 and for product ion MS/MS 100-1800. Ions observed in the TOF-MS scan exceeding a threshold of 100 counts and a charge state of +2 to +5 were set to trigger the acquisition of product ion. The data were acquired and processed using Analyst TF 1.5.1 software (AB SCIEX, Canada).

Fragmentation data were analysed by PEAKS v7.0 (BSI, Canada) software using the S. lalandi Vtg sequences (VtgAa, VtgAb and VtgC) as predicted from the liver
transcriptomic data. PEAKS parameters were as follows: trypsin was used; variable modifications included methionine oxidation, conversion of glutamine/glutamate to pyroglutamic acid, deamidation of asparagine and peptide amidation. Precursor mass error tolerance was set to 0.1 Da and a fragment ion mass error tolerance was set to 0.1 Da. De novo sequencing, sequence search and characterising unspecific post-translational modifications (PTMs) were used to maximise the identifications; false discovery rate (FDR) was set to ≤ 1%, and the individual peptide ion score [-10*Log(p)] was calculated accordingly, where p is the probability that the observed match is a random event. Thus, the proteins and the supporting peptides for the identification of the fraction were obtained and analysed.

4.2.6. Protein gel electrophoresis and Western blot analysis

*S. lalandi* plasma from E2-induced males was diluted 1:500 in molecular grade water while plasma from males prior to E2 treatment were diluted 1:250. Forty microliters of diluted plasma and undiluted pure *S. lalandi* Vtg (350µg/ml) were mixed individually with 20µl of loading buffer dye composed of 24% Tris-Cl pH6.8, 30% glycerol, 30% sodium dodecyl sulphate (SDS), 0.01% bromophenol blue, 16% β-mercaptoethanol (Sigma-Aldrich). The mixtures were heated at 97°C for 7 minutes. Twenty µl of each of the denatured samples were loaded onto a gradient SDS-polyacrylamide gel (4%-15% Mini TGX protean gel; Bio-Rad; Hercules, USA) and electrophoresed at 120V for 45 minutes in Laemmli buffer (Laemmli, 1970). Kaleidoscopic protein ladder (Biorad) was used as size marker following the manufacturer’s instructions. After electrophoresis, the protein bands were analysed either by Coomassie brilliant blue staining (Tao et al., 1993) or by Western blot.

For Western blot analysis, protein bands were transferred onto a nitrocellulose membrane using Trans-blot turbo transfer system (BioRad). The membrane was blocked (1h at room temperature) with 25 ml of Tris-buffered saline (TBS), pH 7.5, containing BSA and Tween-20 at 3% and 0.1%, respectively (Sigma-Aldrich). Membrane was then washed twice with TBS-T (TBS supplemented with 0.1% Triton) (Sigma-Aldrich) and rinsed in TBS for 10 minutes, followed by incubation with the primary antibody (rabbit anti- *M. cephalus* Vtg antibody or rabbit anti-*S. lalandi* Vtg) at 1:20000 dilution for 1h at room temperature (RT) in an orbital shaker. After washing as described above, the
membrane was incubated with the secondary antibody (Goat anti rabbit-IR Dye 678CW; Li-cor Bioscience, Lincoln, USA; diluted 1:1000) for 1hr at RT. Bands were visualized at 800 and 700 channel for the Coomassie-stained gel and the Western blot membrane, respectively, using the Li-Cor Odyssey CLx Infrared Imaging System (Li-Cor Bioscience).

4.2.7. ELISA development

A non-competitive ELISA was first used as a checkerboard titration (Faziellawanie et al., 2011) of the optimal working assay concentration of the antigen (purified S. lalandi Vtg) and antibodies for the competitive ELISA. Anti-S. lalandi VtgA, anti-S. lalandi VtgB or anti-M. cephalus Vtg were tested as primary antibodies. Goat anti-rabbit conjugated to horseradish peroxidase enzyme (Bio-Rad; Hercules, USA) was used as secondary antibody. ELISA plates (Nunc MaxiSorp™ flat-bottom 96 well (eBioscience Affymetrix, Santa Clara, USA) were coated with serial dilutions (5000 to 2ng/ml, 50µl per well) of purified S. lalandi Vtg or M. cephalus Vtg in carbonate-bicarbonate buffer (Sigma-Aldrich) and incubated overnight (18hr) at 4°C. The purified M. cephalus Vtg was obtained from a previous study (Nocilliado et al., 2007). For Non-Specific Binding (NSB), wells were incubated with carbonate-bicarbonate buffer only. The plate was then washed three successive times with 200µl of washing buffer (PBS-T) pH 7.5 composed of phosphate buffered saline and 0.5% Tween-20 (both from Sigma–Aldrich). The blocking step was performed by addition of 50µl/well (except blank wells) blocking buffer pH 8.0 (TENTC) composed of Tris 500mM, EDTA 10mM and NaCl 1.5M containing 2% Casein (all from Sigma-Aldrich) and 0.05% Tween-20 and incubation for 30 minutes at RT. Following 3 consecutive washes (as described above), plates were incubated for 2hrs at 37°C with different dilutions of primary antibody, which ranged from 1:500 to 1:20000 diluted in TENTC blocking buffer. After three consecutive washes, each well received 50µl of enzyme-conjugated secondary antibody at different dilutions ranging from 1:1000 to 1:10000 and incubated for 1hr at 37°C. For substrate development, 50µl of tetramethylbenzidine peroxidase EIA (BioRad) were added into each well and incubated for 30 mins at RT. The reaction was stopped by adding 50µl 1M sulphuric acid (NSW, Australia, Ajax Finechem). Absorbance values were determined using a multimode plate reader (MA, USA, Perkin Elmer) at 450nm, five minutes after the reaction was stopped.
Results of the checkerboard titration using the anti-\textit{S. lalandi} VtgA and anti-\textit{S. lalandi} VtgB raised from the synthesised peptides and pure \textit{S. lalandi} Vtg as antigen did not show acceptable levels of cross reaction, therefore from this part of the study onwards, only the polyclonal \textit{M. cephalus} Vtg antibody was used. Non-competitive ELISA (using only anti-\textit{M. cephalus} Vtg antibody) was performed to establish parallelism between both Vtg purified antigens; \textit{M. cephalus} and \textit{S. lalandi}.

4.2.8. Generation of standard curve

The incubation time and temperature used in each step were optimized in order for the assay to quantify small amounts of plasma Vtg in a small volume of sample (50 µl). All wells excluding the non-specific binding (NSB) and blank wells were coated overnight at 4°C with 50 µl of carbonate-bicarbonate buffer containing 500 ng/ml of purified \textit{S. lalandi} Vtg (25 ng per well). NSB wells were coated with carbonate-bicarbonate buffer only. Blank wells were left empty.

In separate tubes, standards were prepared with pure \textit{S. lalandi} Vtg at initial concentration of 20 µg/ml and serially diluted to 5 ng/ml+ in TENTC blocking buffer. Tubes for \textit{B}_0 and NSB controls were also prepared containing TENTC blocking buffer only. Primary antibody was then added to the tubes (1:1, v/v) to a final dilution of 1:5000 and incubated with gentle shaking overnight at 4°C.

On the next day, the ELISA plate was washed 3 successive times with 200 µl of washing buffer. Blocking was conducted by adding 50 µl/well blocking buffer and incubating it for 1 hr at 37°C. Following the blocking step, the content was removed and the plate was washed as previously described. Fifty µl of standards and controls pre-incubated with primary antibody were added into the plate, which was then incubated for 2 hr at 37°C. After the incubation step, unbound antibody was removed by washing as described above. Following this, 50 µl of goat anti-rabbit HRP antibody previously diluted 1:1000 in TENTC blocking buffer were added, and the plate was incubated for 1 hr at 37°C. As in the previous step, the plate was washed with wash buffer. The substrate development and absorbance values determination were conducted as described in the preceding section.
4.2.9. Validation of the ELISA

Samples from *S. lalandi* males before and after E2 treatment were serially diluted in TENTC buffer from 1:20 to 1:2000 and 1:500 to 1:1x10^6 (v:v), respectively. Samples from maturing *S. lalandi* female were serially diluted in TENTC buffer from 1:60 to 1:8000. One hundred microliters of serially diluted samples, standards and controls were then mixed (1:1, v:v) with primary antibody and pre-incubated overnight at 4°C. The coating, blocking, antibodies incubation and colour development steps were performed as above described. To calculate Vtg levels, the average absorbance value from blank wells was first subtracted from all absorbance measurements (OD). Then, the OD values were converted into percentage binding (B/B0%) by applying the formula: B/B0% = (OD-NSB/(B0-NSB) X 100, where B0 is the maximum binding between the antigen and antibody. Percentage binding values were logit transformed according to the following formula: Logit B/B0 = ln((B/B0)/(100-B/B0)). The amount of Vtg in the plasma samples was calculated using the linear regression formula generated from the standard curve and corrected with the dilution factor.

4.2.10. Assay precision and antibody specificity

The precision of the ELISA was assessed by calculating the intra- and inter-assay coefficient of variation (CV) as previously described (Fazielawanie et al., 2011). On each plate, every point of the standard curve and samples were run in triplicates, from which intra-assay CV was calculated. The inter-assay CV was calculated from the repeated assays conducted on different days (n=7). Parallelism was assessed by analysis of covariance (ANCOVA, p < 0.05) after binding percentages had been logit transformed to achieve a linear transformation of the curves generated by serially diluted *S. lalandi* and *M. cephalus* standards using *M. cephalus* antibody. Moreover, parallelism was also assessed from curves obtained from standard (n=7), *S. lalandi* maturing females (collected at the start of the spawning season, n=3) and from mature *S. lalandi* males [before and after E2 treatment, (n=3)].
4.3. Results

4.3.1. Seriola lalandi Vtg sequences

Three Vtg transcripts were identified from the liver transcriptome corresponding to Vtg Aa (GenBank accession No. KX289700), Vtg Ab (GenBank accession No. KX360749) and Vtg C (GenBank accession No. KX360750). Multiple sequence alignment of the _S. lalandi_ VtgAa amino acid sequence showed a high identity percentage with other teleost sequences including the _M. cephalus_ VtgAa sequence, which shared 82.59 % similarity with _S. lalandi_ (Fig 4.1A and S4.2 Fig A). Multiple sequence alignment of the _S. lalandi_ VtgAb amino acid sequence also showed high identity percentage with other teleost sequences (Fig 4.1B and S4.2 Fig B) including the _M. cephalus_ VtgAb sequence, which shared the 80.59 %. Similarly, the _S. lalandi_ VtgC amino acid sequence showed high identity percentage with other teleost sequences including the _M. cephalus_ VtgC sequence which shared the 82.16 % (Fig 4.1C and S4.2 FigC).

**Fig 4.1. Multiple comparative sequence alignment of _S. lalandi_ Vtg.** Alignment of the _S. lalandi_ (A) VtgAa (B) VtgAb and (C) VtgC Vtg amino acid sequence with other teleost Vtg sequences. Represented is the conserved region contained between the 312 and 411 position of the conserved Vtg_N and LPD_N multidomain. Sequence logo indicates nucleotide conservation. Larger letters and columns indicate higher conservation.
Comparison of the three *S. lalandi* Vtg amino acid sequences showed similarity above 60% between VtgAa and VtgAb. Less than 30% of the VtgC amino acid sequence is identical with VtgAa and VtgAb (Table 4.1). Phylogenetic analysis indicated that the *S. lalandi* VtgAa shares greater homology to that of the *M. cephalus* and *T. thynnus* VtgAa than to the other teleost species tested (Fig 4.2A). Similarly, the *S. lalandi* VtgAb and VtgC also have a higher degree of homology with *M. cephalus* than to any other teleost species tested (Fig 4.2B and C).

Table 4.1. Identity percentage of the predicted Vtg amino acid sequences in *S. lalandi*.

<table>
<thead>
<tr>
<th></th>
<th><em>S. lalandi</em> VtgAa</th>
<th><em>S. lalandi</em> VtgAb</th>
<th><em>S. lalandi</em> VtgC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. lalandi</em> VtgAa</td>
<td>100</td>
<td>63.02</td>
<td>29.07</td>
</tr>
<tr>
<td><em>S. lalandi</em> VtgAb</td>
<td>63.02</td>
<td>100</td>
<td>28.54</td>
</tr>
<tr>
<td><em>S. lalandi</em> VtgC</td>
<td>29.07</td>
<td>28.54</td>
<td>100</td>
</tr>
</tbody>
</table>
4.3.2. Purification and identification of *S. lalandi* Vtg

VtgAa and VtgAb were purified as one from plasma of E\textsubscript{2}-treated *S. lalandi* males by anion-exchange chromatography. A peak was observed when the NaCl gradient was at 90-100% (Fig 4.3) and the protein concentration of this eluted fraction was 350±50 µg/ml. LC-MS/MS analysis (see 4.2.5 for detailed method) of the fraction identified a total of 208 peptides including PTMs (FDR ≤1%); out of these peptide segments, 77 peptides supported the identification of VtgAa (S4.3 Fig), while 131 peptides matched
Moreover, 75 and 129 peptide segments were unique to VtgAa and VtgAb, respectively. In addition, the number of spectra identified for most of the unique peptide was relatively high. Thus, the high coverage and number of spectra identified indicated that the purified fraction mainly contained *S. lalandi* VtgAa and VtgAb. No VtgC was identified in the fraction.

![HPLC of the purified *S. lalandi* Vtg from plasma of E2-induced males](image)

The size of the proteins in the eluted fraction corresponding to the peak was further verified by SDS-PAGE and Western blot analysis. Using the heterologous *M. cephalus* Vtg antiserum, both the Coomassie-stained gel (Fig 4.4A) and the Western blot (Fig 4.4B) revealed three main bands of approximately 118, 132 and 195 KDa. Similar analysis using the anti-*S. lalandi* VtgA and anti-*S. lalandi* VtgB antibodies showed comparable results (Fig 4.4C). The top band of around 195KDa was present in plasma of E2 treated males but not in the *S. lalandi* male samples prior to E2 treatment.
Fig 4.4. SDS-PAGE and Western blot analysis of purified Vtg and plasma samples. Coomassie blue staining (A). Western blot (B) utilizing the polyclonal heterologous *M. cephalus* Vtg antibody and (C) utilizing anti-*S. lalandi* VtgA antibody. White rectangle indicates the band corresponding to *S. lalandi* Vtg. Electrophoresis was done on gradient (4-15%) SDS PAGE gel. Lane 1 - purified *S. lalandi* Vtg; Lane 2 - plasma from *S. lalandi* E₂ induced males and Lane 3 - *S. lalandi* male plasma prior to E₂ treatment. MW; molecular mass markers (MW).

4.3.3. Development of the hybrid competitive ELISA

A checkerboard titration assay was performed in order to identify the optimal antigen coating and antibody concentrations. The combination which yielded an OD of 1 after 30 minutes of colour development was an antigen coating concentration of 500ng/ml and anti-*M. cephalus* Vtg antiserum diluted at 1:5000 (Fig 4.5).

Fig 4.5. Checkerboard titration panel showing the optimal *S. lalandi* Vtg ELISA concentration and antiserum dilution. *S. lalandi* Vtg coating concentration of 500ng/ml and anti-*M. cephalus* Vtg primary antibody 1:5000 dilution were selected as routine assay condition for the competitive ELISA development as this combination (red discontinuous line) gave an OD of 1. Each point represents the mean of triplicate determinations.
Although both antibodies (anti-\textit{S. lalandi} VtgA and anti-\textit{S. lalandi} VtgB) raised from the synthesised peptides showed a positive response on Western blot, binding was very weak (<0.1 absorbance at 450nm) when tested on ELISA even at dilutions as low as 1:500 (S4.5 Fig). In contrast, the heterologous antibody (anti-\textit{M. cephalus} Vtg) showed strong cross reaction with the serially diluted \textit{S. lalandi} Vtg (heterologous antigen), generating the typical sigmoidal curve when tested at the optimal dilution of 1:5000. Furthermore, parallel curves were obtained from the serially diluted homologous and heterologous antigens (Fig 4.6).

![Fig 4.6. Validation of anti-\textit{M. cephalus} Vtg for the hybrid competitive ELISA. Parallelism of curves obtained from serial dilutions of \textit{M. cephalus} (homologous antigen; –) and \textit{S. lalandi} (heterologous antigen; —) Vtg using polyclonal anti-\textit{M. cephalus} Vtg. Each point represents the mean of triplicate determinations. Parallelism was tested by ANCOVA, where lack of significant difference (p > 0.05) between the slopes of the curves indicates parallelism between the curves.]

Parallelism was confirmed by ANCOVA which showed no significant difference (p>0.05) between the slopes of the two curves. Similarly, when serially diluted plasma samples were tested against the anti-\textit{M. cephalus} Vtg, a curve parallel to linearized standard curve was obtained (Fig 4.7A and B). The curve generated by the plasma from \textit{S. lalandi} male prior to E2 treatment was not parallel to the rest of the curves generated including the standard curve (Fig 4.7A and B). These results validated the use of \textit{M. cephalus} Vtg antiserum for the development of a hybrid competitive ELISA for \textit{S. lalandi} Vtg. Plasma dilution of at least 1:20 was necessary to avoid non-specific binding with other proteins present in the plasma.
Antigen concentrations from 20µg/ml to 19ng/ml corresponding to the linear range, as indicated by percentage binding between 20% and 80% (B/Bo %) (Fig 4.7A). The ELISA was optimized to be capable of measuring Vtg with sensitivity down to 19ng/ml, which is the amount of VTG at 80% of binding. The average intra-assay coefficient of variation (CV) from all the different ELISAs was 2% while the average inter-assay CV at 50% binding was 13%.

**Fig 4.7. Hybrid competitive ELISA binding curves.** Binding displacement curves of *S. lalandi* Vtg standard curve (■), serial dilution of plasma from *S. lalandi* females at the start of the reproductive season (△) and *S. lalandi* males before (▼) and after E2 treatment (●). (A) Binding curves B/Bo %. (B) Parallelism of the linearized binding curves. Curves with an asterisk (*) are not significantly different (p > 0.05, ANCOVA) indicating parallelism. Each point represents the mean of triplicate determinations.
4.3.4. Hybrid competitive ELISA validation.

The ELISA was validated by measuring Vtg in plasma of maturing *S. lalandi* females at the start of the reproductive season (n=3) and in *S. lalandi* males before and after E<sub>2</sub>-treatment (n=3). Vtg levels increased in *S. lalandi* males from 0 to 33.41±9 mg/ml (SEM) within 21 days when treated with E<sub>2</sub> (Fig 4.6). *S. lalandi* Vtg levels in females at the start of the reproductive season were 0.65±0.02 mg/ml. The accuracy of the ELISA standard curve was further validated by running a sample of purified *S. lalandi* Vtg of known quantity. At 50% binding, the concentration of 350±50 µg/ml was obtained corresponding to the same concentration of protein as measured by the fluorometer.

4.4. Discussion

The purified *S. lalandi* Vtg contained VtgAa and VtgAb forms of Vtg. Analysis of the *S. lalandi* transcriptome data also showed two different sequences for VtgAa and VtgAb. We aimed to detect both forms in one assay, therefore antibodies were generated on two 12-amino acid regions, which were contained within the Vtg_N and LPD_N multi-domain, reported to be highly conserved (Folmar et al., 1995). Therefore, YTK VtgAa and VtgAb were bioinformatically identified as potential epitopes of the *S. lalandi* VtgAa and VtgAb. The peptides were first synthesized and used to generate the antibodies (anti-*S. lalandi* VtgA and anti-*S. lalandi* VtgB). However, the antibodies generated from the two peptides showed a very weak cross reaction making it impossible to develop the desired ELISA. Therefore, a heterologous antibody (anti-*M. cephalus* Vtg) kindly provided by Professor Akihiko Hara from Hokkaido University was evaluated to develop a Vtg enzyme-linked immunosorbent assay for *Seriola lalandi*.

During Western blot analyses, both homologous antibodies (anti-*S. lalandi* VtgA and anti-*S. lalandi* VtgB) reacted against the purified *S. lalandi* Vtg and plasma from *S. lalandi* E<sub>2</sub>-treated males. Similarly, the heterologous antibody (anti-*M. cephalus* Vtg) also reacted against the purified *S. lalandi* Vtg and plasma samples. However, during ELISA development, both homologous antibodies; anti-*S. lalandi* VtgA and anti-*S. lalandi* VtgB showed a very weak response. In contrast, the heterologous *M. cephalus* Vtg antibody, which was raised against the serum from male *M. cephalus* induced with E<sub>2</sub>, showed a positive response to the *S. lalandi* standard, generating the typical sigmoidal curve when
made to react with the serially diluted heterologous antigen. The cross reactivity of the *M. cephalus* Vtg antiserum is not surprising considering the high similarity between the *S. lalandi* and *M. cephalus* Vtg sequences as well as their apparent common ancestry, as shown by the phylogenetic analysis. Another validation for the use of *M. cephalus* antibody in the ELISA was the parallelism between the curves generated from the cross reaction of this antibody with the purified *M. cephalus* and *S. lalandi* Vtg standards. It has been previously demonstrated that antibodies raised against Vtg from one species can detect Vtg from other fish species and this cross-reactivity was used to develop hybrid ELISAs. This was the case in spotted wolfish (*Anarhichas minor*) Vtg ELISA that used a commercially available polyclonal antibody against Atlantic wolfish (*Anarhichas lupus*) and a spotted wolfish (*Anarhichas minor*) Vtg standard (Maltais et al., 2014). Hybrid Vtg ELISA using Vtg standards from copper redhorse (*Moxostoma hubbsi*) and shorthead redhorse (*Moxostoma macrolepidotum*) was developed using anti-carp Vtg antibody (Maltais et al., 2010), and Hyblack-fin goodeid (*Girardinichthys viviparus*) Vtg was assessed using homologous standard and a heterologous rainbow trout anti-Vtg (Vega-López et al., 2006).

Some authors have previously compared hybrid, heterologous and homologous Vtg competitive ELISAs reporting that the heterologous antibody did not cross-react well and the results were not quantitative (Mylchreest et al., 2003). Nevertheless, the ELISA developed in this study reacted better to the heterologous antibody raised against the *M. cephalus* Vtg than to the homologous antibody raised against peptides developed from predicted epitopes, indicating a higher importance to the quality of the antibody then the specificity of the antigen. Other authors reported a lack of sensitivity by the hybrid assay (Tyler et al., 2002). However, in the present study, our hybrid competitive assay has a sensitivity in the range of that reported for other homologous Vtg competitive ELISA (Heppell et al., 1999) or heterologous ELISA (Palumbo et al., 2009). The assay we developed is capable of measuring as little as 0.95ng of Vtg per well. This ELISA has a wide range of detection (20 µg/ml to 19 ng/ml), which corresponds to between 20% and 80% binding (B/B0 %). The coefficient of variation values (CVs) were also within the acceptable ranges as reported for other teleost Vtg ELISAs (Holbech et al., 2001; Maltais et al., 2014; Maltais et al., 2010). Anti-*M. cephalus* Vtg did not cross-react with any other plasma protein when diluted at least 1:20, pointing to the specificity
of the assay, as in other studies (Ndiaye et al., 2006; Tyler et al., 2002). Parallelism between curves of *S. lalandi* Vtg standard, E<sub>2</sub>-treated males and maturing females validated the assay for quantifying *S. lalandi* Vtg.

The identity of the Vtg peak was confirmed by LC-MS/MS. Only Vtg Aa and Vtg Ab were identified from that fraction. Other studies used VtgAa and VtgAb, without Vtg C, to generate antibodies for the development of a Vtg ELISA (Amano et al., 2007). Results of the Western blot and Coomassie staining of this study revealed comparable bands of the same pattern and sizes that have been reported for other teleost Vtg, such as in spotted wolffish Vtg, which consists of one 166 kDa band identified as the Vtg monomer and two minor ones of approximately 117 kDa and 98 kDa (Maltais et al., 2014). Using a heterologous reaction, anti-seabream Vtg antiserum detected three bands in tilapia plasma at molecular weights of 200 kDa, 140kDa, and 130 kDa (Swart et al., 2009). In our study, the same pattern was also present in samples from plasma of E<sub>2</sub>-treated *S. lalandi* males but not in *S. lalandi* male plasma before E<sub>2</sub>-treatment, confirming that Vtg induction occurred in *S. lalandi* males in response to the E<sub>2</sub> treatment. The 132 kDa and smaller bands could be degradation or breakdown products from the larger Vtg molecule as previously reported (Swart et al., 2009).

Results of our ELISA revealed that *S. lalandi* Vtg levels in males significantly increased from 0 to 33.41±9 mg/ml (SEM) within 21 days when treated with E<sub>2</sub> at 5mg/Kg BW three times at 7 days interval. Same dose of E<sub>2</sub> administrated in male sea bass, 6 times every 2 days, increased Vtg levels up to 50mg/ml (Mañanós, Zanuy, et al., 1994). The higher amount found in sea bass in comparison of that found in *S. lalandi* could be due to the fact that E<sub>2</sub> was administrated more frequently. *S. lalandi* Vtg levels in females at the start of the reproductive season was 0.65±0.02mg/ml, which is within the range of that reported in *Seriola dumerili* at the start of the season before they peak up to 4.93±1.5mg/ml during the season (Mandich et al., 2004).

In summary, we have developed and validated a competitive hybrid ELISA for *S. lalandi* Vtg. During the ELISA development, both homologous antibodies anti-*S. lalandi* VtgA and anti-*S. lalandi* VtgB raised against synthetic peptides based on the conserved regions of VtgAa and VtgAb did not work probably because both the *S. lalandi* antibodies were raised against a very short fragment of the peptide that may not be exposed in the Vtg molecule. In contrast, the heterologous antibody (anti-*M. cephalus* Vtg) raised
against the entire purified *M. cephalus* Vtg molecule showed strong cross reaction with the *S. lalandi* purified Vtg antigen at dilutions 10 times higher than the one used with both homologous antibodies, generating the typical sigmoidal curve when tested at the optimal dilution. For further studies on ELISA development will be interesting to compare the use of homologous antibodies raised against synthetic peptides based on the conserved regions with homologous antibodies raised against the entire purified *S. lalandi* Vtg. Nevertheless, the hybrid competitive ELISA developed using the purified *S. lalandi* Vtg antigen and *C. cephalus* antibody has acceptable parameters of specificity (no cross-reaction with other proteins present in plasma when samples were diluted over 1:10), precision (the inter-assay coefficient of variation at 50% binding was 13%) and sensitivity (19ng/ml). Therefore, this tool can be used to further understand *S. lalandi* reproductive biology and to support the development and commercial growth worldwide of this important aquaculture species.

### 4.5. References


Poortenaar, C., Hooker, S., & Sharp, N. (2001). **Assessment of yellowtail kingfish (Seriola lalandi) reproductive physiology, as a basis for aquaculture development.** *Aquaculture*, 201, 271-86. doi:10.1016/S0044-8486(01)00549-X.


4.6. Supplementary figures

S4.1 Fig. Multiple comparisons of the peptides raised based on suspected epitopes and *S. lalandi* VtgAa and VtgAb sequences. The alignment of the peptides based on suspected epitopes with the *S. lalandi* VtgAa, VtgAb and VtgC amino acid sequences is shown. Sequence logo indicates nucleotide conservation. Larger letters and columns indicate higher conservation.

![Supplementary figure S4.1](image)

<table>
<thead>
<tr>
<th></th>
<th>S. lalandi</th>
<th>M. cephalus</th>
<th>D. labrax</th>
<th>M. americana</th>
<th>P. major</th>
<th>T. thynus</th>
<th>L. mixtus</th>
<th>C. exoletus</th>
<th>M. saxatilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100</td>
<td>82.59</td>
<td>84.9</td>
<td>84.06</td>
<td>82.92</td>
<td>85</td>
<td>76.21</td>
<td>77.92</td>
<td>84.21</td>
</tr>
<tr>
<td>M. cephalus</td>
<td>82.59</td>
<td>100</td>
<td>81.6</td>
<td>80.62</td>
<td>78.42</td>
<td>79.79</td>
<td>78.74</td>
<td>73.06</td>
<td>81.1</td>
</tr>
<tr>
<td>D. labrax</td>
<td>84.9</td>
<td>81.6</td>
<td>100</td>
<td>94.1</td>
<td>84.73</td>
<td>84.36</td>
<td>77.61</td>
<td>76.86</td>
<td>94.87</td>
</tr>
<tr>
<td>M. americana</td>
<td>84.06</td>
<td>80.62</td>
<td>94.1</td>
<td>100</td>
<td>83.2</td>
<td>82.76</td>
<td>78.61</td>
<td>75.28</td>
<td>84.16</td>
</tr>
<tr>
<td>P. major</td>
<td>82.92</td>
<td>78.42</td>
<td>84.73</td>
<td>83.2</td>
<td>82.76</td>
<td>82.76</td>
<td>81</td>
<td>75.28</td>
<td>84.16</td>
</tr>
<tr>
<td>T. thynus</td>
<td>86</td>
<td>79.79</td>
<td>84.36</td>
<td>82.76</td>
<td>100</td>
<td>75.29</td>
<td>75.16</td>
<td>75.16</td>
<td>83.76</td>
</tr>
<tr>
<td>L. mixtus</td>
<td>76.21</td>
<td>73.74</td>
<td>77.61</td>
<td>76.74</td>
<td>75.52</td>
<td>75.15</td>
<td>74.42</td>
<td>76.72</td>
<td>76.72</td>
</tr>
<tr>
<td>C. exoletus</td>
<td>77.92</td>
<td>73.06</td>
<td>76.86</td>
<td>76.74</td>
<td>75.52</td>
<td>75.15</td>
<td>74.42</td>
<td>76.72</td>
<td>76.72</td>
</tr>
<tr>
<td>M. saxatilis</td>
<td>84.21</td>
<td>81.1</td>
<td>94.87</td>
<td>97.22</td>
<td>84.16</td>
<td>85.86</td>
<td>77.47</td>
<td>76.72</td>
<td>100</td>
</tr>
</tbody>
</table>

S4.2 Fig. The *S. lalandi* Vtg amino acid sequence alignment. Identity percentage of *S. lalandi* VtgAa (A), VtgAb (B) and VtgC (C) amino acid sequences with other teleost Vtg sequences.

![Supplementary figure S4.2](image)
S4.3 Fig. Sequence coverage of *S. lalandi* VtgAa using LC-MS. The entire *S. lalandi* VtgAa sequence is shown, with identified peptide segments highlighted.
S4.4 Fig. Sequence coverage of *S. lalandi* VtgAb using LC-MS. The entire *S. lalandi* VtgAb sequence is shown, with identified peptide segments highlighted.
S4.5 Fig. Checkerboard titration using anti-*S. lalandi* Vtg. Absorbance results at 450nm from ELISA using a range from 500ng/ml to 100ng/ml of *S. lalandi* Vtg antigen coating concentration and a range from 1:500 to 1:20000 dilution of anti-*S. lalandi* Vtg antisera is shown. Each point represents the mean of triplicate determinations.

S4.6 Fig. Vtg levels in plasma of *S. lalandi* males prior to (Time 0) and after treatment with E2. Different letters indicate significant difference (p < 0.05; n=3).
Chapter 5: Development of oral delivery technologies to induce spawning using Tilapia as a model animal

Pablo J. Sanchis, Josephine Nocillado and Abigail Elizur

1Genecology Research Centre, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Queensland, Australia.

Abstract

Methods using oral delivery would create a breakthrough in the capacity to control spawning for aquaculture fish. Two encapsulation approaches to orally deliver GnRH were explored; the encapsulated gonadotropin-releasing hormone (GnRH) was incorporated into the feeds and tested in-vivo in tilapia. The first approach, using alginate-chitosan particles, was found to incorporate 1% (w/w) of GnRHa. Complete release of the GnRHa from the particles was observed after 1hr of exposure to phosphate solution (pH 6.8). The second approach used yeast as a delivery vehicle. The recombinant Southern Bluefin tuna gonadotropin-releasing hormone (sbtGnRH1) expression in yeast was confirmed at the DNA level through PCR and at protein level through mass spectrophotometry. Yeast particles where found to contain 0.03% of rsbtGnRH1 (w/w). In-vivo experiments in tilapia showed that doses of 0.6µg GnRHa/g did not stimulate spawning at temperatures under 23°C.

Key words: Oral delivery, GnRH, alginate-chitosan, yeast, *Tilapia*
5.1. Introduction

One of the bottlenecks in broodstock management of large-bodied fish is the delivery of hormones for spawning induction. In this context, methods using oral delivery would create a breakthrough in the capacity to control spawning and reproductive development. This is especially critical for fish that are not amenable to handling such as blue fin tuna species including Southern Bluefin Tuna (*Thunnus maccuroyii*, SBT).

Under captive conditions, a large number of teleosts, including SBT, exhibit reproductive dysfunctions (Zohar et al., 2001). For instance, in females, vitellogenic oocytes may fail to progress to final oocyte maturation, and thus ovulation and spawning are compromised. In males, small volumes or low quality milt are produced. These challenges are the bottlenecks for SBT broodstock held in captivity, thus hindering the successful farming of this species (Bubner et al., 2012).

This study aims to test whether treatments to manipulate reproductive function can be delivered via the oral route, thereby developing a non-invasive, practical, and cheaper methodology, which can also can allow mass treatment regardless of fish size. Other previously studies showed also interest in oral delivery of bioactive compounds for the aquaculture industry (Mylonas et al., 2000; Polk et al., 1994; Schep et al., 1999; Thomas et al., 1989).

The innovation that we are exploring in this research is to orally deliver a recombinant sbtGnRH peptide (rsbtGnRH1) and the GnRH analogue (GnRHa; D-Ala6,Pro9,NEt) (Zohar and Mylonas, 2001). This peptides are of specially interest for the aquaculture of the SBT where one of the challenges in closing the life cycle of the species in captivity is having control on its reproductive development and spawning in a non-invasive way (Bain et al., 2013). GnRH is a neuropeptide secreted by the hypothalamus that stimulates the secretion of pituitary gonadotropins, which are responsible for the final gonadal maturational processes including oocyte maturation and ovulation in females, and spermiation in males (Kim et al., 2006). It is now established that teleosts can express either two or three forms of GnRH isoforms (GnRH1, GnRH2 and GnRH3) (Lethimonier et al., 2004). GnRH1 is expressed by neurons in the ventral telencephalicpreoptic area of the hypothalamus and is the most relevant isoform for
reproductive function (Parhar et al., 2003; Sukhan et al., 2013). Therefore, in the present study, a recombinant version of GnRH1 was utilized.

Two encapsulation methods to orally deliver GnRH were explored. The challenge was to protect the peptide from degradation while in the digestive tract. For GnRHa, the encapsulation method tested involved the use of alginate-chitosan. For rsbtGnRH1, the peptide was to be delivered via the yeast where it was intracellularly expressed.

The alginate-chitosan complex can facilitate the delivery of the encapsulated compound directly from the gut to peripheral circulation without compromising their integrity due to its stability in low pH (Li et al., 2007). The complex is biodegradable, biocompatible, muco-adhesive, pH sensitive, and presents low toxicity and low antigenic potential (George et al., 2006; Li et al., 2007). In addition, it has been reported to have antimicrobial, antioxidant, analgesic and anti-tumoral activity (Fonte et al., 2012). This method has already been evaluated for oral delivery of egg yolk immunoglobulin antibodies for passive immunotherapy application in Lohmann laying hens (Li et al., 2007). The second “encapsulation” method involves the intracellular expression of rsbtGnRH1 in the yeast Pichia pastoris. There is evidence that recombinant peptides expressed and contained in yeast, which is then orally fed, can enter the peripheral circulation. This has been demonstrated in mice where yeast that expressed a recombinant antigen was mixed with the diet and successfully immunized the fed mice (Kim et al., 2014).

The objective of this study was to set up preliminary protocols for oral delivery of bioactive compounds that control the reproductive development and spawning of cultured fish in captivity. As a step towards accomplishing this objective, tilapia (Oreochromis mossambicus), which is a small and robust fish that shares the same gender of many cultured fish and it is suitable to be maintained in lab conditions was first used as an experimental model to assess the experimental approach and the oral delivery formulations.
5.2. Material and methods

5.2.1. Encapsulation of GnRHa in alginate-chitosan based particles

The peptide gonadotropin-releasing hormone analogue (D-Ala⁶,Pro⁹,NEt) was commercially synthesized (China Peptides, Shanghai). The encapsulation protocol of GnRHa in alginate-chitosan based particles was performed as previously reported (Fonte et al., 2012). Briefly, the alginate-chitosan particles were prepared by a two-step procedure based on the ionotropic gelation of polyanion with calcium chloride followed by polycationic cross-linking. Firstly, 12mg of GnRHa were added to 2.0% (W/W) alginate solution and mixed under magnetic stirring at 800rpm for 5min achieving a 0.005% (w/w) final concentration. Then, 12.5 ml of 18mM calcium chloride solution was added dropwise under magnetic stirring (800 rpm) for 60mins in order to obtain an alginate pre-gel. Secondly, 41.7 ml of 0.07 % (w/w) chitosan solution was added dropwise to the alginate pre-gel under magnetic stirring at 800 rpm for 90 mins, giving a final alginate and chitosan concentration of 0.05% and 0.012% (w/w), respectively (alginate–chitosan mass ratio = 4.3:1). After chitosan addition, the solution was stirred at 800 rpm for 30 mins. Then, the solution was centrifuged at 20,000 xg for 45 min at 4°C and supernatant was removed. The GnRHa particles at the bottom layer of the tube were freeze-dried using a ModulyoD-230 (Thermo Fisher Scientific; Waltham, MA, USA) and stored at -20°C until used.

5.2.2. Intracellular expression of recombinant sbtGnRH1 in yeast.

For this study, a recombinant expression construct of the sbtGnRH1, with molecular weight of 1.1 KDa, was already available at the Elizur lab. The expression construct was generated with an insert containing the sbtGnRH1 conserved decapeptide, followed by a cleavage site (GKR) and the GnRH-associated peptide (GAP) DNA sequences, and ligated into the BamHI and NotI sites of the pPIC3.5 (Invitrogen Life Technologies, Carlsbad, CA, USA) expression vector (Fig 5.1). The vector pPIC3.5 does not contain a secretion signal, therefore induction of the tightly regulated promoter of the alcohol oxidase I (AOX1) methanol-dependent gene makes this expression vector suitable for intracellular expression under methanol induction (Daly et al., 2005). Plasmid construct
were produced in *E. coli* and purified using a Qiagen midi-prep kit (Qiagen, Chatsworth, USA).

The sbtGnRH1 expression construct was linearized with *SalI* and purified using PCR purification columns (Qiagen). Nine micrograms of the linearized construct were introduced into SuperMan⁵ strain cells of the yeast (*Pichia pastoris*) (Biogrammatics, Carlsbad, CA, USA) by electroporation (BioRad GenePulser, Hercules, CA, USA). Putative positive yeast transformants were selected in histidine deficient media. Then, the four most resistant clones were selected based on resistance to the antibiotic Geneticin G418 sulphate, Sigma–Aldrich, St. Louis, MO, USA) at concentrations of 0.5–2 mg/ml.

5.2.3. Recombinant sbtGnRH1 verification by PCR and Mass spectrophotometry.

The four most resistant clones selected were further assessed by PCR. The PCR reaction mix at 25 µl volume contained 2.5µl of 10x PCR buffer, 2µl MgSO4 (25mM), 0.5µl dNTPs (10 mM), 0.5µl each forward and reverse primer (10 mM) that contained a *BamH*I and *NotI* cloning site on the 5’end, respectively (*Table 5.1*), 0.1 µl Taq DNA polymerase (Fisher Biotec, West Perth, Australia), 17.9µl of H₂O and 1 µl of yeast colonies. The thermal cycling parameters were: initial denaturation at 94°C (3 min); followed by 35 cycles of denaturation at 94°C (30s), annealing at 60°C (30s) and extension at 72°C (1 min); and final extension at 72°C (7 min). SuperMan⁵ *P. pastoris*
strain that did not contain the sbtGnRH1 construct but vector pPIC3.5 only were used as a negative control.

### Table 5.1. sbtGnRH1-specific primers.

<table>
<thead>
<tr>
<th>Direction</th>
<th>5' to 3' sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>(TACTGGATCCCATATGCTAAAAAGGCAGCCTGTCAT)</td>
</tr>
<tr>
<td>Reverse</td>
<td>(GTATGCGGCGCTTTTTATAATTCTGTG)</td>
</tr>
</tbody>
</table>

Protein expression induction by methanol was carried out according to Invitrogen’s *P. pastoris* expression manual (Invitrogen Life Technologies). Molecular grade methanol (Sigma-Aldrich, St. Louis, MO, USA) was added every 24hrs at 1% final concentration in a 5-liter flask shaker at 30°C containing 1liter of BMM media. Yeast was harvested 72hrs after methanol induction. The harvested yeast cells were freeze-dried using a ModulyoD-230 (Thermo Fisher Scientific) and stored at -20°C. The expression of the r_sbtGnRH at protein levels was further verified by MALDI TOF/TOF mass spectrometry. The cells were homogenized thoroughly in urea buffer (8M urea, 0.8M NH4HCO3, pH 8.0), followed by de-salt using Sep-Pak C18 column (Waters, USA) and dried down in SpeedVac. Either 4-hydroxy-3-methoxy cinnamic acid (ferulic acid [FA], *Sigma-Aldrich*, Cat. 46278-1G-F) or 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapic acid, *Sigma-Aldrich*, Cat. 85429-1G) was employed as the matrix to obtain the optimal spectra; the cell extract dissolved in 50% methanol/50% MQ, or 60% acetonitrile/40% MQ/0.1% TFA, respectively, as a saturated solution. The protein was reconstituted in MQ water at a concentration of 6×10^{-8} mol/mL; 0.25 μL of matrix solution was spotted first on a 192-wells plate (Cat. 4333375, *AB SCIEX* Australia Pty Ltd), and then 0.25 μL of protein solution was spotted on the same spot, followed by another layer of 0.25 μL matrix solution added on to the top of air-dried sample spot prior to introduction into the mass spectrometer. A 4700 MALDI-TOF/TOF mass spectrometer (*AB Sciex Pty. Ltd., USA*) was used. The mass spectra were acquired using the linear positive ion mode of MALDI-TOF MS with laser energy of 5100.
5.2.4. *Pichia pastoris* cell lysis for peptide extraction

*Pichia pastoris* cell lysis was performed in order to extract the intracellular expressed recombinant sbtGnRH1 peptide. Briefly, dried yeast containing rsbtGnRH1 samples were re-suspended in water with 0.1% of trifluoroacetic acid (TFA) (sigma-aldrich) at (10mg/ml). On ice, the re-suspended sample was homogenized with a vortex and sonicated during 1 minute in the presence of ice with an ultrasonic processor (Sonics Vibra-Cell VCX 750, Newtown, USA), followed by 10 min incubation on ice. The samples were then centrifuged at 12,000xg at 4°C for 15min, and the supernatant was collected. The supernatant was filtrated by centrifugation at 4,000xg at 4°C using a centrifugal filter unit 10kDa MWCO membrane (Millipore, Germany). The filtrate was collected and freeze-dried using a ModulyoD-230.

5.2.5. Quantification of GnRH using high-performance liquid chromatography (HPLC).

The amount of GnRHa in the alginate-chitosan particles and rsbtGnRH1 in yeast was determined by HPLC according to (Kafka et al., 2010) based on a standard curve generated using GnRHa of known concentrations. The standard curve was generated from a stock solution (200 μg/ml) of GnRHa in phosphate buffer (PB) pH 6.8 composed of 0.28M disodium hydrogen orthophosphate and 0.21M potassium dihydrogen orthophosphate. From stock solution, samples were taken and diluted with PB to get standard concentrations ranging from 2.5 up to 200 μg/ml. Dried filtrated samples from yeast containing rsbtGnRH1 and dried GnRHa particles were dissolved in PB (10mg/ml) under magnetic stirring at 800 rpm. In order to determine the release time for the GnRHa, particles samples were dissolved in six replicates in PB as above, and 400 μl were collected at 5, 15, 30, 60, 120 and 240 min and added directly into the HPLC autosampler system (Perkin-Elmer Norwalk, CT, USA). The injection volume to adjust the chromatographic conditions was 50 μl. The reverse phase HPLC was carried out using a Perkin Elmer Series 200 Pump with a Flexar photo diode array wavelength detector (λ = 263 nm). HPLC separations were performed on a Fusion-RP column (C18, 75 x 4.60 mm i.d., 4μm particle size, Phenomenex, CA, USA). The mobile phase was a 0.1% (v/v) acetic acid in milli-Q water (solvent A) and 0.1% CH₃CN in solvent A (solvent B). Samples were eluted by a gradient development from 5 to 40% of solvent B in solvent A at a flow rate of 1.2 mL/mins over 12 mins. The detection wavelength was 220 nm. The software used
for the peak identification and peak’s area calculation was the chromatography data system Chromera version 3.4 (Perkin-Elmer). The regression equation was obtained by plotting the peak area against the GnRHα concentration of each standard. After the chromatographic run, quantification of rsbtGnRH1 and quantification and release of GnRHα particles were calculated based on the regression equation obtained from the standard curve by following the formula: GnRH concentration = (Peak area - 26545) / 42344. The encapsulation efficiency of the GnRHα particles was calculated by following the formula: Encapsulation efficiency % = (GnRHα found withing the alginate-chitosan particles) X 100 / (initial GnRHα added to the alginate solution).

5.2.6. Formulation of on-site produced tilapia pellets

Feed pellets for tilapia were prepared under different percentage combination of 4mm commercial fish pellets (Primo Aquaculture, Narangba, Australia), fish oil (Neptune tackle, Adelaide, Australia) and potato starch (sigma-aldrich). Commercial fish pellets were crushed using a mortar, combinations from 20-80% of fish oil and potato starch were added and all ingredients were mixed. The mix was added to a 96-well silicone sealing mats (BioRad), in order to produce single pellets, and then were stored at -20°C. Combination of ingredients that produced a mixture that was too liquid or too solid to be added in the silicone mats were discarded. The reminding mixtures were added to the silicone mats and left to air dry for 1 hr. Then, single pellets were extracted from the silicone mats by inversion. Tilapia were fed with the produced pellets during different days for each of the remaining combination of ingredients. Therefore, tilapia pellets were chosen based in pellet consistency and fish preference. Tilapia were fed with the formulated pellets very slowly (one by one) for assuring that each fish was taking 5 of the pellets produced. The number of pellets taken by each fish was recorded, if a single fish wanted more than 5 pellets it was fed on commercial diets until satiety for letting the rest of the fish take the desired number of pellets. Once all the fish were fed with the formulated pellets, they were feed until satiety.

5.2.7. On-site tilapia pellets containing GnRHα particles and recombinant yeast

The GnRHα pellets and the rsbtGnRH1 yeast pellets were prepared so that the amount of hormone required per fish per day (0.6µg/g BW) was contained within 5
pellets. Therefore, depending of the fish weight the GnRH containing chitosan/alginate and recombinant yeast were mixed with the on-site produced tilapia pellets ingredients at ratio of 22-19µg/mg and 79-73µg/mg, respectively. The feed pellets were stored at -20°C until used. Pellets containing the same proportion of ingredients but no GnRH were used as a control

5.2.8. In-vivo experiments

In-vivo experiments were conducted at the University of Sunshine Coast aquaculture facilities (QLD, Australia). Tilapia were collected from the wild, sorted by size, acclimatized and domesticated to accept commercial pellet feeding. Once domesticated, fish were anaesthetized with Aqui-S (Primo Aquaculture, Coffs Harbour, Australia) for sex determination by optical observation of the genital papila (Popma et al., 1999). Then, male and female tilapia were tagged accordingly with t-bar anchor extenal tags (Hallprint, Hindmarsh Valley, Australia). Tags had an indetification number and two colours; yellow for males and pink for females.

Three experimental groups were randomly assigned, each fish belonging to the experimental groups was fed with 5 GnRHa pellets containing alginate-chitosan particles or recombinant yeast to orally deliver a dose of 0.6µg GnRH /g of fish. Fish belonging to the control group were fed with pellets containing no GnRH. A total of three experiments were conducted:

(I) The first trial was performed with 60±5g tilapia and distributed randomly in three diferent 250 l tanks, each tank contained 2 male and 3 female tilapia. The experiment was performed at 19-20oC during 7 weeks using 12hrs day light photoperiod. One week later, temperature in all groups was increased to 24-25oC. At the start and end of the experiment fish were anesthetized and ovarian condition was examined through biopsy as previously described (Coward et al., 2001). Prior to the start of the second experiment, fish were maintained at 21-22oC during 15 days.

(II) The second experiment was carried out over 2 weeks. It was performed with 52±10g tilapia which were distributed randomly in three different 250 l tanks, each tank contained 2 male and 3 female tilapia. Temperature ranged from 21-22oC and photoperiod of 12hrs day light was used. Oocytes were collected for examination by applying a gentle pressure on the abdomen and cleared in Serra’s fixative; ethanol,
formalin, acetic acid (6:3:1) for 20 mins (Stoeckel, 2000). The position of the germinal vesicle (GV) was determined using a Nikon SMZ800 stereo microscope (Nikon Corporation, Tokyo, Japan) connected to a HD Lite 1080P camera with retina screen monitor (Scientific instrument and optical sales, Queensland, Australia). Then, fish were allowed to recover in a separate tank for 15 mins prior to being returned into the experimental tanks. Prior to the start of the third experiment, fish were maintained at 21-22oC for 21 days.

(III) The third experiment was a repetition of the second experiment, using the same temperature and photoperiod conditions with the difference that GnRHa administration was extended to a 3-week time and that the tilapia were of 58±15g. At the start and end of the experiment fish were anesthetized, oocytes were collected for ovarian condition examination and blood from all female fish was collected from the caudal vasculature using heparinized syringes in 1.5 ml tubes containing 30IU of heparin. Spermiating males were included in the experimental groups as they affect directly female sexual competition and thus spawning. Plasma was extracted from blood by centrifugation (3000xg) at 4oC for 20 mins and stored at minus 80oC until E2 analysis. Plasma levels of E2 at the start and end of the experiment were determined by a commercially available E2 enzyme immunoassay (EIA) kits (Cayman Chemical Company, Ann Arbor, MI, USA). Steroids were first extracted from 200µl of plasma with 5ml of diethyl ether (Sigma-aldrich). After evaporating the solvent, the extracts were resuspended in 400 µl of EIA assay buffer. E2 plasma levels were measured for all females.

5.2.9. Statistical analysis

Body weights (BW) and plasma E2 values are expressed as mean±SEM. Values from these data were analyzed with one-way analysis of variance (ANOVA).

5.3. Results

5.3.1 On-site tilapia pellets formulation

The experimental tilapia showed preference for the on-site tilapia pellets which contained a combination of commercial pellets, fish oil and starch potato percentage at
80%, 10% and 10% respectively. The average weight of each pellet containing recombinant yeast or alginate-chitosan particles was 33±3mg.

5.3.2 GnRHa alginate-chitosan particles and rsbtGnRH1 yeast

Recombinant sbtGnRH1 expression in yeast at DNA level was verified by PCR using specific primers (Fig 5.2).

![Fig 5.2](image)

**Fig 5.2. Verification of most resistant yeast transformants by PCR.** Molecular marker (Mm). Yeast clones expressing sbtGnRH1 in pPIC3.5, showing the expected product size of 250bp (A,B,G,H). Negative control; vector pPIC3.5 only (C,D). Negative control; SuperMan strain yeast (P. pastoris) strain cells only (E,F).

The expression was further verified at the protein level by mass spectrometry, which revealed a peak corresponding to the estimated 1.1 KDa molecular weight of the rsbtGnRH1 decapeptide (Fig 5.3).

![Fig 5.3](image)

**Fig 5.3. MALDI TOF mass spectrum of yeast (P. pastoris) expressing rsbtGnRH1.** Red line points to the peak corresponding to the 1.1 KDa molecular weight of the rsbtGnRH1.
HPLC analysis of the standards and experimental samples (alginate-chitosan particles and yeast) showed no peak when blank control was tested (Fig 5.4 A-C). However, a peak at the same retention time (7.8 min) and UV absorbance (200 mAU) was observed in the standards and GnRH (GnRHa or rsbtGnRH1) containing samples (Figure 5D, E and F).

**Fig 5.4. GnRH HPLC profiles.** GnRH Profiles generated by HPLC. All samples used the same volume (50 µl) and same buffer [phosphate buffer (PB) at pH 6.8]. Red ellipse represents the GnRH peak found at 7.8mins. PB buffer only (A), alginate-chitosan particles only (B) and yeast only (C). 5µg of GnRHa standard (D) 500 µg of alginate-chitosan particles containing GnRH (E) and 500 µg of yeast extract containing GnRH (F). Y axis; Absorbance (mAU). X axis; retention time (mins).

Quantification of the GnRH within the samples was facilitated by the standard curve generated when plotting the peak area generated by HPLC against known GnRH concentrations (standards) (Fig 5.5).
Fig 5.5. GnRHa standard curve. The standard curve (black dotted line; n=3) obtained by plotting the peak area generated by HPLC (Y axis) against known concentrations of serially diluted GnRHa (X axis). Each line represent a different chromatographic run (n=3). Dots and error bars represent mean and SEM (n=3).

Yeast cells were found to contain 0.03% (w/w) of rsbtGnRH1. Therefore, the amount of rsbtGnRH1 expressed intracellular was 0.3 µg of GnRH1 per mg of yeast when produced in a 5l flask (Fig 5.6). Total amount of GnRHa recovered from alginate-chitosan particles was 6.5±0.25mg contained in 650 mg of particles. Comparison of the initial GnRHa added at the start of the encapsulation procedure (12mg) with that found by HPLC within the alginate-chitosan particles at the end of the encapsulation (6.5mg) showed that 45.9% of the GnRHa was lost during the encapsulation procedure, providing an encapsulation efficiency within the alginate-chitosan particles of 54.1%.

Fig 5.6. GnRH quantification of encapsulated GnRH. The Y axis represents the GnRH concentration calculated from the peak area generated by HPLC (GnRH concentration = (Peak area-26545)/42344; Fig5.5). The X axis shows the GnRH content within 500µg of yeast cells (A) and the GnRHa content within 500µg of alginate-chitosan particles. Columns represent mean (n=3).
Alginate-chitosan particles exposed to phosphate buffer released all of their GnRH content within one hour, as no higher amounts were found in the buffer after 4h of exposure, indicating that 100% of GnRHa from the particles was released (Fig 5.7).

![Graph](image)

**Fig 5.7.** GnRHa release from alginate-chitosan particles over time. Representation of GnRHa amounts released from the alginate-chitosan particles (Y Axis) at different time points (X axis) when exposed to phosphate buffer (pH 6.8). Columns and error bars (n=3) represent mean and SEM, respectively. Different letters indicate significance when p<0.05.

5.3.3. *In-vivo* tilapia feeding experiment of alginate-chitosan encapsulated GnRHa and rsbtGnRH1 in yeast

Summarized results from the three experiments conducted can be found in the following table (Table 5.2).

**Table 5.2.** *In-vivo* experiments from GnRH oral administration at 0.6µg/g.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Fish weight (g)</th>
<th>Duration exp</th>
<th>Fish maturational stage start exp</th>
<th>Temp prior exp</th>
<th>Temp during exp</th>
<th>Temp after exp</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>7 weeks</td>
<td>Very immature stage</td>
<td>/</td>
<td>19-20°C during 7 weeks</td>
<td>23-25°C for 6 days</td>
<td>Treatment and control (male and female) spawn after exp</td>
</tr>
<tr>
<td>2</td>
<td>52</td>
<td>2 weeks</td>
<td>Spermiating males Maturing females</td>
<td>21-22 °C during 2 weeks</td>
<td>21-22 °C during 2 weeks</td>
<td>/</td>
<td>No spawn. No differences on maturational stage (GV position)</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>3 weeks</td>
<td>Spermiating males Maturing females</td>
<td>21-22 °C during 2 weeks</td>
<td>21-22 °C during 3 weeks</td>
<td>/</td>
<td>No spawn. No differences on maturational stage (GV position) or E₂ levels</td>
</tr>
</tbody>
</table>
A total of three experiments were performed: (I) Biopsy at the beginning of the experiment suggested stage 0 of development, since it was very hard to obtain a gonadal sample without damaging the fish, indicating a very immature stage. Every fish was fed with 5 GnRHa pellets containing alginate-chitosan particles or recombinant yeast to orally delivery a dose of 0.6µg GnRH /g of fish. Although tilapia ate the GnRH pellets containing alginate-chitosan particles, they did not like the yeast pellets as they refused to swallow them. During the 7 weeks of the first experiment at 19-20°C, fish showed no response to treatment. However, male behavior such as nest construction and coloration change, was observed during this period in all the groups including the control (contained no GnRH) and the two treatment groups; alginate-chitosan particles and the yeast pellets (both contained a GnRH dose of 0.6µg/g BW). Six days following temperature raise to 24-25°C, simultaneous spawning was observed in 3 of 3 females from the control group, 1 female from the yeast experimental group and 3 of 3 females from the Alginate experimental group. Regarding to the males, all of them (from both treatments and control) released sperm when a gentle pressure was exerted on the abdomen. Males and females indicated temperature response but not treatment response.

(II) During the second experiment, fish were maintained at 21-22 °C for 2 weeks to stimulate gonadal development before treating the experimental fish with GnRH. The yeast group was removed based on the negative response from experiment one. The treatment group was fed with alginate-chitosan pellets at the same dose of experiment 1 but during 2 weeks. The control group was feed with the same pellets containing no GnRH. Oocytes from all females (except from one female of each group that had no developed oocytes) were collected at the start and end of the experiment by simply applying gentle pressure on the abdomen. When this pressure was applied in the males, milt was released in all males from both groups at the start and end of the experiment). Microscopy observation revealed that all female’s oocytes GV were undergoing migration, indicating that fish were nearing final maturation as previously reported (Stoeckel, 2000). This stage of the oocytes was confirmed at the start and end of the experiment in all females that released oocytes at the start of the experiment including treatment and control groupo. No differences were found in the females that did not release oocytes at the start of the experiment, as they neither release oocytes at the
end of the experiment. Visual observation confirmed no spawning during the trial in the control.

(III) The third experiment was a repetition of the second experiment using the same temperature and photoperiod conditions with the difference that GnRHa administration was extended to a 3-week time. Also during this experiment plasma samples were collected at the start and end of the experiment to identify a possible E₂ response to GnRHa administration in female fish. Visual observations confirmed no female spawning during the course of the trial in the control or treatment groups. However, males from both groups were spermiating at the start and end of the experiment. Microscopy of the oocytes confirmed the same stage of GV that was found at the beginning and end of the experiment in all the females from both groups. No significant differences were found at the beginning and end of the experiment in plasma E₂ levels from treatment groups when compared with control group (Fig 5.8).

![Fig 5.8. E₂ levels in Tilapia after GnRH oral treatment (Experiment 3). Mean plasma E₂ levels of Tilapia females at the start of the trial (grey) after 3 weeks (white). Tilapia fed on pellets containing no hormone, (control; n = 3). Tilapia fed on pellets containing Alginate-Chitosan GnRHa particles at dose of 0.6µg/g (n=3) (GnRHa pellets). Column and bar represent the mean and SEM, respectively.](image)

5.4. Discussion

Many fish species, when reared in captivity exhibit reproductive dysfunctions (Zohar et al., 2001). GnRHa treatment has been effective for inducing spawning in Bluefin tuna as in many other species (Zohar et al., 2001). However, the current method of GnRH administration in large fish is very stressful for fish and handlers alike. Also, it can cause
lethal infections for these highly valuable broodstock and is labor-intensive for workers. Therefore, one of the bottlenecks in managing reproduction in large bodied fish is the delivery of hormones for controlling reproductive dysfunctions. Consequently, the main objective of this study was to combine the application of gonadotropin-releasing hormone (GnRH) and oral delivery technologies to manipulate fish reproduction. This combined methodology can be considered especially interesting for the SBT aquaculture industry as it can allow GnRH treatment to manipulate the reproductive process regardless of fish size.

We explored two encapsulation methods to orally deliver GnRHs. The first approach was found to contain 1% of GnRHα (w/w) in an alginate-chitosan complex, with an encapsulation efficiency of 54.1%. Complete release of the GnRHα from the particles was observed after 1hr of exposure to phosphate solution (pH6.8) at the same pH as that reported for intestinal fluid (Li et al., 2007). The second approach used yeast as a delivery vehicle. Recombinant sbtGnRH1 expression in yeast was confirmed at DNA and protein levels by PCR and mass spectrophotometry, respectively. Yeast particles where found to contain only 0.03% of rsbtGnRH1 (w/w).

We assessed the in-vivo effect of these two encapsulation methods in tilapia as a model animal for SBT. Unfortunately, the in-vivo experiment was compromised because of the lack of availability of Tilapia at the appropriate size. In Australia, tilapia cannot be purchased freely because it is illegal, as tilapia is considered an introduced pest. Therefore, a special permit was needed for tilapia to be collected from the wild. However, they can only be found at certain times of the year. It was very difficult to find sufficient numbers of similar size fish to perform the experiments; hence we were limited to three female Tilapia per treatment. The effectiveness of the experiments was assessed through visual observation of spawning, oocyte GV position identification and measurement of plasma E₂. Treatments were planned with pellets containing recombinant yeast and pellets containing alginate-chitosan particles for the oral delivery of GnRH, however only alginate-chitosan pellets were used as tilapia did not like the yeast pellets. This statement was established as fish were spitting the pellets after taking them with the mouth; the fact that fish were not swallowing the pellets made it impossible to perform the experiment using the yeast treatment. Producing the recombinant GnRH in a fermenter system can increase the protein expression in yeast.
*Pichia pastoris* from mg to g amount (Cereghino et al., 2002). Therefore, fermenter production could be a future solution to increase the amount of GnRH/g yeast in order to reduce the overall yeast content in the pellets without compromising the amount of GnRH delivered in the treatment.

During the first *in-vivo* trial, spawning of control and treatment fish was observed at 23-26°C. However, no spawning was observed in any of the fish (treated or untreated) when temperature was below 23°C, indicating a spawning response to temperature but not to treatment. During the second and third experiments, performed at temperatures below 23°C, no spawning was observed in control or treated fish, neither differences in the GV position or E2 levels were found between the start and end of the experiment or when comparing control and treatment groups, indicating no response to the treatments.

It has been reported that GnRHa with or without a dopamine antagonist, should stimulate the synthesis and release of endogenous gonadotropins, thus spawning (Yaron et al., 2009). In spotted seatrout (*Cynoscion nebulosus*) it was demonstrated that oral administration of 0.2–2.5 mg LHRHa/kg to females resulted in successful spawning 32–38 h later (Thomas et al., 1989). Therefore, oral administration of 0.6µg GnRHa/g was expected to lead to a response in tilapia. However species such as tilapia and cyprinids exhibit a very strong dopaminergic inhibition (Levavi-Sivan et al., 2004; Zohar et al., 2010). Therefore, a successful spawning agent like GnRHa (very efficient in other fish species), in this species for example has to contain a dopamine antagonist (Yaron, 1995).

Spawning experiments showed that successful ovulation in the common carp can be achieved by a single administration of 10 µg/kg GnRHa combined with 20 mg/kg of the water-soluble dopamine receptor antagonist, metoclopramide (MET) (Drori et al., 1994). Other species such as Grey mullet (*Mugil cephalus*), where GnRHa alone was able to induced to spawn, the combined treatment with MET was found to be more potent in inducing ovulation and spawning (Aizen et al., 2005; Zohar et al., 2010). The fact that our tilapia did not spawn in response to the oral delivery could be because the levels of GnRHa administrated were not sufficient to overcome dopamine inhibition. For further studies, it will be better to either add an antagonist such MET or use species that are not under dopamine inhibition as model animals to test the efficiency of GnRHS therapies and oral delivery technologies.
In conclusion, this preliminary work set up the bases to further develop oral delivery therapies using tilapia as a model species. Both encapsulation methods presented in this study; alginate-chitosan particles and intracellular expression in yeast, can potentially be an effective vehicle for the oral delivery of reproductive related hormones such as GnRH. Both methods appeared to hold successfully the desired hormones, although optimization of the GnRH load is needed. Hormone release was confirmed under a similar pH of that reported for intestinal fluid for the alginate-chitosan encapsulated GnRH. This system can also be adapted to deliver other relevant bioactive recombinant peptides.

5.5. References


Chapter 6: Conclusions

This PhD project reports on the investigations aimed at the long term enhancement of the aquaculture of yellowtail kingfish (*Seriola lalandi*, YTK) and Southern Bluefin tuna (*Thunnus maccocyii*, SBT). Each of the species cultured presents a number of bottlenecks that affect the reproductive performance of the broodstock. Relevant biotechnological methods were applied to manipulate directly or indirectly (using model animals) the endocrine regulation of reproduction of these species, which are the main species farmed by the industry collaborator of this PhD research; CleanSeas Tuna Ltd (CST, Arno Bay, South Australia).

In a large number of aquaculture species, such as YTK and SBT, undergoing puberty in captivity is a major challenge. The problem presented concerns either early, late or all together failure to enter puberty (Taranger et al., 2010). SBT is the most late maturing among the Bluefin tunas, taking 9-12 years to become mature (Gunn et al., 2008). High costs and risks are associated with maintenance until maturation of these large fish, therefore shortening the time towards puberty will have significant commercial applications. YTK females take around 3-4 years to reach sexual maturity while YTK males are maturing in just 1-2 years old (Poortenaar et al., 2001). Thus, late maturing YTK females affect the generation time, which is causing a major bottleneck when trying to conduct selection and genetic breeding programs. Therefore, CST is interested on shortening the breeding period in order to increase the genetic gain of genetically selected YTK broodstock (Knibb et al., 2016). The first step towards shortening the breeding period time is the advancement of the onset of puberty, which in many teleost species, such as YTK and SBT, may be controlled and managed by manipulation of hormonal parameters (Okuzawa, 2002).

This PhD research aimed to address approaches towards the advancement of puberty in fish, as well as develop tools towards this process and towards the assessment of the procedures. Two different approaches were used (Chapter 2 and 3), Kisspeptin slow release implants, that operate at the neural system level and intraperitoneal injections of recombinant YTK follicle stimulation hormone (rytkFsh), which operate directly at the gonad level. Two of the chapters of this thesis deal with
the methodologies from both approaches. The first approach (Chapter 2) explored the use of slow release EVAc implants to deliver Kiss1-15 and Kiss2-12 to immature female YTK (1-2 years-old). The dose used was 50µg/kg, administrated in-vivo five times at an interval of 15-17 days. Analysis of the results from the Kiss trial showed that significant difference was found in the 17β-estradiol (E2) levels within the Kiss1-15 treatment group, at the start and end of the trial. The results of this study with YTK females were consistent with previous studies in YTK males (Nocillado et al., 2013) providing evidence of a stimulatory effect of Kiss1-15 treatment in female YTK.

The second procedure towards the long term aim of advancement of puberty was through the use of rytkFsh (Chapter 3). Using the Pichia pastoris expression system, a single chain recombinant YTK Fsh was produced. The biological activity of the recombinant hormone was tested by in-vivo and in-vitro bioassays (Sanchis-Benlloch et al., 2016). Results of the in-vitro Fsh receptor assay showed that rytkFsh activated the cAMP signal transduction system in a dose-dependent manner. The rytkFsh also stimulated secretion of E2 and 11 ketotestosterone (11KT) from YTK female and male gonad fragments, respectively. When tested in-vivo, rytkFsh increased plasma E2 and stimulated oocyte development in immature female YTK. In maturing YTK males, rytkFsh advanced spermatogenesis, however it reduced plasma 11KT. Further optimization of the hormonal doses, frequency and duration of the rytkFsh administration should enable accomplishing a commercially relevant protocol for inducing the advancement of the onset of puberty particularly in farmed female YTK.

One of the tool development outputs of this project is an enzyme linked immunosorbent assay (ELISA) for YTK vitellogenin (Vtg) (Chapter 4). As circulating Vtg is a direct indication of the maturational stage of the fish, this is an ideal tool to be used to assess both maturational stages and effectiveness of hormonal treatments as shown in the Chapter 2, where plasma levels from Kisspeptin treated fish were assayed using this tool. The hybrid competitive YTK Vtg ELISA developed in this project has acceptable parameters of specificity, precision and sensitivity as previously reported for other teleost Vtg ELISAs; Palumbo et al., 2009). This ELISA will help to understand further the Seriola lalandi reproductive biology and could be used to assess maturation and hormone therapy effectiveness not only in YTK but also in Mullet (Mugil cephalus) due
to the similarity of the Vtg nucleotide sequences of these two species with that of YTK Vtg, and most likely other related species.

At the present time, hormonal therapy by injection or implantation to achieve spawning is the most common hormone delivery system in the aquaculture industry. These techniques can be labor intensive and stressful to the fish due to the handling and anesthetic application involved. In the case of the tunas, the current method of hormone administration for spawning induction is a very labor-intensive and risky method for the workers and stressful method for the fish, as need to be done using spear guns. In contrast to injections and implantations currently used, delivery of bioactive compounds (peptides, genetic constructs and recombinant hormones) via the oral route could be used as a non-invasive, practical, and cheaper technique which also can allow mass treatment for manipulating the reproductive process regardless of fish size. As part of this PhD, two diets containing two hormone encapsulation methods using alginate-chitosan particles and yeast as vehicle were developed. The encapsulation methods were meant to protect the hormone from degradation while in the digestive track until its release to the blood stream. Alginate-chitosan particles, were found to incorporate 1% (w/w) of GnRHa and they were observed to release GnRHa from the particles after 1hr of exposure to the same pH of that reported for intestinal fluids (Li et al., 2007). The recombinant sbtGnRH1 expression in yeast was confirmed at the DNA level through PCR and at protein level through mass spectrophotometry. In-vivo experiments showed that pellets containing yeast were not eaten by tilapia. Although the pellets containing alginate-chitosan particles were eaten, the doses of did not stimulate E2 secretion or gonadal development at temperatures under 23°C. This could be due to the fact that dose tested was not strong enough to overcome tilapia dopamine inhibition (Zohar et al., 2010). Nevertheless, both of the encapsulation methods appeared to hold successfully the desired hormones. This study has set up the basis for further studies in exploring this technique as a potential effective vehicle for the oral delivery of GnRHs and other relevant peptides for the aquaculture industry. For further studies, a higher dose of GnRH in the alginate-chitosan particles or the addition of a dopamine antagonist could help to overcame the dopamine inhibition presented in tilapia. Regarding the yeast as an encapsulation methodology, fermenter techniques (Cereghino et al., 2002)
could help to increase the expression of sbtGnRH1 allowing to deliver a high sbtGnRH1 concentration within less quantity of yeast cells making the pellets attractive to the fish.

6.1. References


Poortenaar, C., Hooker, S., & Sharp, N. (2001). Assessment of yellowtail kingfish (Seriola lalandi lalandi) reproductive physiology, as a basis for aquaculture development. Aquaculture, 201, 271-86. doi:http://dx.doi.org/10.1016/S0044-8486(01)00549-X.


In-vitro and in-vivo biological activity of recombinant yellowtail kingfish (Seriola lalandi) follicle stimulating hormone

Pablo J. Sanchís-Benlloch a,1, Josephine Nocillado a,1, Claudia Ladisa a, Joseph Aizen a, Adam Miller b, Michal Shpilman c, Berta Levavi-Sivan d, Tomer Ventura a, Abigail Elizur a,⇑

a Genecology Research Centre, Faculty of Health, Education and Engineering, University of the Sunshine Coast, Queensland, Australia
b Clean Seas Tuna Ltd, Port Lincoln, SA, Australia
c Department of Animal Sciences, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot 76100, Israel
d Genecology Research Centre, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Queensland, Australia

1 Equal contributions.

E-mail address: AElizur@usc.edu.au (A. Elizur).

Abstract

Biologically active recombinant yellowtail kingfish follicle stimulating hormone (rytkFsh) was produced in yeast Pichia pastoris and its biological activity was demonstrated by both in-vitro and in-vivo bioassays. Incubation of ovarian and testicular fragments with the recombinant hormone stimulated E2 and 11-KT secretion, respectively. In-vivo trial in immature female YTK resulted in a significant increase of plasma E2 levels and development of oocytes. In males at the early stages of puberty, advancement of spermatogenesis was observed, however plasma 11-KT levels were reduced when administered with rytkFsh.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

The genus Seriola includes highly active pelagic fish belonging to the Carangidae family and are found in the Atlantic, Indian and Pacific Oceans. The culture of Seriola began in Japan over 70 years ago (Nakada, 2008). Yellowtail kingfish (Seriola lalandi, YTK) is one of the larger members of the genus and its importance for the aquaculture industry is growing worldwide due to its fast growth, high flesh quality and suitability for farming in both cage and recirculating aquaculture systems (RAS) (Chen et al., 2006; Orellana et al., 2014; Poortenaar et al., 2001). YTK is a gonochoristic species with an asynchronous oocyte development, which provides the capacity for multiple spawning within a reproductive season, between spring and summer (Poortenaar et al., 2001). YTK puberty age can vary between male and female fish, and between geographical locations. According to observations in farm conditions in South Australia, precocious males can start to spermiate from 12 months old onwards, however females take 4–5 years to reach sexual maturity. Currently, a genetic selection program is being undertaken for YTK (Whatmore et al., 2013), and there is interest to shorten its generation time to increase genetic gain. Advancing pubertal development is the first step towards shortening generation time, and as such has been the focus of many studies (Carrillo et al., 2009; Taranger et al., 2010). Strategies to advance the onset of puberty range from the control of environmental parameters, as reported for Atlantic salmon (Salmo salar) where the control of salinity and photoperiod modulates pubertal development (Melo et al., 2014), to hormonal therapies such as in female red sea bream (Pagrus major), where continuous administration of GnRHa resulted in precocious induction of puberty (Kumakura et al., 2003).

The development of gonads in fish is mainly controlled by two pituitary gonadotropins: follicle stimulating hormone (Fsh) and luteinizing hormone (Lh) (Levavi-Sivan et al., 2010). Evidence suggests that Fsh plays a key role in the onset of puberty and early stages of reproductive development while Lh plays a role at the advanced stages of maturation (Yaron et al., 2003). However, studies in Japanese eel and goldfish reported recombinant Fsh activates the later stages of maturation (Kobayashi et al., 2006; Hayakawa et al., 2008). Gene knockout studies of fsh and lh in zebrafish have confirmed that the fsh-fshr signalling pathway is essential in puberty onset and gonadal growth in male and female fish (Zhang et al., 2015, 2014). Until recently, knowledge gaps still exist in relation to the exact actions of Fsh and Lh (Mazón et al., 2014) in most perciforms. This is partly due to the lack of biologically active pure forms of these hormones (Yu et al., 2010; Zhang et al., 2014). This
problem has recently been circumvented with the production of recombinant gonadotropins in heterologous expression systems resulting in pure forms of the hormones with biological activity (Levavi-Sivan et al., 2010). To date, piscine recombinant Fsh have been produced successfully for 12 species using different heterologous expression systems (Levavi-Sivan et al., 2010; Yu et al., 2010; Kobayashi et al., 2010; Hayakawa et al., 2009; Chen et al., 2012; Mazón et al., 2014). However, recombinant Fsh has been tested in vivo in six species only. Recombinant goldfish (Carassius auratus) Fsh (Hayakawa et al., 2008; Kobayashi et al., 2006) and recombinant Manchurian trout (Brachymystax lenok) Fsh (Ko et al., 2007) successfully induced milk production in goldfish. Also, recombinant goldfish Fsh induced ovulation in betterling (Rhodeus ocellatus ocellatus) and promoted the initiation of spermatogenesis in sexually immature male Japanese eel (Anguilla japonica) (Hayakawa et al., 2008, 2009). Recombinant Japanese eel Fsh induced spermatogenesis and oocyte maturation in sexually immature Japanese eel (Kobayashi et al., 2010). Recombinant orange-spotted grouper (Epinephelus coioides) Fsh administrated to juvenile grouper increased serum sex steroid levels and induced early ovarian development (Chen et al., 2012). In zebrafish (Danio rerio) it was found that the native form of the recombinant Fsh had higher activity when compared to its 6His-tagged form (Yu et al., 2010). In prepubertal European sea bass male (Dicentrarchus labrax), recombinant Fsh (rFsh) was able to trigger the process of spermatogenesis (Mazón et al., 2014). In the same species, rFsh was tested for its in vivo stability, allowing the comparison of the recombinant hormones produced in two different expression systems (Molès et al., 2011).

In the present study, we have generated a single chain recombinant yellowtail kingfish Fsh (rytkFsh) using the yeast (Pichia pastoris) expression system (Hollenberg and Gellissen, 1997). The recombinant hormone was tested both in vitro and in vivo for its efficacy in inducing sex steroid secretion and promoting gonadal development in immature YTK females and YTK males at the onset of sexual maturation.

2. Materials and methods

All experiments were conducted with approval from the Animal Ethics Committee of the University of the Sunshine Coast (Queensland, Australia) under approval number AN/A/12/69.

2.1. Isolation of full-length fshb and fsha cDNA sequences

Total RNA was extracted from frozen YTK pituitaries previously stabilised in RNALater (Ambion Life Technologies, Carlsbad, CA, USA) with Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA quality and quantity was established using Agilent’s (USA) with Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) stabilised in RNALater (Ambion Life Technologies, Carlsbad, CA, USA) under approval number AN/A/12/69.

All experiments were conducted with approval from the Animal Ethics Committee of the University of the Sunshine Coast (Queensland, Australia) under approval number AN/A/12/69.

2.1. Isolation of full-length fshb and fsha cDNA sequences

Total RNA was extracted from frozen YTK pituitaries previously stabilised in RNALater (Ambion Life Technologies, Carlsbad, CA, USA) with Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA quality and quantity was established using Agilent’s 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). First-strand cDNA was generated from 1 µg total RNA using 5’ and 3’-SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). Synthesised 5’ and 3’ cDNAs were tested by PCR amplification of the reference gene acidic ribosomal phosphoprotein P0 (ytkARP) (Nocillado et al., 2012).

Gene-specific RACE PCR primers (Table 1) for fshb were designed from the previously isolated partial YTK fshb sequence (GenBank Accession No. HQ449731). The primers used for the first and semi-nested 3’ RACE PCR were Primer 1 and Primer 3, respectively. The first and semi-nested 5’ RACE PCR reverse primers were Primer 2 and Primer 4. In both reactions, the fshb primers were paired with Nested universal primer (NUP). The first round PCR reaction mix contained 1.5 µl 10× PCR buffer, 1.2 µl MgSO4 (50 mM), 0.3 µl dNTPs (10 mM each), 0.3 µl of NUP (10 µM), 0.3 µl of each forward and reverse primer (10 µM), 0.06 µl Platinum high fidelity Taq DNA polymerase (Invitrogen Life Technologies) and 1 µl of the first-strand cDNA. The semi-nested PCR amplification reaction mix contained 2.5 µl 10× PCR buffer, 2 µl MgSO4 (50 mM), 0.5 µl dNTPs (10 mM each), 0.5 µl of NUP (10 µM), 0.3 µl of each forward and reverse primer, 0.1 µl Platinum high fidelity Taq DNA polymerase and 1 µl of the first round PCR product (diluted 20-fold). No template control reactions were performed in order to rule out contamination. The thermal cycling parameters for the first and semi-nested reactions were: initial denaturation at 94 ºC (3 min); followed by 35 cycles of denaturation at 94 ºC (30 s), annealing at 53 ºC (30 s) and extension at 72 ºC (1 min); and final extension at 72 ºC (5 min). PCR product purification and cloning were as previously described (Nocillado et al., 2012). Plasmid DNA from positive colonies were sequenced by the Australian Genome Research Facility (Brisbane, Australia).

Sequences were analysed using NCBI’s Basic Local Alignment Search Tool (Altschul et al., 1990) and Sequencer 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA).

The full length fshb cDNA sequence was isolated by 3’RACE PCR. Primers 6 and 7 (Table 1) were designed according to the conserved regions of Dicentrarchus labrax (GenBank Accession No. AF269157) and Scomber japonicus (GenBank Accession No. JF495131) glycoprotein alpha sequences. The first and semi-nested PCR amplification was performed as for fshb. The thermal cycling parameters for both reactions were: initial denaturation at 94 ºC (2 min 30 s); followed by 30 cycles of denaturation at 94 ºC (30 s), annealing at 60 ºC (30 s) and extension at 72 ºC (45 s); and final extension at 72 ºC (7 min). PCR products were purified, cloned, sequenced and sequence analysed as described for fshb. The signal sequences of fshb and fsha were identified using SignalP 4.1 software (Petersen et al., 2011).

2.2. Synthesis of a single chain fshba and ligation into the pPIC9K expression vector

The design of the rytkFshba was generally according to Kasuto and Levavi-Sivan (2005) and Aizen et al. (2007). The cDNA sequences encoding for YTK fshb and YTK fsha (excluding the signal sequence) were linked with the nucleotide sequence encoding for GSGSHHHHHHHHGS and were then codon-optimised according to the yeast (P. pastoris) coding preference. The codon-optimised sequence was synthesised and ligated into the EcoRI-NotI sites of pPIC9K (GenScript, Piscataway, NJ, USA). Sufficient quantity of the construct plasmid DNA was generated in JM109 Escherichia coli. Plasmid DNA was purified with QIAprep spin midiprep columns (Qiagen, Hilden, Germany). The purified plasmid DNA was linearized with Sall (New England Biolabs, Ipswich, MA, USA). Nine microgram of the linearized construct were transformed into SuperManHis+ strain of the yeast (P. pastoris) cells (Biogammatrics, Carlsbad, CA, USA) by electroporation (BioRad GenePulsar, Hercules, CA, USA).

2.3. Selection of high expressing clones and induction of protein expression

Positive yeast transformants were selected in histidine-deficient media. High copy number colonies were screened based on the resistance to the antibiotic Geneticin (G418 sulphate, Sigma–Aldrich, St. Louis, MO, USA) at concentrations of 0.5–2 mg/ml. Using this protocol, 10 most resistant clones were selected from 800 colonies. From these 10 clones, the highest expressing clones were identified by methanol induction in 125 ml cultures. Protein expression induction by methanol in 1 litre (L) volume was carried out in 5 L shaker flask at 30 ºC. Molecular grade methanol (Sigma–Aldrich) was added every 24 h at 1% final concentration. Yeast was harvested 72 h after induction. The rytkFsh was purified from the culture media using nickel
nitrilotriacetic acid-agarose (Ni-NTA, Qiagen) that binds His-tagged proteins. Media from yeast without recombinant DNA served as negative control. All the mentioned steps including electroporation protocol, selection in histidine-deficient media, antibiotic resistance, and protein expression induction by methanol were carried out according to Invitrogen’s P. pastoris expression manual (Invitrogen Life Technologies).

2.4. Purification of rytkFsh by dialysis and Western blot analysis

The recombinant protein was dialyzed against 0.1 PBS in Slide-A-Lyzer G2 Dialysis Cassettes that had a molecular weight cut-off of 20 kDa (Thermo Fisher Scientific, Pittsburgh, PA, USA), following the manufacturer’s protocol. The expected size of the deglycosylated rytkFsh was 23–35 kDa. For Western blot analysis, 25 μl of the purified recombinant hormone was deglycosylated at 37 °C overnight with N-glycosidase F (PNGase F, New England Biolabs) following the supplier recommendations. SDS–PAGE was performed using 20 μl of the deglycosylated protein on a 12% Mini gel (BioRad) at 160 V for 1 h. 6XHis protein ladder (Qiagen) was used as a marker (4 μl). The recombinant protein was mixed with loading buffer composed of Tris-Cl, glycerol, 20% SDS, Bromophenol blue and DTT at final concentration of 0.045 M, 10%, 1%, 0.01 and 0.05 M, respectively (Sigma–Aldrich). Then the sample and the protein ladder were heated to 98 °C (7 min) prior to loading into the gel. Proteins were transferred onto a nitrocellulose membrane using Trans–blot turbo transfer system transfer pack (BioRad). The membrane was blocked (1 h at room temperature) with 25 ml of Tris-buffered saline (TBS), pH 7.5, containing BSA with loading buffer composed of Tris-Cl, glycerol, 20% SDS, Bromophenol blue and DTT at final concentration of 0.045 M, 10%, 1%, 0.01 and 0.05 M, respectively (Sigma–Aldrich). Then the sample and the protein ladder were heated to 98 °C (7 min) prior to loading into the gel. Proteins were transferred onto a nitrocellulose membrane using Trans–blot turbo transfer system transfer pack (BioRad). The membrane was blocked (1 h at room temperature) with 25 ml of Tris-buffered saline (TBS), pH 7.5, containing BSA and Tween-20 at 3% and 0.1%, respectively (Sigma–Aldrich). Membrane was then washed twice with TBS-T (TBS supplemented with 0.1% Triton) (Sigma–Aldrich), followed by incubation with primary antibody (mouse Penta-His antibody, Qiagen) at 1:2000 dilution for 1 h at room temperature in an orbital shaker, followed by two consecutive washes as described above. Then incubation with secondary antibody diluted 1:5000 (Goat anti mouse-IR Dye 800CW: Li-cor Bioscience, Lincoln, USA), Bands were visualized at 800 channel using the Li-cor Odyssey CLx Infrared Imaging System (Li-cor Bioscience).

2.5. Prediction of rytkFsh 3D structure

To predict the folding of rytkFsh, a 3D structure of was generated using the iterative online software I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). Visualization and labelling of the model were performed using Discovery Studio 4 and Adobe Photoshop CC2014.

2.6. In vitro heterologous receptor binding assay

To study the receptor binding of the novel rytkFsh, we utilised the tilapia Fsh receptor (tiFshr) as heterologous receptor. The assay used a light sensitive luciferase (LUC) reporter, with cAMP response element (CRE-LUC; Invitrogen) that was previously demonstrated as a useful tool for discriminating cAMP signalling pathway (Biran et al., 2008). Fsh is known to exert its effect via cAMP upon binding to its cognate receptor (Levavi-Sivan et al., 2010). The entire coding region of tiFshr was inserted into pcDNA3.1 (Invitrogen). Three microgram of tiFSHR-pcDNA3.1 construct with three microgram of luciferase reporter plasmid were transiently transfected into COS-7 cells (American Type Culture Collection). Forty-eight hours after transfection, cells were treated with increasing doses of rytkFsh (16, 80, 400, 2000 and 5000 ng/ml) or tiFsh (0.8, 4, 20, 100 and 275 ng/ml). The hormone treatment and the subsequent measurement of luciferase activities were carried out as previously described (Biran et al., 2008). The EC50 values were calculated from concentration response curves by means of computerized nonlinear curve fitting with Prism version 6 software (GraphPad).

2.7. In vitro bioactivity assay using YTK gonads

The bioassay procedure was performed according to Aizen et al. (2007) with the following modifications. Briefly, testes from male YTK at the start of spermatogenesis/onset of puberty (3.2 kg body weight (BW), 59 cm fork length (FL) and 0.07 gonadosomatic index (GSI)) or immature ovaries from immature YTK female (2.4 kg BW, 49 cm FL and 0.23 GSI) were divided into uniformly sized fragments (20 mg each). The fragments were pre-incubated at 21 °C with gentle shaking in a 24-well culture plate that had 1 ml/well of basal medium eagle (BME) containing NaHCO3 (2.5 g/L), penicillin (50 iu/ml), streptomycin (0.05 mg/ml), and nystatin (1.25 iu/ml) and 0.05% BSA buffered to pH 7.4 with 2.1 mM of Hepes (all from Sigma–Aldrich). The culture media was replaced every hour during pre-incubation. After the 3 h pre-incubation, the fragments were incubated in the same medium containing recombinant rytkFsh at doses of 800, 400, 200 and 100 ng/ml. As a positive control, gonadal fragments were incubated in the same medium containing YTK pituitary extracts. Freshly dissected pituitaries from YTK male at the start of the spermatogenesis (3.12 kg BW, 59 cm FL and 0.08 GSI) and immature YTK female (3.5 kg BW, 60.5 cm FL and 0.29 GSI) were homogenized in 0.1% PBS and stirred for 30 min at 4 °C. The mixture was centrifuged at 3000g for 4 min and 50 μl from the top layer were used as a positive control. Negative control wells contained culture medium only. Incubation with rytkFsh, YTK pituitary homogenate and media alone was conducted for 18 h at 21 °C. These incubations were performed in triplicate wells per treatment. The incubation medium was collected and stored at −80 °C until assayed for 11-ketotestosterone (11-KT) or estradiol (E2) concentrations.

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>5′–3′ sequence</th>
<th>Direction</th>
<th>Sequence amplified</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1</td>
<td>CCATATGGACGGZCACTGGTACC</td>
<td>Forward</td>
<td>fshh</td>
<td>53</td>
</tr>
<tr>
<td>Primer 2</td>
<td>ACATCCGAGCTTTGGTCACTT</td>
<td>Reverse</td>
<td>fshh</td>
<td>53</td>
</tr>
<tr>
<td>Primer 3</td>
<td>GAGGTAACACATCCTGGATGT</td>
<td>Forward</td>
<td>fshh</td>
<td>53</td>
</tr>
<tr>
<td>Primer 4</td>
<td>GGTAAACCTGCTGCACATATGG</td>
<td>Reverse</td>
<td>fshh</td>
<td>53</td>
</tr>
<tr>
<td>NUP</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Primer 6</td>
<td>GGAAATTTCTCTAGACGACG</td>
<td>Forward</td>
<td>fshh</td>
<td>53</td>
</tr>
<tr>
<td>Primer 7</td>
<td>GCTGCAACCACAGTGCCACGGTCATGG</td>
<td>Forward</td>
<td>fshh</td>
<td>53</td>
</tr>
</tbody>
</table>

* NUP: Nested Universal Primer.

To predict the folding of rytkFsh, a 3D structure of was generated using the iterative online software I-TASSER (http://zhan-glab.cmb.med.umich.edu/I-TASSER/). Visualization and labelling of the model were performed using Discovery Studio 4 and Adobe Photoshop CC2014.

2.8. In vivo bioassay in immature female YTK and maturing male YTK

Eighteen months old YTK were used as experimental animals for the in vivo experiments at CleanSeas Tuna Ltd (Arno Bay, South Australia). At the start of the trial, average BW and FL were...
2.79 ± 0.10 kg and 52.26 ± 0.72 cm (mean ± SD), respectively. Fish were reared in a 6000 L tank using ambient sea water. In order to determine whether there were spermatizing males in the group, gentle pressure was applied on the abdomen, however no fish released sperm. Gonadal biopsy was not conducted as it is technically difficult to obtain a gonadal biopsy of very immature gonad without damaging the fish. At the end of the trial, fish BW and FL were 3.2 ± 0.14 kg and 58.36 ± 0.80 cm for females (n = 17) and 2.88 ± 0.08 kg and 56.86 ± 0.52 cm for males (n = 24), respectively.

The experiment commenced on October 2014 and was terminated on December 2014, which coincides with the spawning season for YTK in South Australia. The experimental animals were tagged and randomly assigned to 2 experimental groups. During the experiment, the fish were under natural photoperiod which ranged from 12L:12D (October) to 14L:10D (December). Water temperature ranged between 15 °C and 20 °C. Experimental animals were injected as described by Mazón et al. (2014) with the following modifications. YTK was injected intramuscularly in the right epaxial muscle anterior to the dorsal fin. Animals belonging to control group (n = 22) were injected saline only. Animals belonging to treated group (n = 19) were injected at doses of 10–20 µg/kg with lympholished rytkFsh resuspended in sterile saline (0.9% NaCl). Injections were repeated 6 times with 10 day’s interval. At the end of the trial, fish were terminally anesthetized with Aqui-S (Aquatic anaesthetic) at a dose of 1 ppt. Then, BW and FL were recorded. Fish were bled by caudal puncture using heparinised syringes (heparin 30 IU/mL, Sigma). Two millilitre of blood per fish were collected. All fish gonads were then dissected out and weighed. In addition, male gonads were inspected for the presence of sperm when processed for gonadal fragment collection. Both male and female gonad fragments corresponding to the medial area were fixed in 4% paraformaldehyde for histological analysis. Plasma from the experimental animals was extracted from blood by centrifugation at 3000g at 4 °C for 20 min and then stored at −80 °C until 11-KT and E2 analysis.

2.9. Sex steroids analysis

Levels of 11-KT and E2 were determined by commercially available enzyme immunoassay (EIA) kits (Cayman Chemical Company, Ann Arbor, MI, USA), which are known to be highly specific for these steroids, as detailed in their specifications. For the in-vitro assay, the intra-assay coefficient of variation (CV) was 7.3% and 9.8%, while the inter-assay CV was 10.7% and 13.9% for E2 and 11-KT, respectively. For the in-vivo assay, the intra-assay CV was 9.8% and 9.6% while the inter-assay CV was 13% and 11.8% for E2 and 11-KT, respectively.

Steroids were extracted from 200 µl of plasma with 5 ml of diethyl ether. Solvent was evaporated and extracts were resuspended in the EIA assay buffer. Plasma levels of 11-KT and E2 were measured for males and females, respectively. For E2 assays, the plasma was diluted 2 times while for 11-KT, 10 times dilution was required to optimise the sensitivity of the assay defined as 90% binding.

2.10. Histological analysis

Right and left gonads were removed and weighed from each male (n = 24) and female (n = 17) fish. Cross section samples were taken from the medial area of both gonads as being representative for the whole lobe and fixed in 4% paraformaldehyde for 24 h and then transferred to 70% ethanol until tissue processing for histology. All samples were processed for histological investigation. The gonad samples were embedded in paraffin and sectioned transversely at 6 µm width. Sections were stained with haematoxylin and eosin. The classification of developmental stage of oocytes was according to Gillanders et al. (1999). Histological analysis was performed under a DM5500B Microscope equipped with a DFC550 camera system (Leica Microsystems, Wetzler, Germany). Measurements (n = 600 oocytes/group) of the main oocyte diameter were taken from each sample using an eye piece micrometre mounted on a light microscope (BS51, Olympus, Tokyo, Japan).

2.11. Statistical analysis

Body weights (BW), fork length (FL) and GSI were expressed as mean ± SD. Levels of E2 and 11-KT from the in-vitro and in-vivo experiments were expressed as mean mean ± SEM. Values from these data were analysed with one-way analysis of variance (ANOVA) followed by a multiple comparison test (Tukey). Differences were reported as statistically significant when p < 0.05 (Zar, 1999).

3. Results

3.1. Yellowtail kingfish Fsh and predicted structure of single chain rytkFsh

Multiple sequence alignment showed 97% similarity of ytkFshb (GenBank accession No. HQ449731) with those of amino acid sequences from the same genus (Seriola dumerili and Seriola quinqueradiata) (Fig. 1A). Similarity dropped to 57% when compared to other perciforms, such as Oreoichromis niloticus, and to 37% when compared to mammals, such as Homo sapiens. However, ytkFsh (GenBank accession No. KT364712), the gonadotropin alpha subunit, was 100% identical to that of Seriola dumerili and 99% with Seriola quinqueradiata (Fig. 1B). The percentage similarity dropped to 85% when compared to other perciform fish, such as Oreoichromis niloticus, and to 53% when compared to mammals, such as Homo sapiens. The construct of the ytkFsh is presented in Fig. 2A. Modelling of the single chain recombinant rytkFsh predicted a properly folded 3D structure (Fig. 2B). The model showed 3 loops in each subunit, the expected binding sites and the linker sequence does not obstruct the alpha and beta subunits.

3.2. Recombinant single chain rytkFsh (rytkFsh)

The single chain ytkFsh construct was successfully electroporated into the yeast (P. pastoris) genome and recombinant Fsh was produced. Yield ranged from 400 to 900 µg/L, depending on the specific yeast clone used (n = 6). Western blot analysis of deglycosolated rytkFsh using the His tag antibody showed 3 bands: the expected 23 kDa and >30 kDa bands representing rytkFsh and the enzyme PNGase F, respectively, and a third band below 15 kDa, that might be due to partial degradation of the recombinant protein (Fig. 3).

3.3. In-vitro bioactivity of rytkFsh

The tilapia Fsh receptor was activated by both rytkFsh and recombinant tilapia FSH (rtiFsh) in a dose-dependent manner using the cAMP signal transduction system as a reporter. The rtiFsh was 7-fold more effective than rytkFsh in inducing tifsh activity (EC50 73.78 ng/ml for rtiFsh and 530.5 ng/ml for rytkFsh) (Fig. 4). In-vitro bioassay using YTK gonadal fragments assessed the efficiency of rytkFsh in inducing E2 secretion from immature female gonad fragment or 11-KT secretion from testicular fragment taken from males at the onset puberty. In females, the rytkFsh stimulated a dose response in terms of E2 secretion (Fig. 5A). However, only the highest dose (800 ng/ml) was significant when compared with the negative control. In males, 11-KT secretion from...
Fig. 1. Multiple comparative sequence alignment of ytkFshb (A) and ytkFsha (B) with other vertebrate Fsh sequences. Sequence logo indicates similarity conservation with bigger letters indicating higher conservation.

Fig. 2. Structure of the single chain rytkFsh construct. (A) Construct of the expression vector for ytkFshb linked by the sequence GSGSHHHHHHGS to ytkFsha and introduced into the EcoRI and NotI sites of the expression vector pPIC9K. The vector contains the 5' and 3' promoter transcription-termination sequences of the alcohol (methanol) oxidase gene (AOX1) and a yeast alpha mating factor signal peptide (AMF). (B) Predicted 3D structure of the rytkFsh construct. The ytkFshb (blue) linked to ytkFsh alpha (green) and the Histidine linker (GSGSHHHHHHGS) in red. Beta L1,2,3 represents the loops within the ytkFshb subunit and Alpha L1,2,3 represents the loops within the ytkFsh alpha subunit.
YTK testicular tissues treated with increasing doses of rytkFsh also showed a response in a dose-dependent manner (Fig. 5B). As in the YTK pituitary extract was used as positive control. Column and bar represent the mean and the SEM, respectively. Different letters indicate significant difference (p < 0.05, N = 3).

3.4. In-vivo bioactivity of rytkFsh

In-vivo assays in sexually immature female YTK resulted in a significant increase in plasma E₂ in treated fish when compared with the control (Fig. 6A). In males, plasma levels of 11-KT showed significantly lower levels of 11-KT compared with control group (Fig. 6B). Although the GSI of females was slightly higher in the treated group (0.2 ± 0.03 vs. 0.23 ± 0.05), the difference was not significant. In males, the GSI in control group was slightly higher than in the treated group (0.07 ± 0.02 vs. 0.054 ± 0.03), however the difference was not also significant.

Histological analysis of the cross section from the middle area of the right and left gonads showed no developmental differences between the two lobes, either in males or in females. Oocyte diameter did not significantly differ between the two groups, with means ± SEM of 40.55 ± 0.59 vs. 41.41 ± 0.83 μm for control and treated females, respectively. Oocytes at the chromatin nucleolar and perinucleolar stages of development (Stages 1 and 2, respectively) were present in both rytkFsh-treated and untreated fish (Fig. 7A and B). However, oocytes at early cortical alveolar stage (Stage 3) were present only in the rytkFsh treated group, suggesting the onset of gonadal development.

YTK males presented an asynchronous pattern of spermatogenesis, where all germ cell stages were present at the same time. However, within each spermatocyst, germ cell development was synchronous. Stages observed were mature (stage 1), developing (stage 2) and mature (stage 3). Stages 1 and 2 were present in both control and treated groups (Fig. 7C). Stage 3, which is characterised by spermatozoa in lumen of the lobules, was found only in the treated group (Fig. 7D).

4. Discussion

Yellowtail kingfish is of growing importance in aquaculture around the world, and as such there is interest in having better control over its reproductive development, particularly the onset of sexual maturation in female broodstock. We have explored the use of recombinant ytkFsh in manipulating reproduction in 18 month old YTK in South Australia. Age at sexual maturation varies between male and female YTK. It also varies between geographical locations as well as within populations. In South Australia, males may start to spermiate from 12 month old onwards based on observations under farm conditions. In contrast, females take 4–5 years to reach sexual maturity. In New South Wales (Australia), where temperatures are higher, males can reach sexual maturity at the age of one year and females at 3+ years (Gillanders et al., 1999). In New Zealand, where it is colder, it can take longer than in either South Australia and New South Wales (Poortenaar et al., 2001). Earlier sexual maturation has been reported for YTK raised in Western Australia under commercial conditions when compared to wild YTK in this area (Kolkovski and Sakakura, 2004). These differences suggest that YTK age of puberty can be influenced by
environmental parameters such as temperature and photoperiod as reported in other fish species (Taranger et al., 2010).

As a first step towards developing the tools to controlling reproductive function in farmed YTK, we cloned the alpha and beta subunits of YTK fsh. Alignment of the alpha and beta ytkfsh sequences showed that, as in other fish species, fshb subunit is less conserved than the fsha subunit, indicating species specificity of the beta subunit and a high conservation of the alpha subunit over vertebrate evolution from fish to mammals, as reviewed by Yaron et al. (2003). We used the alpha and beta ytkfsh sequences to design a single chain construct, with a linker composed of 2x(GSGS) and 6His in between, which is slightly modified from that designed in tilapia (Aizen et al., 2007). Although other literature demonstrated that the native form of Fsh has higher activity when compared to 6His-tagged recombinant hormone (Yu et al., 2010), we have utilised 6His tagging to facilitate the purification and detection of the recombinant hormone as there are no specific antibodies produced yet for the YTK Fsh. The prediction of the three-dimensional structure of the single chain protein confirmed that the linker sequence did not disturb the proper folding of the recombinant hormone, and therefore was not expected to interfere with its biological function, as reported in other recombinant glycoproteins (Xing et al., 2004). We generated a single chain rytkFsh hormone in the yeast (P. pastoris), which is capable of post-translational modifications typically associated with higher eukaryotes, such as the addition of N-linked high mannose moiety (Cereghino and Cregg, 2000). The consensus sequence for N-linked glycans in yeast is Asn-Xaa-Ser/Thr, which is critical for bioactivity of the recombinant hormone (Cereghino and Cregg, 2000). The deglycosolation of rytkFsh with PNGase F to the nascent translated protein implies

Fig. 6. rytkFsh in-vivo trials on YTK. (A) Mean plasma E2 of rytkFsh-treated YTK females (n = 5) and control (n = 12) injected with saline only (B) mean plasma 11-KT levels of rytkFsh treated YTK males (n = 14) and control (n = 10). Treatment includes doses of 10–20 μg/kg rytkFsh. Column and bar represent the mean and SEM, respectively. Different letters indicate significant difference (p < 0.05).

Fig. 7. Histological sections of YTK ovary and testis from the in-vivo trial. Ovary (A) and testis (C) from YTK injected with saline only (control) while B and D are ovary and testis, respectively, from YTK treated with rytkFsh. Oocytes at chromatin nucleolar stage of development (Stage 1) and perinucleolar stage (Stage 2) were present in both rytkFsh treated and untreated groups. However, oocytes at cortical alveolar stage (Stage 3) were only present in the rytkFsh treated group. Mature testes (stage 3) were observed only in rytkFsh-treated fish (D). (SG = spermatogonia; SD = spermatids; SZ = spermatozoa; CNS = chromatin nucleolar stage; PS = perinucleolar stage; CAS = cortical alveolar stage.) Scale bar represents 50 μm.
that the carbohydrate modification of the mature rytkFsh protein occur exclusively through the N-linked glycosylation as suggested for tilapia and Japanese Fsh (Aizen et al., 2007).

Western blot analysis of the deglycosylated rytkFsh, using antibodies raised against the histidine tag, revealed the expected 23 kDa band indicating that the hormone was successfully synthesized, secreted into the media and harvested using the Ni-NTA beads. The sizes of other reported deglycosylated recombinant Fsh range from 23 kDa in tilapia (Aizen et al., 2007) to 35 kDa in grouper (Chen et al., 2012) and Manchurian trout (Ko et al., 2007). Western blot analysis also revealed a >30 kDa band representing the deglycosylation reagent PNGase F, and an additional band which might represent partial degradation of the recombinant protein, as described by (Kasuto and Levavi-Sivan, 2005).

In-vitro receptor assay and gonadal bioassay confirmed that the recombinant single chain ytkFsh is biologically active. The receptor assay confirmed bioactivity of the rytkFsh showing that tilapia Fsh receptor was activated by both rytkFsh and rtiFsh dose-dependently in the cAMP signal transduction system. As expected, the efficacy of rytkFsh activation was not as potent as the rtiFsh, as the two Fshb subunits only share 57% similarity at the amino acid level, however confirming that the rytkFsh is biologically active. When using the rytkFshs to activate YTK testicular and ovarian sections examined through 11-KT and E2 secretion, a dose response to the hormone was detected, significantly for the highest dose (800 ng/ml), which was as potent as the YTK pituitary extract. These results further confirm the biological activity of rytkFsh. In a study in tilapia, where the sex steroid levels were assayed using an enzyme-linked immunoassay, it was found that the rtiFsh stimulated the secretion of E2 and 11-KT in mature gonads at the same level as that of the tilapia pituitary extract (Aizen et al., 2007). In the present study, the levels of E2 and 11-KT secreted were found to be lower when compared with other in-vitro studies using recombinant Fsh in tilapia (Aizen et al., 2007) and European sea bass (Mazón et al., 2014), probably due to the difference in the maturational stage of the tested gonads.

The effect of the rytkFsh was examined in-vivo by administering 6 times, at 10 day’s intervals, rytkFsh to 18-month old YTK and following the changes in circulating sex steroid levels and changes in gonadal development at the end of the trial. Augmenting E2 plasma levels is a prerequisite for advancing reproductive development and vitellogenin synthesis (Pankhurst, 2008). In a short term study, the E2 levels found in immature rainbow trout and mature goldfish were significantly elevated 24 h after a single injection of recombinant Fsh (Ko et al., 2007; Kobayashi et al., 2006). The E2 levels found in mature goldfish were 2 times higher than the levels observed in the present study while the levels reported for immature rainbow trout were 2 times lower. In juvenile grouper treated with recombinant Fsh, at a dose of 200 µg/kg for five times at 48 h intervals (Chen et al., 2012), the E2 levels were also within the range found in our study for immature YTK. While the grouper trial used a 10-fold higher dose of recombinant Fsh, was of shorter duration and was on juvenile fish, the outcome in terms of E2 levels were similar, confirming the biological activity of the YTK Fsh recombinant hormone.

Histological analysis of the female gonads revealed that the oocytes at the choratin nucleolar and perinucleolar stages (Stages 1 and 2 of development) were present in both rytkFsh-treated and untreated fish. However, oocytes from the treated group exhibited onset of development as indicated by the presence of oocytes at early Stage 3 or cortical alveolar stage. Although differences in mean oocyte diameter were not statistically significant between the treated and the control groups, there was a trend showing higher oocyte diameter in rytkFsh-treated fish compared with the control fish. The plasma E2 levels together with the ovarian histology suggest that the administration of rytkFsh to immature female YTK promoted gonadal development although the response was only detectable at the early stages of manifestation and would have benefited from a longer duration of the treatment.

In male teleosts, 11-KT is recognised as the major androgen responsible for testicular development (Dahle et al., 2003). In this study, the effect of rytkFsh administration at 10 day’s intervals for 6 times was assayed in 18-month old males through measurement of 11-KT levels in plasma and by histological analysis of the testes. Histological examination revealed that, Stage 3, characterised by spermatozoa in lumen of the lobules, was found only in the treated group. In accordance, sperm was found within the gonads of the majority of the rytkFsh-treated males indicating the activation of a late stage of spermatogenesis. Plasma testosterone levels showed a lower trend in the rytkFsh-treated males compared with the control (data not shown). The significantly lower levels of plasma 11-KT in the rytkFsh-treated fish, compared with the control group, suggests the fish were already towards final stages of maturation, which also have been reported to correspond to YTK to decreasing plasma 11-KT levels (Poo̜tenaar et al., 2001). Such a reduction in 11-KT was also observed exactly seven days after spermatiation in the three-spined stickleback (Gasterosteus aculeatus) (Pål et al., 2002). Juvenile European sea bass treated twice with recombinant Fsh, resulted in increased 11-KT plasma levels and testicular development, however they did not reach final stages of maturation within the trial period (Mazón et al., 2014). Other long term studies showed that recombinant goldfish Fsh (5–6 administrations at 7 day intervals) induced milt production in male goldfish and initiation of spermatogenesis in sexually immature male Japanese eel, although no differences were found in size of the eel testicular lobes or in the levels of 11-KT (Hayakawa et al., 2008). When recombinant Fsh was administrated in the Japanese eel at a higher dosage and frequency (8 times, at 2–5 days interval), the treatment was more effective increasing the size of the testicular lobes and plasma 11-KT levels dropped compared with the controls (Hayakawa et al., 2009; Kobayashi et al., 2010).

In summary, this study demonstrated that biologically active recombinant yellowtail kingfish Fsh can be produced in yeast P. pastoris and its biological activity was confirmed by both in-vitro and in-vivo bioassays. In future studies, further research on administration at different periods of gonadal development could help define the most appropriate time frame of administration. Also, optimisation of dosage, frequency and duration of administration could facilitate achieving commercially applicable protocol for inducing early maturation in farmed female YTK and other late maturing species.

**Funding**

This work was supported by the Australian Seafood Cooperative Research Centre and the Fisheries Research and Development Corporation grant 2008/745 to AE and JN, and 2013/700 to AE, and the University of the Sunshine Coast PhD stipend to PS.

**Acknowledgments**

We would like to acknowledge Clean Seas Tuna for the generous provision of fish and facilities to carry out these experiments. We are grateful to the Clean Seas team, especially to Michael Harrison for their support and looking after the fish. We thank Dr. Craig Foster for the opportunity to work with the company, and Mr. Andrea Miccoli for his work on the recombinant yeast.
References


Zhang, Z., Lai, S.-W., Zhang, L., Ge, W., 2015. Disruption of zebrafish follicle-stimulating hormone receptor (fshr) but not luteinizing hormone receptor (lhcr) gene by TALEN leads to failed follicle activation in females followed by sexual reversal to males. Endocrinology. 1039.

Please cite this article in press as: Sanchis-Benlloch, P.J., et al. In-vitro and in-vivo biological activity of recombinant yellowtail kingfish (Seriola lalandi) follicle stimulating hormone. Gen. Comp. Endocrinol. (2016), http://dx.doi.org/10.1016/j.ygcen.2016.03.001