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Contents

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2 Executive summary

Giant grouper is a significant commodity in the live reef food-fish trade in Asia. Wild populations of giant grouper have declined significantly due to exhaustive fishing of both market-size fish and fingerlings and the destruction of their reef habitats. Farming of giant grouper is vital to resolve these pressing issues. The overarching **aim** of this project was to establish a sustainable aquaculture industry for giant grouper in Vietnam, the Philippines and Australia. The **specific objectives** were: **1**. Develop reliable giant grouper captive-breeding technologies; **2**. Explore the potential of germ-cell transplantation and surrogate technologies as alternative approaches to giant grouper seed production; **3**. Develop reliable larval-rearing technologies for giant grouper; **4**. Apply genetic approaches to broodstock management and hybridisation; and **5**. Build capacity in the form of knowledge transfer and training in larval rearing, genetics and biotechnology as it applies to giant grouper aquaculture.

The project objectives were successfully achieved with impacts going well beyond the project and partner countries. As a spinoff of the successful breeding program, Vietnam has a new crop, the grouper hybrid, a cross between male giant grouper and female tiger grouper. This new crop contributed 50% of total grouper production in 2018, up from 5% in 2013. About two thirds of the current supply for hybrid grouper fingerlings now comes from domestic sources. Farmers find the hybrids desirable because they inherit valuable traits from the male giant grouper, such as fast growth, good meat quality and beautiful colouration while also retaining the resilience from the female tiger grouper. This remarkable success was enabled by the establishment of giant grouper broodstock through this project. Sourcing male giant grouper is one of the major challenges in giant grouper aquaculture. It was presumed that as protogynous hermaphrodites, all individuals mature first as females, at 20 to 40 kg in body weight, and then change into functional males when body weights already range from 40-60 kg. We discovered that about one in three individuals mature directly as males (a trait called diandry), at body weights between 16 and 35 kg. Using the hormonal treatment we developed, mature males of less than 10 kg can be obtained. As an alternative approach to obtaining even smaller broodstock, we have optimised a protocol to use smaller species of groupers (100 g to 5 kg in body weight) as surrogate giant grouper broodstock. In parallel with the successful breeding of groupers, we developed genetic tools to prevent inbreeding and species misidentification, and these were applied to manage the giant grouper broodstock in partner countries as well as in Australia.

We applied innovative molecular approaches to address a suite of questions regarding giant grouper larval culture. We identified the genes regulating appetite and digestion in early developing giant grouper larvae, and these will serve as the foundation for optimising early stage diets. DNA barcoding is employed to identify the food preference of grouper larvae. We established the origins of NNV infections and found that biosecurity measures are essential to prevent the disease from local and imported viral strains.

Additional impacts of this project come from the application of tools and technologies generated here to develop the culture of a variety of commercially important marine fish, as well as endangered species of sea cucumbers and frogs; with collaborators already using the tools we developed to study these animals. Scholars and trainees via this project are extending their expertise in their home countries in Southeast Asia, Africa, the Middle East and the Pacific Islands.

In light of the expansion of the hybrid grouper industry, further work includes: (1) Ensuring a reliable supply of giant grouper milt for hybrid production. This involves sourcing of males as well as preservation of gametes to facilitate hybrid production; (2) Optimising survival rates and growth of hybrid groupers through diet development, genetic and disease management. This will help sustain and build upon the current achievements, extend the impacts and enable the industry to respond to future challenges and expand.

3 Background

Supply and demand for giant grouper

Groupers are a major component of the live reef food-fish trade (LRFFT) in Southeast Asia, an industry valued at US$1 billion per year (Sadovy et al. 2013). Within this market, giant grouper is one of the most heavily exploited species of groupers. Giant grouper commands a high market price, reaching as high as US$169/kg, mainly due to its cultural significance as a symbol of abundance. As a result, the demand for this fish is high throughout China, Hong Kong, Japan, Singapore and Taiwan. There is considerable pressure on wild giant grouper populations, it is already difficult to source them in Southeast Asia (Donnelly 2009). Giant grouper are the largest reef-dwelling bony fish in the world, reaching 2.3 m in total length and 400 kg in body weight (Craig and Hastings 2007). As in other large groupers, giant groupers inhabit reefs and aggregate during the spawning season. Such behaviour renders them highly vulnerable to destructive fishing practices such as the use of cyanide and explosives, and also destroys their reef habitat. The large size of giant groupers indicates a long life span and, accordingly, late sexual maturity (Freitas et al. 2015; Wakefield et al., 2013). Wild populations are estimated to have a doubling time of about 14 years (Froese and Pauly 2018). These scenarios have placed the giant grouper populations in a precarious state and the species was classified as vulnerable in 2013 by the IUCN (Sadovy et al. 2013). Since 1995, the supply of all groupers for the LRFFT has been insufficient to meet demand (Johannes and Riepen, 1995). In the intergovernmental forum on LRFFT held in Thailand in 2013, it was reported that demand for groupers still outpaces the predicted supply (Sadovy, 2013), indicating that the pressure on grouper populations in the wild, including that of giant grouper, will continue.

Giant grouper aquaculture

Due to the giant groupers' fast growth and high price, there have been investments to develop technologies to breed giant grouper in captivity. Taiwan was the first country to report mass seed production of giant grouper (Ho et al. 1997) followed by China, Indonesia, Thailand and Vietnam (Kongkeo et al. 2010), followed by Malaysia (Cheng et al. 2015), Hawaii (Garcia-Ortega et al. 2012) and Australia (Knuckey 2014). The lack of reliable captive breeding technology and poor larval survival have been the biggest challenges, as a result, culture of giant groupers is still largely dependent on wild-caught fingerlings or juveniles (Rimmer and Glamuzina, 2019).

Another constraint to giant grouper aquaculture has been the extremely low survival rate of larvae. Compared with other grouper species, giant grouper larviculture has low success rates and inconsistent yields. Giant grouper larvae have low survival due to small mouth gape at first feeding; extreme sensitivity of larvae to physical disturbance; and the requirement for mass production of suitable first prey in terms of quality and quantity (Rimmer and Glamuzina 2019). Giant grouper larvae are also highly susceptible to nervous necrosis virus (NNV), with mass mortalities being a common occurrence at the early larval stages (Pakingking et al. 2010).

Giant grouper biology

Prior to this project commencing, there were no published studies regarding giant grouper reproductive biology. The large size at sexual maturity and the solitary nature of mature individuals, except during spawning aggregations, make them difficult to study in the wild (Lau and Li, 2000; Shuk Man and Wai Chuen, 2006). It was presumed that as in other epinephelines, giant grouper is a protogynous hermaphrodite, maturing first as females and then changing into functional males (Sadovy and Liu, 2008). There was also a lack of

knowledge regarding larval development, including digestive physiology. These knowledge gaps impeded the development of reliable breeding technologies for the species.

Key issues addressed by the project

Giant grouper is a high-value, fast-growing species with significant aquaculture potential. In addition to its importance in the LRFFT, it is also an ecologically important member of the reef ecosystem (Mouillot et al. 2014). The outlook for supply of all wild groupers is bleak, as nearly 95% of the coral reefs in Southeast Asia where groupers live are at heightened risk from human activities (Burkett et al. 2011). In the Philippines, which make up part of the Coral Triangle, more than 50% of coral reefs are degraded (Wilkinson 2008) and fishers have reported that giant grouper have disappeared from their catches in the last decade (Lavides et al. 2009, 2016). Captive breeding of giant grouper is therefore crucial to meet market demand, and vital in relieving the pressure on wild stocks.

Reliable full cycle aquaculture production of giant grouper has been most challenging in Vietnam and the Philippines. This limitation is due to a lack of knowledge on their maturation and spawning behaviour as well as low larval survival. Giant grouper farming has been hampered by the high level of investment required to hold and maintain giant grouper broodstock, which in turn has constrained research into this species. This project addressed these bottlenecks by combining resources through a cooperative, multilateral approach. The commissioned institution was the University of the Sunshine Coast (USC) in Australia, in collaboration with Queensland DPI Northern Fisheries Centre (NFC, who were later dissolved and withdrew from the project). In Vietnam, the leading partner was Research Institute for Aquaculture No. 3 (RIA3), in collaboration with RIA1. In the Philippines, the country partner was the Southeast Asian Fisheries Development Center, Aquaculture Department (SEAFDEC/AQD).

Project justification – Australian, Vietnamese and Philippine context

With fishing of giant grouper being unsustainable, captive production is the only sustainable means of meeting the growing demand. The only major producer of captive-bred giant grouper in Asia is Taiwan. Production methods are established and functional; however, they have not been disseminated beyond the Taiwanese facilities. Additionally, they suffer from low larval survival and lack of genetic management, leading to inbreeding. China and Vietnam have resorted to importing fingerlings and broodstock from Taiwan. Since genetic management has not been implemented in Taiwan, there has been no assurance as to the species identity or genetic integrity of the of exported groupers. During the SRA phase of this project (FIS/2012/037), we genotyped broodstock in Vietnam and discovered species misidentification in some of the giant grouper broodstock imported from Taiwan. Another concern of imported groupers from Taiwan is the lack of effective biosecurity in their aquaculture facilities, as observed during the SRA phase of this project, which likely contributes to the widespread occurrence of viral diseases.

This project provided Australia with the opportunity to take a leadership role in the South-East Asian region through collaboration on the development and application of innovative aquaculture technologies. Aquaculture is a growth industry dominated by Asian production and Australia's capacity to compete on large scale market supply of fingerlings or market size fish is unrealistic. However, we do have an opportunity to establish ourselves as technological leaders and feeders of knowledge, expertise, training and innovation to the industry. In Australia, grouper aquaculture is small but very advanced, with The Company One (formerly FinFish Enterprise Pty Ltd, formally NFC) developing technologies and know-how for giant grouper culture and extending these to industry production. Land-based aquaculture farms in Australia are now (2019) producing and selling around 15 tonnes (t)/year of giant grouper on the domestic market and as fingerling exports to Asia.

The techniques developed in this project are beneficial for the development of giant grouper aquaculture in Australia. The outputs can also be applied to other grouper species that will become a priority in the years ahead, adding to the return on investment.

The White Paper 'Australia in the Asian Century' highlighted the impending shift of world economic power to Asia, therefore it is more imperative than ever for Australia to contribute meaningfully to the Southeast Asian economic growth. The paper specifically states that Australia's commercial success in the region requires that highly innovative, competitive Australian firms and institutions develop collaborative relationships with others in the region. With the exception of China, the remaining world aquaculture production occurs within Vietnam, Indonesia, Thailand, Myanmar, the Philippines, India and Japan. This project situates Australia in a position to develop long-term and productive relationship with both the Philippines and Vietnam and to lead the innovative development of technologies allowing for production of a high-value food commodity within key regional players in the aquaculture marketplace. It also contributes to meeting the foreseeable demand for higher quality produce and protein rich foods throughout Asia as household incomes rise with the growing economy.

Priorities for aquaculture development were similar in both the Philippines and Vietnam. One of the priority research areas at SEAFDEC/AQD is 'Quality seed for sustainable aquaculture'. This thematic area addresses issues related to improvement, maintenance and availability of aquatic seed that constrain the growth of the aquaculture industry, not only in the Philippines but also in the wider Southeast Asian region. This research area focuses on needs associated with the environmental and genetic requisites for robust broodstock technology, biomarkers for egg quality and larval survival as well as important culture traits such as fast growth and hardiness. At RIA3/RIA1, the identified short-term priority for marine aquaculture in Vietnam is to develop breeding technologies for high-value aquaculture species while the long-term research priority is improvement of broodstock and seed quality through genetic approaches and selective breeding programs. Production of grouper hybrids is now a priority with the domestic market for this seafood expanding.

Although facilities and production methods are in place in both Vietnam and the Philippines for a variety of other grouper species, giant grouper aquaculture is in its infancy in these countries, and species-specific methodologies and logistics need to be developed to address issues concerning larval rearing, grow-out and broodstock management, as well as genetic management to address inbreeding and future selection programs. There are also issues with genetic bottlenecks and disease vulnerability that prevent the Taiwanese giant grouper industry from providing viable broodstock as a starting point. For these reasons, it was considered prudent to begin the aquaculture of giant grouper in Vietnam and the Philippines from the outset with wild-caught broodstock and modern technologies.

The investment in creating giant grouper production systems in Vietnam and the Philippines provides benefit to these countries on multiple levels. Overall, although China has been the biggest producer of cultured groupers in recent decade, fingerlings and even subadults are still partly sourced from the wild and imported either from Taiwan or from other Southeast Asian countries (reviewed by Rimmer and Glamuzina, 2019). The rapid expansion of hybrid grouper aquaculture justified the investment in giant grouper aquaculture as the fast growing and disease-resistant hybrid, sired by giant grouper males, is significantly increasing profits from marine aquaculture for Vietnamese farmers, increasing the economic value of the industry as a whole. With the successful spawning of giant groupers in the Philippines, a similar scenario is expected in the Philippines. These outcomes will decrease pressures on the remaining wild giant grouper populations and continue to underpin innovative industry developments.

4 Objectives

Aims and objectives

The development aim of this project was to establish a sustainable aquaculture industry for giant grouper in the South-East Asian region.

The specific aims were to:

Objective 1: Develop reliable giant grouper captive-breeding technologies

- Determine earliest age for sex reversal
- Develop protocols for synchronised spawning

Objective 2: Explore the potential of germ-cell transplantation and surrogate technologies as alternative approaches to giant grouper seed production

- Determine age of larvae amenable to germ-cell transplantation and carry out transplantation trials
- Determine optimal stage for collecting germ cells (type A spermatogonia) for transplantation
- Develop cryopreservation protocols for male giant grouper germ cells
- Develop molecular methods to advance sexual maturation, induce spawning and sex reversal

Objective 3: Develop reliable larval-rearing technologies for giant grouper

- Develop optimal larval-feeding techniques
- Characterise larval development using transcriptomics[1] and stable isotopes

Objective 4: Apply genetic approaches to broodstock management

- Apply genetic markers to prevent inbreeding and to enable genetic selection
- Explore hybridisations to achieve desirable traits

Objective 5: Build capacity in the form of knowledge transfer and training in larval rearing, genetics and biotechnology as it applies to giant grouper aquaculture

- Ensure full technology transfer and extension with all project partners

[1]Transcriptomics is a molecular technique that enables the characterisation of the full range of genes expressed in a particular tissue type at a specific developmental stage of an organism.

5 Methodology

This section is presented following the Project Objectives and the Activities within each objective. When outputs/milestones in an Activity are interrelated, the methods are presented as one section. Detailed methodologies for specific experiments are described either in the Appendices or in the published articles directly generated from this project and other publications enabled by this project.

Objective 1: Develop reliable giant grouper captive-breeding technologies

At the beginning of the project, RIA1 in Cat Ba had 70 available giant grouper broodstock ranging in body weight from 32-91 kg (Fig 1A; Table 1 of Appendix 1). Another 20 fish (25-40 kg; 3-4 years old) were purchased for Cat Ba RIA1. In RIA3, 16 wild-caught broodstock (33-62 kg) were procured and stocked in concrete tanks at the Mariculture Research and Development Center (MRDC) (Fig 1B; Table 2 and 3 of Appendix 1). At SEAFDEC/AQD, a total of 24 wild-caught broodstock (9-35 kg) were bought and stocked in cages at Igang Marine Station (Fig 1C; Table 4 of Appendix 1). From these broodstock, the outputs and milestones for Activities 1.1 and 1.2 were achieved (detailed in the sections below).

Fig 1. Giant grouper broodstock at (A) RIA1, (B) RIA3 and (C) SEAFDEC/AQD Igang Marine Station. (Images: A-J. Nocillado; B-T.Q. Thai; C-P. Palma)

Activity 1.1 Determine the earliest age for sex reversal

1.1.1 Sex reversed broodstock through the administration of slow-release methyl testosterone (MT) (Vietnam, Australia)

Sex-reversed broodstock were successfully obtained after MT treatment of broodstock at Finfish Enterprise (FFE), Australia. FFE was subsequently acquired by another private company, The Company One, and their protocols became proprietary; therefore, the method used cannot be described here.

At MRDC RIA3, 5 broodfish (28-39 kg) were reared in 90 m^3 concrete tank and fed daily with trash fish containing MT (Fluka) at 50 mg/kg feed. After 4-5 months, 2 fish (28 and 30 kg) were confirmed as males.

1.1.2 Identification of the earliest point at which successful sex reversal can be induced (Philippines)

Sex reversal was induced with MT in experiments that commenced in December 2015 at SEAFDEC/AQD's Igang Marine Station. MT was administered by feeding via gelatin capsules inserted into fish by-catch and squid supplemented with Vitamin C (0.1%) and Vitamin E (0.05%) (Fig 2A). The control group (n=6) was fed fish by-catch and squid with Vitamins C and E supplement only. All fish were examined monthly for presence of milt by application of slight abdominal pressure or cannulation (Fig 2B, 2C). In addition, mucus

vitellogenin was checked monthly during full moon to monitor sex change or ovarian development.

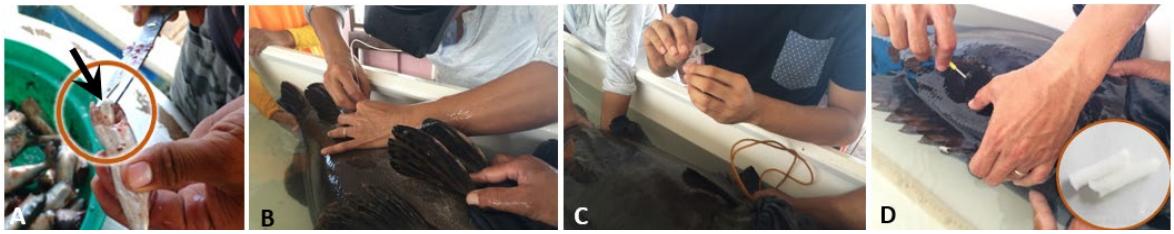


Fig 2. MT administration to induce sex reversal in giant grouper broodstock. (A) Gelatin capsule containing MT being added into feed. Monitoring of gonadal stage by cannulation (B) and application of gentle pressure on the abdomen (C). MT treatment using silastic implants (D). (Images: F. Ayson)

Other modes of administration assessed were injection (1 mg/ kg BW dose) every 15 days and silastic implantation (1 mg MT/kg BW dose) introduced every month for 6 months (Fig 2D). Regardless of the mode of administration tested, fish stopped eating resulting in weight loss; therefore, the experiment was terminated. Fish resumed feeding in about 2 weeks after the treatment was stopped.

Vitellogenin level from mucus samples of experimental and control fish were determined using an enzyme-linked immunosorbent assay (ELISA), developed as part of this project (Palma et al. 2019).

1.1.3 Sex reversal by inhibition of the aromatase enzyme with Fadrozole (Australia)

Research to determine when cypa19a1a (the gene responsible for encoding gonad aromatase in fish) is expressed was conducted. Commencement of cypa19a1a gene expression indicates the biosynthesis of oestradiol (E_2), which is responsible for ovarian differentiation in fish (Guiguen et al. 2010). Expression of gonad aromatase was confirmed by polymerase chain reaction (PCR) in hatchery-bred giant groupers 1 kg in body weight (BW).

Following the confirmation of aromatase expression, an experiment inducing precocious sex change was conducted at FFE, who also kindly provided the experimental fish. The trial commenced on 13 Nov 2014 and concluded on 3 June 2015 (approximately 200 days). The weight of the fish at the start of the trial ranged from 2442 -3791 g with an average weight of 3021 g.

Three experimental groups, each with 12 fish, were allocated across six tanks. Twelve fish received a treatment of Fadrozole via intramuscular cholesterol implant (Lee, et al., 1986) at 1 mg.kg BW and a second group of 12 received 3 mg.kg BW. The final group was a control (12) and was given a blank cholesterol implant. All treatments were run as duplicates with six fish per tank, two tanks per treatment.

Biometric data (weight and length) and blood sampling (caudal vein) were conducted at commencement (Day 0) to establish the baseline. At 50-day time points, one fish per tank was sacrificed and tissues preserved for histological, physiological and molecular analysis. Brain, pituitary, liver and gonad samples were collected for molecular and histological analysis. Re-implantation of the remaining fish was carried out at each time point. At the conclusion of the experiment, the remaining fish were weighed, sacrificed, and tissue samples were collected as in the 50-day time points. Gonadosomatic index (GSI) was determined from the ratio of the gonad weight to the total body weight.

Plasma levels of E_2, T and 11-KT were determined using commercially available ELISA kits (Cayman Chemical, USA). Steroids were extracted from 200 µl plasma with diethyl

ether. Gonad tissue samples for histology were fixed in 10% neutral buffered formalin or 4% paraformaldehyde overnight then transferred to 70% ethanol for storage. Samples were embedded in paraffin, sectioned (5-7 µm) and stained with Haematoxylin and Eosin (H&E) following standard protocols.

Transcriptomics was employed to determine differentially expressed genes in the gonads of fish that were developing either as female or transitioning into male (Table 1). The protocols for RNA extraction, transcriptome sequencing, quality verification of the transcriptomic data, assembly, gene and protein annotations and determination of differentially expressed genes followed those described by Bar et al. (2016) and the references therein.

Table 1. Gonad status and biometrics of fish where samples were taken for differential gene expression analysis. Gonad status was based on histological observation. SSC= spermatogenic cyst.

Sample	Treatment	Gonad status	Weight (g)	Length (mm)
4.2	Control	Female	3279	520
4.4	Control	Female	3986	512
3.4	1mg FAD	Female with SSC	3415	552
6.1	1mg FAD	Female with SSC	3797	560
2.6	3mg FAD	Mid-Transition to male	4788	578
5.3	3mg FAD	Late-Transition to male	3932	550

In addition to transcriptomics, real-time quantitative PCR (QPCR) was employed to characterise the expression level of female-specific genes gonad aromatase (*Cyp19a1a*) and forkhead-box L2 (*foxl2*) transcription factor, and the male-specific gene double-sex and mab-3 related transcription factor 1 (DMRT1). The validated QPCR gene-specific primers and probes for each gene are shown in Table 5 (Appendix 1). QPCR analysis followed the absolute quantification method (Wong and Medrano 2005).

Activity 1.2 Develop protocols for synchronised spawning

1.2.1-1.2.3 Synchronised spawning, optimal sex ratio and nutritional supplements for giant grouper broodstock (Vietnam, Philippines, Australia)

General husbandry procedures

Broodfish were reared either in concrete tanks (180 m^3) or sea cages (50m^3) and fed with trash fish (mainly pilchards and scad) (size 100-200 g/fish) and squid (size 70-200 g/individual) every day. Broodstock feeds were supplemented with vitamin C (60 g/kg trash fish) and E (3600 mg/kg trash fish), minerals (5 g/kg trash fish) and omega 3,6,9 fatty acids (Alaska) (1%/kg trash fish) every 3-5 days. Seawater temperature in Cat Ba ranged from 25-29°C.

In tanks, culture water was changed 50 – 100% every 5 days. Tank water parameters were 27-28°C and 32-33‰ salinity. Cages were cleaned of debris and attached growing organisms once or twice a month.

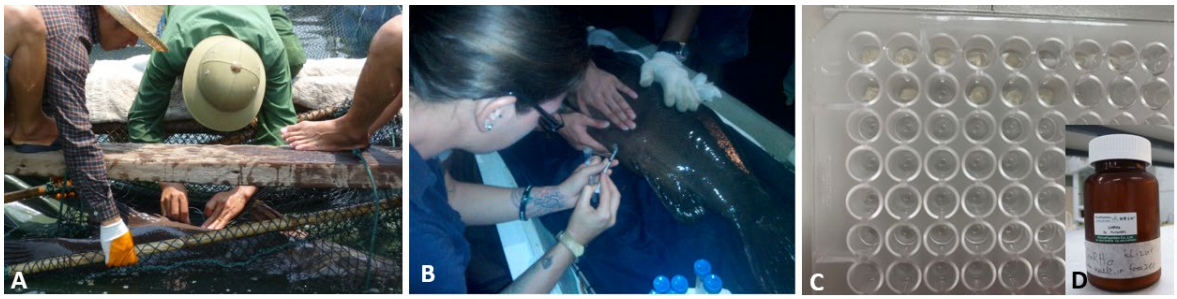


Fig 3. Conducting a biopsy on female giant grouper in Cat Ba RIA1 (A); collecting milt from male giant grouper broodstock in SEAFDEC Igang Marine Station (B); implants prepared at USC (C) containing GnRHa (D). (Images: A,C,D – J Nocillado; B – K. Anderson)

Hormonal induction of spawning

To determine timing for hormonal induction, broodstock were examined monthly, 4-5 days before the full moon (Fig 3A), as giant groupers spawn by the lunar cycle. Maturity in males was confirmed by the release of milt through the gonadal pore when gentle pressure on the abdomen was applied (Fig 3B). Gonadal biopsy was conducted on females to collect oocytes for observation and measurement. Oocyte diameter was determined under a microscope. Mean oocyte diameter of greater than 400 µm indicates readiness for spawning induction.

Slow release implants containing gonadotropin-releasing hormone (GnRHa) were used to stimulate spawning at a dose of 40-60 µg/kg in females and 20-30 µg/kg in males. GnRHa implants were prepared at USC laboratories (Fig 3C, 3D). An established GnRHa ELISA was used to assess the release rate of the implants (Mylonas and Zohar, 2007). A commercially available salmon GnRHa (Syndel, USA) in pellet form and human chorionic gonadotropin (hCG) were also trialed for spawning induction.

Sex ratios tested for spawning in sea cage and concrete tanks were: 1 male and 1 female; 2 males and 2 females; 2 males and three females.

Objective 2: Explore the potential of germ-cell transplantation (GCT) and surrogate technologies as alternative approaches to giant grouper seed production

This objective was aimed at producing giant grouper gametes in a “smaller, faster-maturing surrogate grouper broodstock”. The general methods include identifying a suitable host, determining when the host larvae is amenable to transplantation and then optimising the transplantation protocol.

Activity 2.1 Determine sterility in hybrid grouper (Vietnam, Philippines)

It has been previously demonstrated that functional donor gametes can be generated in a sterile host (Okutsu et al. 2007; Li et al. 2017; Xu et al. 2019). Sterility can be achieved using two traditional approaches: hybridisation or triploidy. Hybrid giant grouper x tiger grouper and hybrid giant grouper x orange-spotted grouper were produced by fertilising tiger grouper and orange-spotted grouper eggs with giant grouper sperm. Hybrids were examined by dissection to determine extent of gonadal development. The giant x tiger hybrids were 3-15 kg in body weight (1-4 years old) while the giant x orange-spotted hybrids were 2.5 kg in body weight (about 1.5 years old).

Triploidy is being developed for small honeycomb grouper *E. merra,* where “cold shock” is being applied directly after fertilisation of the honeycomb grouper eggs, producing triploid fish.

Activity 2.2 Determine age of larvae amenable to germ-cell transplantation (Australia; Philippines)

2.2.1 Appropriate stage at which germ cells can be introduced to host larvae

Orange spotted and tiger grouper larvae were collected daily from 3 to 15 days after hatching and fixed for histological examination following standard procedures. Serial sections (5 µm) were stained with haematoxylin and eosin (H&E) and examined under a compound microscope. Germ cells migrating towards the anterior of the peritoneal cavity of the larvae were identified from serial sections of larvae oriented crosswise.

2.2.2 Optimised protocol for germ cell transplantation and transplantation trials

The germ cell transplantation (GCT) protocol developed for Southern bluefin tuna (Bar et al. 2016) was optimised for groupers. To test the suitability of host larvae for injections and to determine compatibility between species, GCT experiments utilising donor cells from orange spotted grouper were carried out using both giant grouper and orange spotted grouper larvae as hosts, using methodologies detailed in Takeuchi et al. (2003) and Yoshizaki et al. (2010). This was the inverse donor-host relationship from what was desired however it was a way to validate the GCT methodology prior to sacrificing male giant grouper. Orange spotted grouper donor germ cells were prepared from fresh testis for each batch and labelled prior to transplantation with the fluorescence dye PKH26 (MINI26-1KT; Sigma) at a concentration of 10 µM. Larvae were collected from the tank and anaesthetized with ethyl 3-aminobenzoate methanesulfonate salt (A5040; Sigma-Aldrich) in seawater. Each larva was then transferred using a wide bore Pasteur pipette to a petri dish coated with 4% agar for transplantation. Germ cells in L-15 were collected from a deep cavity slide and transplanted to the peritoneal cavity of the larvae, under a dissecting microscope. Transplantation needles were prepared by pulling glass capillaries (GD-1; Narishige, Tokyo, Japan) using an electric puller (PC-10; Narishige). The tips of the needles were sharpened with a grinder (EG-400; Narishige) to an angle of 35°, with an opening of 40-60 µm. The needle was attached to a micro-injector (1M-9B; Narishige) and was directed to the larvae peritoneal cavity using a micro-manipulator (M3301R; World Precision Instruments). After cell transplantation, the larvae were transferred to a recovery tank inside a 900 L larval rearing tank or directly back into a dedicated larval rearing tank. Control larvae (i.e. non-transplanted larvae) were kept in their original larval rearing tanks.

Activity 2.3 Determine optimal stage for collecting germ cells (spgA) for transplantation (Australia)

Molecular markers for primordial germ cells and spermatogonia A

Primordial germ cells are the precursors to gametes; however they are difficult to isolate and low in numbers in mature fish. In contrast, spermatogonia type A (spgA) are abundant in mature male fish and are able to migrate through the genital ridge, colonise the primordial gonad, retain cell plasticity and differentiate into functional gametes within the host (Okutsu, et al., 2006). As such, a study was directed towards developing molecular markers for spgA (methods described in 1.1.3 milestone of Objective 1).

Activity 2.4 Develop cryopreservation protocols for male giant grouper primordial germ cells (Philippines)

Standard cryopreservation protocols for indefinite viable preservation of germ cells

Due to the rarity of male giant grouper, a protocol was tested using tiger grouper testis. Gonad tissues (800-1000 mg) were minced in a glass cavity slide using Wecker scissors.

Minced tissue was transferred into 2 mL cryogenic vials with 1 mL of cryopreservation media containing:

- 1.5% (v:v) bovine serum albumin (Sigma, A2153)
- 100 mM trehalose dihydrate (Sigma, T9531)
- 9% (w:v) dimethyl sulfoxide (DMSO) (Sigma, D4540)

Vials were placed into a pre-chilled (4°C) BioCell (Nihon Freezer Co. Ltd, Japan) and transferred to -80°C for 90 min, before long term storage into liquid nitrogen.

Sperm cryopreservation

To address the constraint regarding the lack of male giant groupers for breeding trials, the use of cryopreserved milt was evaluated. The cryopreservation protocol by Fan et. al (2014) was modified. Briefly, MPRS (Ringer solution) diluent was mixed with 10% DMSO, where sperm was added at 1:1 ratio by volume. The diluted sperm was dispensed into cryovials at a volume not exceeding 1.0 mL. Cryovials were suspended 6 cm above the surface of liquid nitrogen for 10 min, and then on the surface for 5 min, and finally immersed in liquid nitrogen for storage. When frozen sperm was used for quality evaluation or hybridization trials, it was thawed at 37°C in a water bath for 2 minutes.

Initial trials on cryopreservation were done using tiger grouper sperm. Fish were sampled and gonadal biopsy was performed. Spermiating males with milt score of +2 or +3 were injected with hCG (1000IU/kg BW). Stripping followed 36 hours after induction (Fig 4A). A collector was specially designed to prevent air and water exposure of the collected sperm and also to prevent the sperm being sucked in by the person performing the collection (Fig 4B-D). Motility test was performed by suspending 1 µL of thawed sperm in 199 µL of seawater. Sperm motility was calculated as the percentage of progressively moving sperm. Those that did not show forward movement were not considered motile. Only sperm with ≥80% motility was used in the experiments. Sperm viability test was determined by adding 30 µL of eosin-nigrosin dye and 30 µL of the thawed sample on a pre-labelled glass slide (NAFA and ESHRE-SIGA, 2002). The mixture was incubated for 30 sec then a smear was made, air-dried and viewed under oil immersion objective. Viability was assessed by estimating the percentage of viable (white) and non-viable (pink/red cells) (Fig 4E).

For cryopreservation at -80^{o}C biofreezer, similar preparation of MPRS-DMSO (9:1 v/v) was used but with different concentrations of Ficoll 70 (0%, 5%, 10%, 15%). Ficoll 70 preserves cellular integrity by effectively inhibiting ice recrystallization at -80^{o}C (Yuan et al., 2016). After mixing grouper sperm into equal volume of MPRS-DMSO in cryovials, samples were immediately placed in the biofreezer.

Fertilization capacity was used as an additional indicator of sperm quality. Artificial fertilization was done by gently mixing 100 µL post-thaw extended sperm with 2 mL eggs (~3000 oocytes) for 3 min. Twenty mL of fresh seawater was added, mixed and incubated for 10 min. Fertilized eggs were washed four times and immediately transferred to 4-L incubation tanks with gently aerated seawater as described in Kiriyakit et al. (2011).

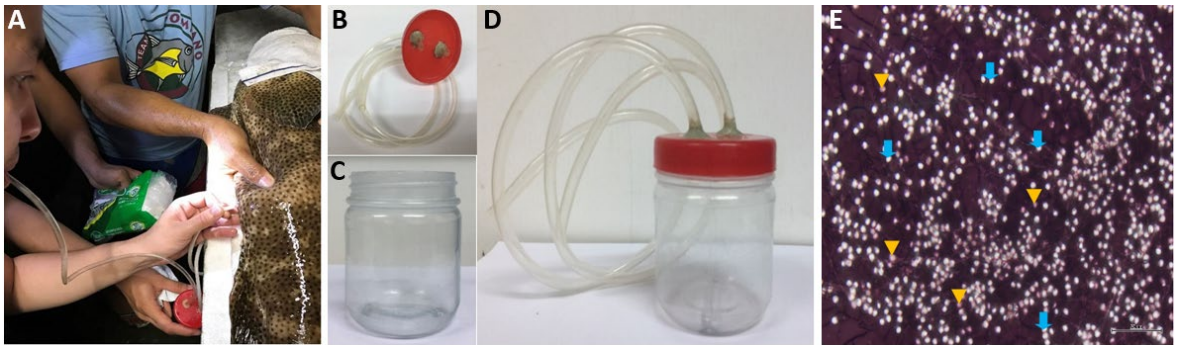


Fig 4. Collection of sperm from tiger grouper (A). Sperm collector designed by partners at SEAFDEC/AQD (B-D). Viable sperm (white, blue arrows) and non-viable sperm (pink/red; yellow arrowheads) (E). (Images: J. Superio)

Activity 2.5 Develop molecular methods to advance sexual maturation, induce spawning and sex reversal (Philippines, Australia)

Oral delivery methods for the introduction of genes encoding for reproduction-related peptides and aromatase inhibitors

Synthetic molecular technologies were applied to generate recombinant hormones, either in protein or DNA form, that comprise the key hormonal regulators of the giant grouper reproductive axis. The recombinants included the mature gonadotropin-releasing hormone (GnRH) decapeptide, and single chain follicle stimulating hormone (FSH) and luteinizing hormone (LH). These hormones are known to control various reproductive processes in fish and have been used to overcome reproductive dysfunctions associated with the rearing of broodstock in captivity, where the natural cues for maturation and spawning are diminished or are altogether lacking (Zohar and Mylonas, 2001). The recombinants we produced in protein form using the yeast (*Pichia pastoris)* expression system are either secreted from the yeast (FSH and LH) or contained within the yeast cells (GnRH). Detailed procedures are described in our publications (Palma et al. 2019b; Nocillado et al. 2019). The DNA sequences encoding for the hormones were obtained from the transcriptomic data derived from Objective 1, Milestone 1.1.3. Expression constructs were designed, synthesised and introduced into the yeast. Bioactivity of generated recombinants was confirmed in both *in vitro* and *in vivo* assays.

Recombinant hormones in DNA form (FSH and LH) were generated by inserting the encoding sequence of the gene of interest into an expression vector that enables it to be transformed into the active protein form once the construct is introduced *in vivo* (Palma et al. 2019c). We developed methods to deliver the recombinant hormones in DNA form via the oral route in collaboration with Prof Ming-Wei Lu of the National Taiwan Ocean University (outlined in our published paper: Palma et al. 2019c). For the oral delivery of recombinant GnRH contained in yeast, the lyophilised yeast cells are simply mixed with feed (Palma P, Nocillado J et al. *in preparation*).

In addition to generating the recombinants, we developed enzyme-linked immunosorbent assays for FSH, LH and vitellogenin (VTG) (described in Nocillado et al. 2019; Dennis et al. *in preparation* and Palma et al. 2019a, respectively). These tools were used to measure the circulating levels of these proteins and helped in understanding the reproductive biology of giant grouper.

Objective 3: Develop reliable larval-rearing technologies for giant grouper

Activity 3.1 Develop optimal larval feeding techniques (Australia, Vietnam, Philippines)

3.1.1 Determination of the benefit, if any, of the inclusion of copepods in the early larval diet of giant grouper, as compared to rotifers (Australia); 3.1.2 Determination of the benefit, if any, of the inclusion of copepods for extended periods during larval development

The benefit of adding copepods to a rotifer diet at the first feeding of giant grouper larvae was assessed at FFE. Hatched larvae (from 30 eggs/L) were stocked following standard grouper rearing procedures. The experiment tested 3 treatments with 4 replicates. The three treatments were as follows: (1) rotifers only; (2) rotifers + copepods at 4 ind/ml; and rotifers + copepods at 10 ind/ml. Algae were added to all tanks daily throughout the experimental period at a ratio of 50:50 T.ISO:Nannochloropsis. All 12 tanks were stocked with SS-rotifers (10/mL) at day 2. Copepods were added as a single addition immediately after rotifers were added for Treatments 2 and 3. Live prey was counted twice daily during the morning and afternoon and rotifer numbers were maintained at ≥20/mL after day 2; unenriched until water exchange on day 6 and enriched thereafter. The experiment lasted for 12 days and samples were taken at Days 3, 6, 9,12 were fixed in formalin for length measurement. On day 12, larvae in each tank were counted for survival analysis.

In RIA1, copepods were cultured in ponds and fed to grouper larvae from days 8-20 post-hatch. Copepods were cultured in outdoor ponds (500-1500 m^3) where water temperature ranges from 20-29°C, salinity 20-30 ppt, and pH 7.5-8.5 (Fig 5A). Pond water was inoculated with a mixture of microalgae (*Nanochloropsis oculate, Dunaliella tertiolecta, Chaetoceros calcitrans, Isochrysys galbana, Tetraselmis sp*), maintaining a density of 10^4/ml. The diameter of copepods at early stages were small enough for the small mouth gape of giant grouper and hybrid larvae (Fig 5B, C).

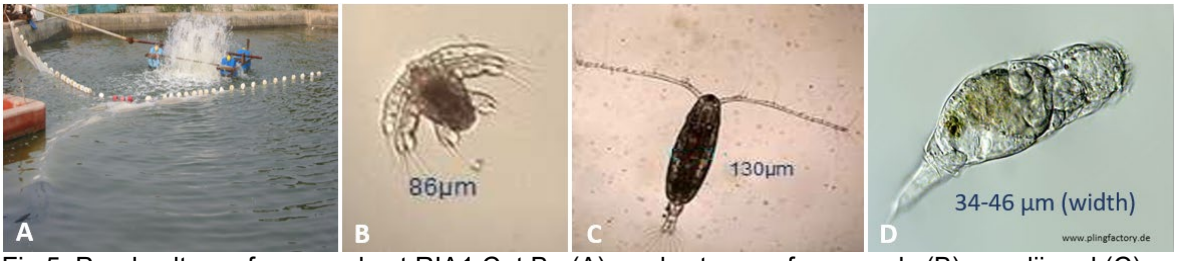


Fig 5. Pond culture of copepods at RIA1 Cat Ba (A); early stages of copepods (B) nauplii and (C) copepodite; *Proales similis* (D) (Images: Cao Van Hanh)

Proales similis, a minute rotifer, was evaluated at SEAFDEC/AQD as a first feed for giant grouper larvae (Fig 5D). *Proales similis* has a length of 83±11 µm and body width of 40±6 µm, making it 38% smaller and 60% narrower than SS-type rotifer *Brachionus rotundiformis*, and is therefore a good candidate for fish larvae with very small mouth gapes (Wullur et al. 2009). Experiments were conducted to determine the benefit of adding *Proales similis* as first feed to giant grouper larvae. Giant grouper larvae were stocked at 20 ind/L. Treatments were (a) Brachionus only, (b) Brachionus + Proales, and (c) Brachionus + mixed copepods. Initial feeding of Brachionus (≤60 µm) started at day 1 at 1 ind/ml. Proales (0.2-1 ind/ml) was fed in the morning from day 2 until day 10. Copepod supplementation (0.1-.05 ind/ml) was done from day 2 to day 10. Because Proales are primarily benthic, modified trials were conducted supplying Proales either twice a day or via a drip method. Growth and survival rates were used to assess the benefit of the minute rotifer.

Activity 3.2 Characterise larval development through transcriptomics and stable isotopes (Australia)*

3.2.1 Identification of the genes of the digestive system expressed by giant grouper larvae throughout their developmental stages

There is a lack of knowledge regarding larval developmental physiology in giant grouper particularly relating to ontogenesis of genes that regulate appetite and digestion. This complicates delivery of appropriate larval food. To address this issue, a transcriptomic study was conducted on giant grouper larvae between 1 and 14 days post-hatch. This was conducted in collaboration with The Company One, formerly FFE, who provided the larval samples (Outlined in our published paper, Anderson et al. 2018a). Transcriptomics analysis was also carried out on orange spotted grouper in larvae at days 12, 18 and 50 post-hatch to obtain further insight regarding grouper digestive development (Anderson et al. 2018b); the transcriptomic data for these were provided by our collaborator from Taiwan. Detailed methods are described in Anderson et al. (2018a, 2018b) (Appendix 2, 3).

3.2.2 Identification of the feeding habits of giant grouper larvae through DNA barcoding (This aim replaced the study using stable isotopes; variation was approved.)*

Mixed copepod samples, as well as other invertebrates for analysis have been collected from various sources, including Port Stephens Fisheries Institute in New South Wales, brackish water earthen ponds in Dumangas, Iloilo, Philippines, RIA3&1 in Vietnam and FFE in Cairns. Several DNA extraction methods were tested to enable extraction from individual copepod samples and among those tested, the One-Tube Tissue DNA Extraction Kit (Bio Basic, Canada) enabled extraction of DNA from all single whole copepods tested according to manufacturer's instructions, except step 3 was substituted for a 56 °C incubation for 10 min. PCR optimisation was then conducted using degenerate Cox1 primers (Table 2). Each possible primer combination was tested using DNA extracted from adult copepods collected in the Philippines. The PCR reaction contained 1.25 µL 10× PCR buffer, 0.25 µL 10 mM dNTPs, 0.5 µL 50 mM $MgCl_2$, 250 nM forward primer, 250 nM reverse primer, 0.05 µL TAQ, 0.7 µL DNA, and molecular grade water to a final volume of 12.5 µl. 'Touchdown' PCR cycling conditions were used which consisted of: initial denaturation at 94 °C for 60 s, then 14 cycles of: denaturation at 94 °C for 20 s, annealing at 60 °C (-1 °C per cycle), and extension at 72 °C for 60 s, followed by 27 cycles with an annealing temperature of 46 °C, and a final extension at 72 °C for 10 min. Using these parameters, DNA amplification was observed for some samples using the primer combinations mlCOIintF + LEP-R1, mlCOIintF + HC02198, and LEP-F1 + LEP-R1. PCR optimisation was then achieved by adjusting the PCR reaction components to: 2.5 µL 10× PCR buffer, 0.5 µL 10 mM dNTPs, 1.0 µL 50 mM MgCl2, 500 nM forward primer, 500 nM reverse primer, 0.1 µL TAQ, 2.0 µL DNA, and molecular grade water to a final volume of 12.5 µl. When the optimised reaction composition was used in combination with the touchdown cycling conditions above, significant Cox1 amplification was observed for all copepods tested from the Philippines using the primer combinations mlCOIintF + LEP-R1 and mlCOIintF + HC02198, but not LEP-F1 + LEP-R1

Table 2. Universal primers for the PCR amplification of Cox1 mitochondrial gene

Primer	Direction	Sequence (5' to 3' direction*)	Source
LCO1490	F	GGTCAACAAATCATAAAGATATTGG	Folmer et al. 1994
HC02198	R	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. 1994
LEP-F1	F	ATTCAACCAATCATAAAGATATTGG	Watson et al. 2015
LEP-R1	R	TAAACTTCTGGATGTCCAAAAAATCA	Watson et al. 2015
mlCOIintF	F	GGWACWGGWTGAACWGTWTAYCCYCC	Leray et al. 2013
jgHCO2198	R	TAIACYTCIGGRTGICCRAARAAYCA	Geller et al. 2013

*Degenerate base codes: W = AT, Y = CT

In order to eliminate the possible contamination by grouper DNA in prey DNA collected from the gut, a blocking primer strategy was implemented during PCR amplifications (Leray et al. 2013). This is illustrated in Fig 6.

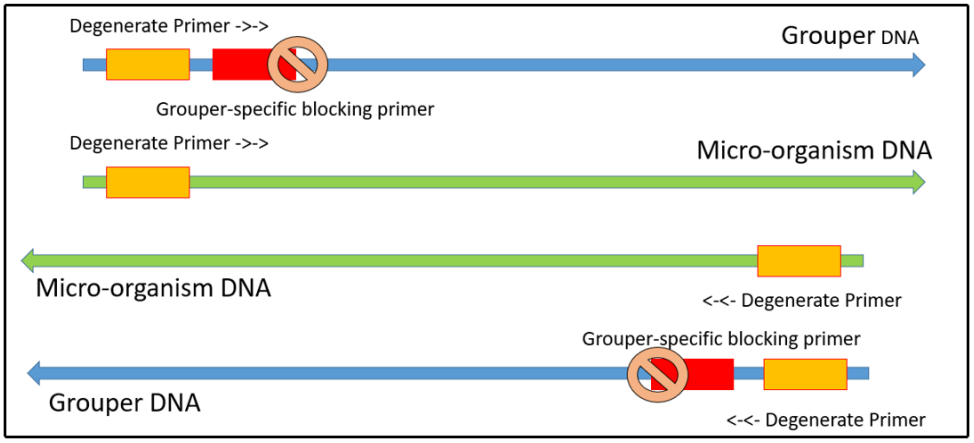


Fig 6. Diagram of PCR primers for the amplification of COX1 from organisms ingested by grouper larvae. The degenerate primer (yellow) can anneal to both grouper DNA and DNA of eaten food organisms. Amplification cannot proceed with grouper DNA template due to the grouper-specific blocking primer (red).

Objective 4: Apply genetic approaches to broodstock management

Activity 4.1 Apply genetic markers to prevent inbreeding and enable genetic selection (Australia, Vietnam, Philippines)

4.1.1 Genetic markers for identification of parentage and pedigree establishment; identify additional microsatellite markers to have at least 16 that can be used in a multiplexed reaction; 4.1.2 Implement of a genetic management program at the three sites; 4.1.3 Implement a genetic program for selection of improved giant grouper

Previously reported microsatellite markers in giant grouper (Rodrigues et al. 2011; Yang et al., 2011; Zeng et al. 2008) were validated by touchdown PCR using M13-labelled primers (Schuelke 2000). The validated microsatellite markers were utilised to genotype giant grouper broodstock in Vietnam, the Philippines and Australia. The applicability of the markers for parentage assignment was confirmed in giant grouper spawns from FFE. Detailed methods describing the validation of the microsatellite markers and data analysis are outlined in our published paper, Bright et al. (2016a; Appendix 4).

Studies on genetic selection focused on resistance to the viral nervous necrosis (VNN) disease, which is a serious threat to grouper aquaculture (Pakingking et al. 2010). Nervous necrosis virus (NNV) sequences available from public databases, as well as NNV sequences isolated from grouper samples collected from Vietnam and Taiwan, were analysed to determine (a) if there is a genetic basis for resistance to NNV and (b) whether there are location-specific specific strains of NNV. Sequence isolation methods and analyses of sequences are described in our published paper, Knibb et al. (2017, Appendix 5).

Activity 4.2 Explore hybridisations to achieve desirable traits (Vietnam, Philippines)

4.2.1 Tiger grouper and giant grouper hybrids with increased disease resistance; 4.2.2 Orange spotted (aka gold spot grouper) and giant grouper hybrids that mature faster without further intervention

Successful hybridisation between male giant grouper and female tiger grouper was achieved by the country partners in Vietnam. RIA3 has 60 mature tiger grouper broodstock (>4 years old; 46 females) and another 100 potential broodstock of more than 2 years old (October 2019). RIA3 also has 16 giant grouper broodstock, of which 3 are mature diandric males and 2 were sex reversed from female with MT. Tiger groupers spawn from February to September during the new moon. Females are considered ready for spawning induction when their oocytes have an average diameter of 600 µm. To induce spawning, females were treated with 2 injections of hCG at a dose of 500 IU/kg for the first treatment, followed by 1000 IU/kg for the second treatment (administered 18 hrs apart). Eggs were stripped 48-50 hrs after the second injection (Fig 7A). When used as a source of sperm, a male giant grouper was injected once with hCG at 500 IU/kg. Sperm was collected 24 hrs post treatment by applying pressure on the abdomen (Fig 7B, C). Once collected, eggs and sperm were mixed manually with a feather to enable fertilisation (Fig 7D).

Fig 7. Standard protocol for hybrid grouper production in RIA3 MRDC: (A) stripping of eggs from female tiger grouper; (B) collection of sperm from male giant grouper; (C) tubes with collected giant grouper sperm; (D) mixing of eggs and sperm for fertilisation. (Images: T.Q.Thai)

Fertilised eggs were incubated in fiberglass tanks at a density of 25-30 eggs/L in full seawater (30-32 ppt) and at a temperature of 28-29°C. Dissolved oxygen (DO) was maintained at more than 5 mg/L and pH is from 7.0-8.0. Using these standard procedures, fertilisation rate ranges from 47-75% while hatching rate was from 56-80%. Fertilised eggs can also be sold directly to nursery farmers.

Larval rearing until the fry stage was conducted in concrete tanks or lined tanks (8-12 m^3). Larvae were stocked at a density of 10-12 individuals/L (Fig 8A). Details of microalgae tank conditioning, live and pellet food throughout the nursing stage are shown in Table 3. Water parameters were: salinity 30-32 ppt; temperature 28-29°C; DO >5mg/L; pH 7-8. Daily water exchange was at 10% from days 10 to 20 and 50-100% from day 21 to 40.

Grading was performed once a week until the fry stage when they were 1.5-2 cm in length.

Table 3. Feeds provided to hybrid groupers until day 35 post-hatch

Days post hatching	Feeding	
	Food	Density
1-20	Microalgae (*Nannochloropsis*)	$3x10^5$ CFU/ml
1-20	Rotifer nauplii	5-12 ind/ml
18-30	Artemia nauplii	8-10 ind/ml
25-35	Biomass Artemia	
25-35	Pellet food NRD 2-3 (200-300 µm)	
27-35	Pellet food NRD 5-8 (500-800 µm)	

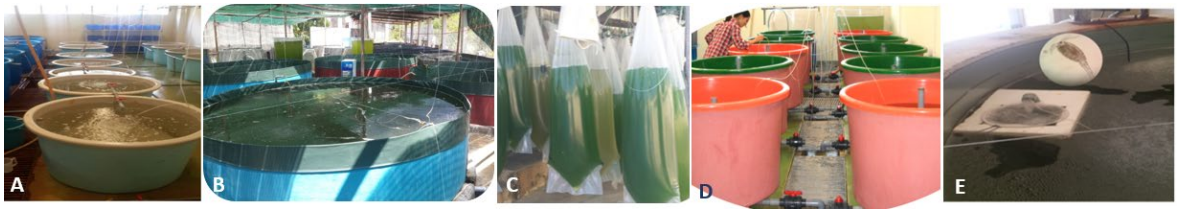


Fig 8. Larval and fingerling production of grouper hybrids. Hatching tanks for fertilised hybrid grouper eggs (A); lined tanks for rearing until the fry stage (B); microalgal cultures for larval rearing tank conditioning (C); monitoring live food cultures (D); copepod culture in tank (E) (Images: T.Q. Thai)

Culture of hybrids from fry to fingerling stage was carried out in concrete or lined tanks (8-12 m^3) at a stocking density of 0.5-1 ind/L (Fig 8B). Cultures of microalgae (Fig 8C), Artemia biomass (Fig 8D) and copepods (Fig 8E) supported the larval and fingerling production. Fry were fed with biomass Artemia as well as pellet food, namely: NRD8 (800 µm); NRD-Inve P16 (1600 µm) at 5-7% of total body weight. Water was changed 100% daily. Water parameters were: salinity 30-32 ppt; temperature 28-29°C; DO >5 mg/L; pH 7-8. Grading was conducted once a week. At the end of this phase, the fingerlings or juveniles were 5-7 cm in length and ready for sale to grow-out farmers.

The larval rearing protocol in RIA1 has minor differences compared to that applied in RIA3. Fertilized eggs were hatched in 1 m^3 indoor tank provided with continuous aeration. Hatched larvae were then transferred to 500 m^2 outdoor concrete tank. After about 4 weeks, the larvae were transferred to 8 m^3 indoor tanks. Size grading was done during transfer to minimise the incidence of cannibalism, which is high at this stage. The food supply to larvae and water management schedule during larval rearing are shown in the tabular diagram below (Fig 9):

Crosses between male giant grouper and female orange spotted grouper have been produced in both Philippines and Vietnam. The production procedures are similar to those for producing the male giant grouper x female tiger grouper hybrid.

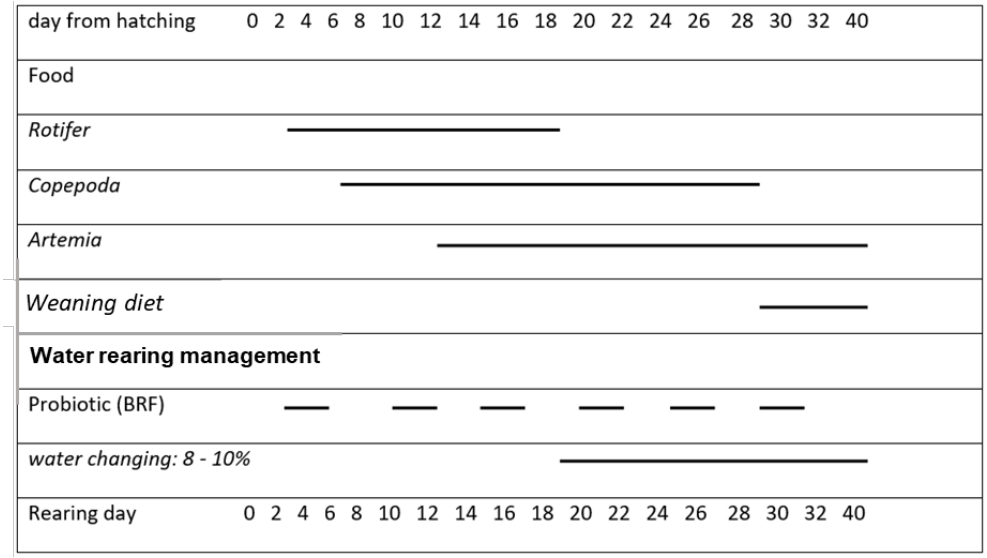


Fig 9. Diagram of the feeding and water management regimes for larval rearing of hybrid grouper in Cat Ba RIA1.

4.2.3 Socio-economic impact studies of the hybrid grouper industry in Vietnam (Vietnam, Australia)

This additional milestone was accomplished during the one-year extension of the project. A bio-economic analysis of the hybrid grouper industry in Vietnam was conducted to determine the profitability of the farms, given the rapid rise of the industry in the last 2-3 years of the project. A survey method was utilised together with bioeconomic modelling, which is commonly used in fisheries and aquaculture as a tool to evaluate changes in technology and policy on natural resource use and household welfare (e.g. Affholder et al. 2010; Anderson & Seijo 2010; Petersen et al., 2013; Sinh et al. 2003). Detailed methods of the bioeconomic survey are described in Dennis et al. (under revision) (Appendix 6).

In addition to the bio-economic study, the community impacts of the hybrid grouper industry in Vietnam was assessed using a photovoice study. Photovoice is an emerging visual social research methodology involving photography, and in the context of grouper farming, enabled the farmer to directly tell their stories (Pierce, 2018). The full report of the photovoice study is shown in Appendix 7.

6 Achievements against activities and outputs/milestones

Objective 1: To develop reliable giant grouper captive-breeding technologies

no.	activity	outputs/ milestones	completion date	comments
1.1	Determine earliest age for sex reversal	Sex-reversed broodstock through administration of slow-release methyl testosterone (MT)	Dec. 2014	Using MT implants, sex reversal was achieved at FinFish Enterprise (FFE) and at RIA3. *A, PC Vietnam*
		Identification of the earliest point at which successful sex reversal can be induced in the giant grouper	Dec. 2015	Trialled MT in 9-26 kg fish; evidence of reduced estrogen activity was observed, however the experiment had to be terminated because the fish ceased to feed whenever MT was administered. From continued monitoring, proof of diandry in giant grouper was obtained **(Palma et al. 2019a)**. *PC Philippines.*
		Sex reversal by inhibition of the aromatase enzyme	Dec. 2016	In a trial at FFE that lasted 200 days, giant groupers (4-5 kg) treated with the aromatase inhibitor Fadrozole (1-3 mg/kg doses) transitioned to males **(Bright et al. 2016b)**. Evidence of diandry in giant grouper was obtained from the Control group of this experiment. *A.*
1.2	Develop protocols for synchronised spawning	Synchronised spawning of male and female giant grouper broodstock	Dec. 2015	Spawning consistently observed after GnRHa implantation at SEAFDEC/AQD; spawning at RIA3&1 achieved with similar hormonal treatment; diandry was also observed among the maturing giant groupers (data are included in **Palma et al. 2019a**). *A, PC Vietnam, Philippines.*
		Optimal sex ratio for successful spawning and fertilisation	Dec. 2016	A ratio of 2 males and 3 females were found optimal at FFE. A 1:1 female to male ratio did not result in spawning. *A, PC Vietnam, Philippines*
		Nutritional supplements for improved reproductive performance	Dec. 2016	Broodstock diet is supplemented with vitamins, minerals and PUFAs. Fresh squid and fish by catch are fed prior to and during spawning. *PC Vietnam, Philippines.*

PC = partner country, A = Australia

Objective 2: To explore the potential of germ-cell transplantation and surrogate technologies as alternative approaches to giant grouper seed production

no.	activity	outputs/ milestones	completion date	comments
2.1		Confirmation of sterility in hybrid grouper	June 2019	Hybrids were grown and then gonads were dissected and examined visually and histologically. Tiger x orange-spotted grouper hybrids were devoid of gonads even at 2.5 kg body weight. Gonadal development was not observed in 3-15 kg giant x tiger hybrids. *PC Vietnam, Philippines*
2.2	Determine age of larvae amenable to germ-cell transplantation	Appropriate stage at which germ cells can be introduced to host larvae	June 2015	Serial sections of potential host larvae (between 8 and 15 days post-hatch) were analysed to determine migration of primordial germ cells. Results indicated that the window is 9-12 days after hatching for tiger grouper and orange spotted grouper larvae. *A.*
		Optimised protocol for germ-cell transplantation and carry out transplantation trials	June 2016	The germ cell transplantation protocol developed by the Elizur lab for yellowtail kingfish as surrogate for Southern bluefin tuna was optimised in tiger grouper and orange spotted grouper larvae as hosts. Transplanted germ cells were observed in host larvae 22 days post-transplantation, indicating applicability of the protocol. *A, PC Philippines*
2.3	Determine optimal stage for collecting germ cells (spermatogonia A) for transplantation	Molecular markers for primordial germ cells and spermatogonia A	Dec. 2015	Markers were identified by transcriptomics. Germ cell markers found were Vasa, Nanos$^{1}/_{2,}$ Notch1, CxCR4, WT1a/b, LY75; spermatogonia A marker: DND. *A.*
2.4	Develop cryopreservation protocols for male giant grouper primordial germ cells.	Standard cryopreservation protocols for indefinite viable preservation of germ cells	Dec. 2017	Confirmed viability of sperm stored at 4°C and -80°C. Stocks of giant grouper, tiger grouper and orange spotted grouper sperm are also cryopreserved in liquid nitrogen **(Palma et al. 2019d)**. *PC Philippines*

2.5	Develop molecular methods to advance sexual maturation, induce spawning and sex reversal	Oral-delivery methods for the introduction of genes encoding for expression of reproduction-related peptides, and aromatase inhibitors	June 2018	Recombinant DNA technologies and encapsulation methods were applied generating synthetic reproduction-related hormones that could be delivered via the oral route. Using tiger grouper as a model, sexual maturation and sex reversal were demonstrated after oral delivery of the recombinant hormones **(Palma et al. 2019b; Palma et al. 2019c; Nocillado et al. 2019; Palma et al. 2019e.; Palma et al. 2017; Nocillado et al. 2016.** *A, PC Philippines.*

PC = partner country, A = Australia

Objective 3: To develop reliable larval-rearing technologies for giant grouper

no.	activity	outputs/ milestones	completion date	comments
3.1	Develop optimal larval-feeding techniques	Determination of the benefit, if any, of the inclusion of copepods in the early larval diet of giant grouper, as compared to rotifers	Dec. 2014	A feeding trial at FFE showed that providing copepods (4 individuals/ml) at first feeding was essential to achieve better growth and survival rates of giant grouper larvae. *A*
		Determination of the benefit, if any, of the inclusion of copepods for extended periods during larval development	June 2016	With the success of producing hybrid giant and tiger grouper larvae, prolonged feeding with copepods was no longer required. A minute species of rotifers, *Proales similis*, was found to improve larval survival in giant grouper. *PC Vietnam, Philippines*
		Identification of the genes of the digestive system expressed by giant grouper larvae throughout their developmental stages	Dec. 2016	Using transcriptomics, clusters of differentially expressed genes at different larval stages were identified **(Anderson et al. 2018a,b).** Annotated genes include those responsible for appetite regulation and digestion. *A*.
		Identification of the feeding habits of giant grouper larvae through investigation of 16S RNA and cytochrome oxidase, as well as stable isotopes present within their gut contents	June 2019	DNA barcoding is being employed as an alternative method to identify the preferred live food of giant grouper larvae reared in green water system. *A*

PC = partner country, A = Australia

Objective 4: To apply genetic approaches to broodstock management

no.	activity	outputs/ milestones	completion date	comments
4.1	Apply genetic markers to prevent inbreeding and to enable genetic selection	Genetic markers for identification of parentage and pedigree establishment; identify additional microsatellite markers to have at least 16 that can be used in a multiplexed reaction	Dec. 2014	Reliable giant grouper microsatellite and mitochondrial DNA markers were successfully validated and used to assign parentage of series of spawns. Primers of these markers can be multiplexed in PCR reactions. **(Bright et al. 2016a)**. *A, PC Vietnam, Philippines.*
		Implementation of a genetic management program at the three sites	Dec. 2015	The microsatellite and mitochondrial DNA markers generated in this project were used to genotype giant grouper broodstock in partner countries and in Australia and assessed their genetic diversity. *A, PC Vietnam, Philippines*
		Implement a genetic program for selection of improved giant grouper	Dec. 2018	A combined genetic and molecular approach was utilised to determine resistance and susceptibility to viral nervous necrosis (VNN) of groupers. Mortalities resulting from VNN were found to correlate with genetic family background. Regional groupings of VNN strains were observed suggesting susceptibility of groupers to endemic reservoirs of the virus. **(Knibb et al. 2017)**. *A, PC Vietnam*

4.2	Explore hybridisations to achieve desirable traits	Tiger grouper and giant grouper hybrids with increased disease resistance	June 2016	Hybridisation was achieved by fertilising tiger grouper eggs with giant grouper sperm. The resulting hybrid is now a new and very successful crop variety in Vietnam. Farmers confirm the fast growth and disease resistance qualities of the hybrid. The hybrid grouper industry has rapidly expanded and in a bio-economic study, profitability of farms was confirmed although constraints were also identified **(Dennis et al. under revision, *Aquaculture*)**. Hatcheries and nurseries in Vietnam supply 2/3 of the required fingerlings by grow-out farmers. In a photovoice study, farmers, including women, conveyed the socio-economic benefits they derive from the growth of the hybrid grouper industry. *PC Vietnam***.**
		Orange spotted grouper and giant grouper hybrids that mature faster without further intervention; collect fin clips from giant grouper in the Philippines	June 2017	Orange spotted grouper and giant grouper hybridisation was achieved following the method for tiger and giant grouper hybrids. The new hybrids are already provided to farmers in Central and North Vietnam. Orange spotted grouper and giant grouper hybrids have also been produced by SEAFDEC/AQD in the Philippines, however only on small scale. *PC Vietnam, Philippines*

PC = partner country, A = Australia

7 Key results and discussion

The project achieved its stated objectives, significantly advancing knowledge regarding giant grouper reproductive biology and developing cutting edge technologies for captive breeding of this species. These advances are reinforced with genetic management tools that were also generated and validated as part of the project. The suite of technologies developed for broodstock management and larval rearing are applicable to other species and broader areas in fish reproductive endocrinology and aquaculture. These advances are published in high impact journals (a total of 7; one in Nature Publishing), with 71% "first-authored" by Vietnamese or Filipino staff, while almost all publications included them as co-authors". The project's capacity building efforts enabled the hybrid grouper industry in Vietnam to rapidly expand, providing grouper farmers significant socio-economic gains. In the Philippines, capacity building supported SEAFDEC/AQD's training of stakeholders within the country, the Southeast Asian region, and extra-regional fish farmers in the Middle East and Africa.

The key results are presented and discussed in this section: giant grouper reproductive biology, larval development and genetic management are unifying themes as these were identified as the most significant bottlenecks to full life-cycle aquaculture at the outset of the project.

Reproductive biology of giant grouper: sex differentiation, sexual maturation and sex reversal

Sex reversal by inhibition of aromatase with Fadrozole (Australia)

Large size at sexual maturation and sex reversal is one of the limiting factors for the captive breeding of giant grouper. We addressed this issue by monitoring the reproductive development (by periodic gonadal biopsy) of giant grouper of various sizes (9-43 kg), which were acquired for the project in partner countries. We also took advantage of data obtained from untreated controls of experiments (examined by histology and PCR) conducted in smaller sized giant groupers (1-5 kg) in Australia. We found that *cyp19a1a*, the gene encoding for gonad aromatase, is already expressed in gonads of giant grouper as small as 0.8 kg – 1 kg in body weight (Fig 10A). This result indicated that the gonads are already capable of the biosynthesis of sex steroids, importantly oestradiol (E_2), which is responsible for ovarian differentiation (Guiguen et al. 2010). This further implied that inhibition of E_2 synthesis can be applied at this early stage, which would be one way of inducing precocious sex reversal. Indeed, in an experiment that lasted 200 days, utilising Fadrozole as aromatase inhibitor, gonads transitioned to males after three treatments given at 50-day interval and a dose of 3 mg/kg (Fig 10B-D).

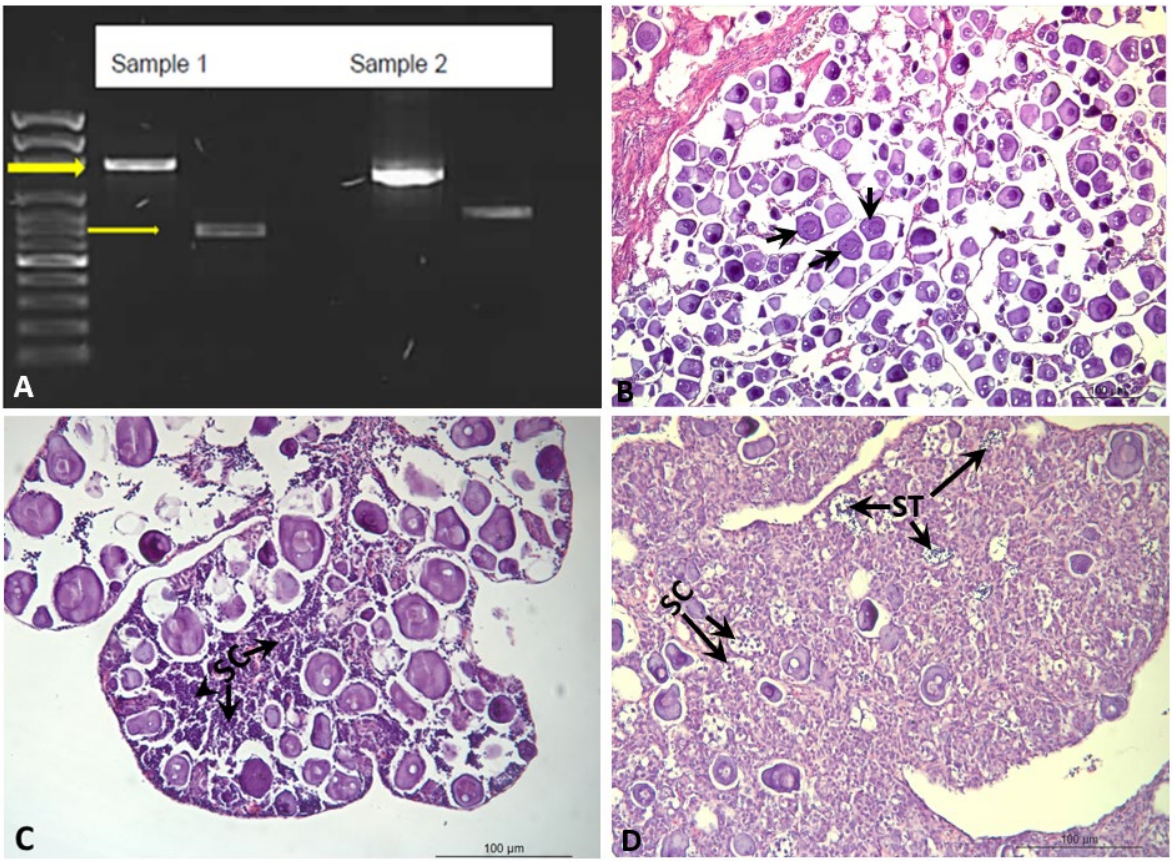


Fig 10. PCR amplification of gonad aromatase transcript in giant grouper about 1 kg in body weight (A). The yellow arrows point to the expected products amplified by two pairs of gene-specific PCR primers. B, C, D are representative sections of gonads from control (B) and Fadrozole-treated fish (C and D). Arrows in B point to primary growth oocytes, which are abundant in the section. In contrast, the number of primary growth oocytes was significantly reduced in fish treated with 3 mg/kg Fadrozole (C and D), where the male gametes spermatocytes (SC) and spermatids (ST) were observed. Scale bar 100 µm. (Images: D. Bright)

Further evidence that inhibition of E_2 synthesis by Fadrozole treatment results in masculinisation was obtained by examining the level of expression of female- and male-associated genes in the gonad. Expression of the female-associated genes (Cyp19a1a, Cyp19a1b and Foxl2) was significantly downregulated by Fadrozole (Fig 11A,B). In contrast, the expression of DMRT1, a male-associated gene, was not significantly changed but showed a trend of upregulation (Fig 11C).

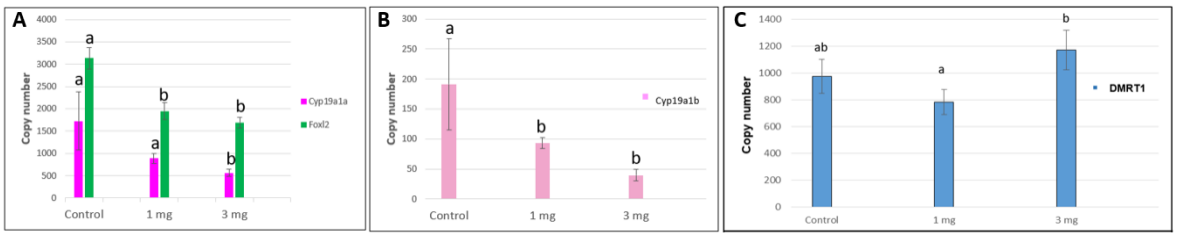


Fig 11. Expression level of female-associated genes Cyp19a1a, Foxl2 (A) and Cyp19a1b (B), and male-associated gene DMRT1 (C). Different letters indicate significant difference ($p<0.05$; N=8).

Plasma concentrations of sex steroids, E_2, 11KT and T, appeared contradictory. Whilst plasma E_2 was significantly reduced with Fadrozole treatment as was expected (Fig 12A), 11KT was also reduced in the treated group (Fig 12B). Further analysis of the 11KT levels was conducted to determine whether the data are being skewed by outliers. Results showed that indeed there were individuals in some replicate tanks that affected the mean

per group (Fig 13A). Similar results were obtained for plasma T (Fig 13B). The histological evidence shown in Fig 10C and D were obtained from gonads which were collected at the termination of the experiment. It must be noted that not all of the fish in the Control group were sacrificed. Two individuals were spared as they exhibited a territorial behaviour, which is characteristic of dominant males and TCO chose to keep them as future broodstock (a video file is included in this report). Of those that were sacrificed, significant differences were observed between the Control and treated group's gonadosomatic index, GSI (Fig 14). Mean GSI in Control fish was higher (more than double) than those of treated fish despite similar body size range. Bigger gonads in Control fish were expected, as ovaries grow faster in size than testis. In addition, the colour and higher vascularisation of the gonads in Control fish indicate ovarian differentiation. Altogether, the results confirmed that sex reversal can be carried in giant grouper less than 5 kg in body size (Bright et al. 2016b). On the other hand, whilst Fadrozole was effective as an aromatase inhibitor, its high cost and unreliable supply make it impractical to use in a commercial large scale.

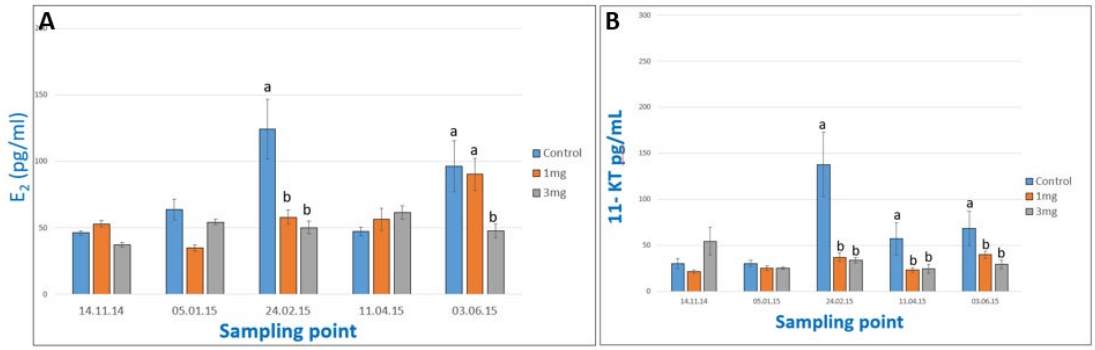


Fig 12. Plasma concentrations of E_2 and 11KT in Control and Fardozole-treated fish determined at the different sampling points. N = 12, 12, 10, 8 and 8 per sampling point. Different letters indicate significant difference ($p<0.05$).

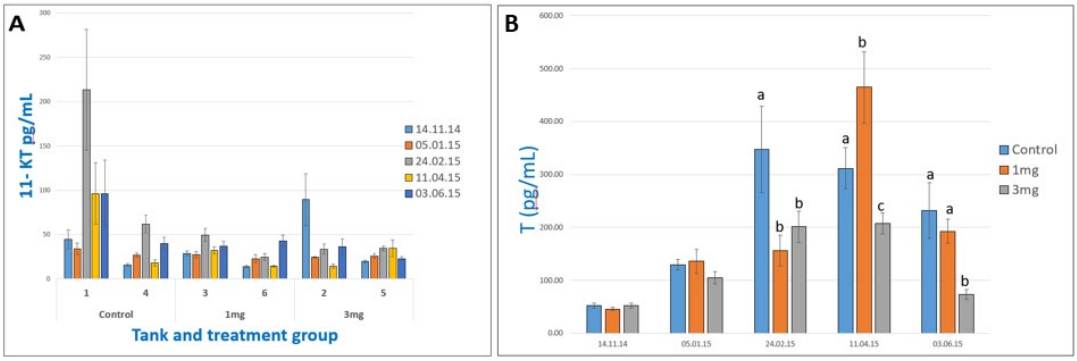
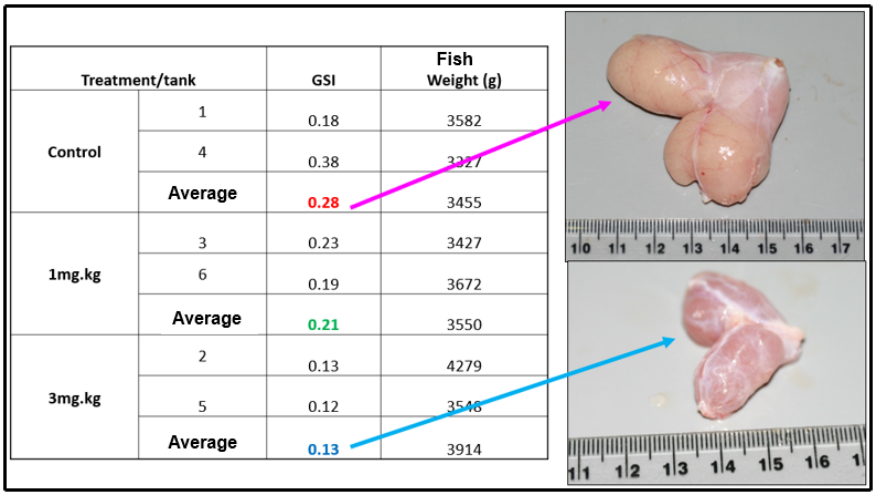


Fig 13. Mean plasma concentration of 11KT calculated per replicate tank per treatment group (A). Plasma T levels expressed as mean per treatment group per sampling point (B). N = 12, 12, 10, 8 and 8 per sampling point.



Treatment/tank		GSI	Fish Weight (g)
Control	1	0.18	3582
	4	0.38	3327
	Average	0.28	3455
1mg.kg	3	0.23	3427
	6	0.19	3672
	Average	0.21	3550
3mg.kg	2	0.13	4279
	5	0.12	3548
	Average	0.13	3914

Fig 14. Gonadosomatic index (GSI) and visual characteristics of gonads from dissected Control and Fadrozole-treated fish.

Sexual maturation of immature giant grouper broodstock (Philippines, Vietnam, Australia)

Immature giant grouper of various body sizes were monitored to determine at which point developmental reproductive milestones occur. Gonadal differentiation in giant grouper was observed in fish around 2.5 kg, as indicated by the gonads exhibiting oocytes at primary growth stage (Palma et al. 2019a). Sexual maturity in females, indicated by oocytes >400 µm in diameter, was observed in fish with an average weight of 23.5±1.5 kg in the Philippines and 33.5±2.5 kg in Vietnam. A ground-breaking finding in the course of the regular monitoring of the giant groupers in this project was the occurrence of primary males. Evidence for this was also obtained in Vietnam and the Philippines, which was consistent with the observations during the aromatase inhibition experiment conducted in Australia. Primary males matured without going through a functional female phase. What is more significant was the fact that the primary males were not necessarily the biggest fish in the group. In the Philippines, the average weight of mature primary males was 17.1±2.1 kg while in Vietnam they weighed 34.3±0.9 kg (Palma et al 2019a), compared with males obtained following natural or induced sex reversal, which can be 50-100kg and a great challenge to handle. Details of these data are described in our paper Palma et al. (2019a) (Appendix 8). These primary males have already contributed to the production of hybrid groupers in Vietnam and pure line giant grouper production in the Philippines (Fig 15).

Fig 15. Collection of milt from a primary male (A) to fertilise stripped eggs (B) for the production of pure line giant grouper (SEAFDEC/AQD, Igang Marine Station). The primary male on the left (A) was 18 kg while the female on the right (B) was 30 kg at the time of sampling. (Images: P. Palma)

One of the approaches tested to stimulate sex reversal was treatment with methyl-testosterone (MT), a synthetic androgen that has higher potency than naturally occurring testosterone (Piferrer and Donaldson 1991). At SEAFDEC/AQD in the Philippines, different methods of administration were tested, such as through the feed, injection and implantation, however none of them resulted in sex reversal although the level of VTG detected from the skin mucus indicated reduced E_2 synthesis (Palma et al. 2019a). In contrast, sex reversal was achieved in RIA3 in Vietnam, but the dose was tenfold higher (5 mg/kg at SEAFDEC/AQD vs. 50 mg/kg in RIA3). A disadvantage associated with MT is being a synthetic androgen, it is quite stable and therefore can persist and accumulate in the aquatic environment as an endocrine pollutant. With the discovery of the occurrence of primary males in giant grouper, we have a strategy to obtain giant grouper males without androgen treatment. This novel finding can revolutionise grouper aquaculture, both for pure line and hybrid grouper production. Sexual maturation in diandric males can be advanced using the hormonal treatment developed in this project (described in Palma et al. (2019b, c; Appendix 9, 10), making it feasible to have mature male giant grouper broodstock less than 10 kg in body weight.

Biotechnological strategies to advance sexual maturation and sex reversal (Philippines, Australia)

The ability to advance sexual maturation is highly desirable from the farming perspective, particularly in species that are late maturing or large in size at maturity (Taranger et al. 2010). Giant grouper matures in three to four years, at which time they are about 20 kg in body weight. Many individuals undergo sex change to males several years thereafter, hence male broodstock are even much heavier. The prolonged broodstock conditioning prior to spawning is both logistically and financially challenging. This project addressed these issues by generating tools, technologies and assays that can be applied to induce precocious maturation and enable documentation of the physiological changes at the molecular, cellular and organismal levels.

The underlying mechanisms triggering puberty in fish are not well understood (Taranger et al. 2010) let alone in a diandric protogynous hermaphrodite. Nevertheless, as in other vertebrates, the brain-pituitary-gonadal axis in fish integrates the various signals controlling reproductive function (Zohar et al. 2010). The key hormones are gonadotropin-releasing hormone (GnRH) produced in the hypothalamus, and the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) produced in the pituitary gland. GnRH stimulates the production and secretion of FSH and LH, which in turn stimulate the

gonad to synthesise the sex steroids testosterone (T) and E_2. T and E_2 mainly drive spermatogenesis and oogenesis in testis and ovaries, respectively.

We applied recombinant DNA technology (Glick et al. 2010) and the yeast (*Pichia pastoris*) expression system (Byrne 2015) to generate recombinant giant grouper GnRH (rggGnRH), recombinant FSH (rggFSH) and recombinant LH (rggLH). The rggGnRH was designed to be contained within the yeast cells, making oral delivery possible by incorporating the yeast cells themselves into pelleted food. The rggFSH and rggLH are in two forms: protein, produced in the yeast; and DNA, produced in bacteria. The rggFSH and rggLH proteins are administered by injection while their counterpart in DNA form can be orally delivered.

Expression of rggGnRH was confirmed by mass spectrometry (Fig 16). *In vitro* biological activity was confirmed using a receptor cell assay utilising the tilapia GnRH receptor (Fig 17).

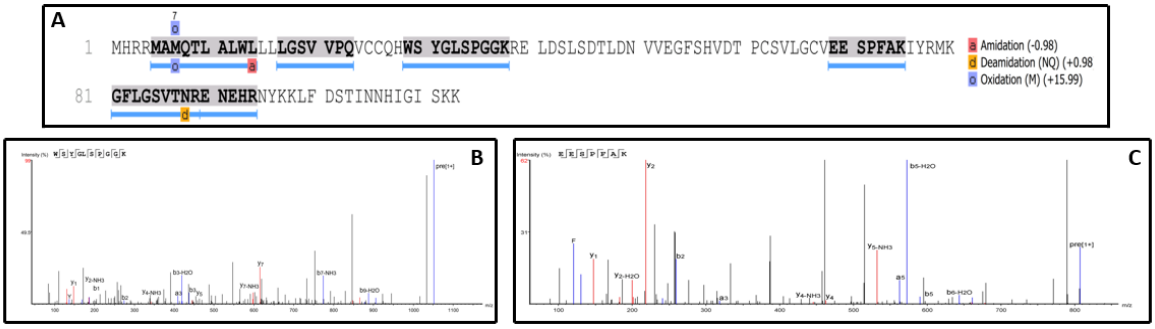


Fig 16. Mass spectrometry data confirming the expression of recombinant GnRH decapeptide and GnRH-associated peptide in yeast, *Pichia pastoris*.

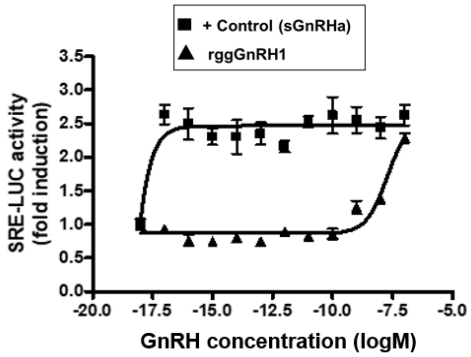


Fig 17. Luciferase activity in response to stimulation of GnRH receptor with recombinant GnRH peptide (rggGnRH1) in an *in vitro* assay using COS-7 cells.

In vivo biological activity of rggGnRH was assessed in juvenile orange spotted grouper (*E. coioides*) as model species. Results showed the physiological effect of rggGnRH. Compared with the control, there were individuals in the group fed with 1x_rggGnRH and 10X_rggGnRH that had increased plasma levels of FSH (Fig 18A), LH (Fig 18B) and E_2 (Fig 18C). The GSI in most of the fish in the treated group was also elevated (Fig 19A) although oocyte diameters were similar to controls (Fig 19B). Gonadal histology showed majority of fish in the rggGnRH-treated group had gonads in a more advanced stage of development compared with the control (Fig 20A,B).

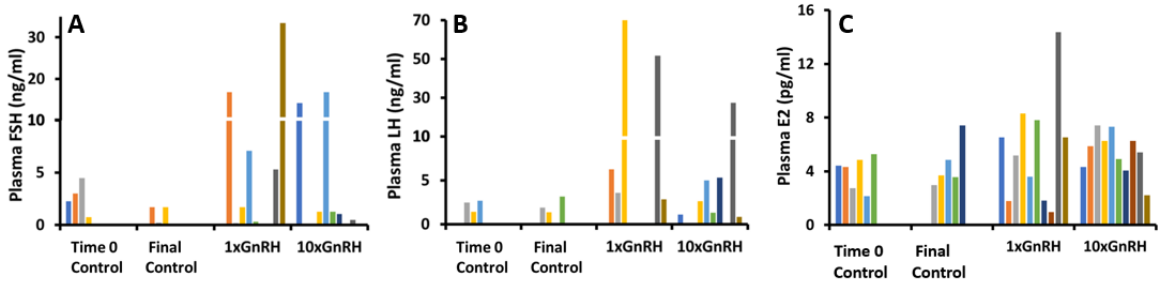


Fig 18. *In vivo* biological activity of rggGnRH in immature orange spotted grouper as indicated by increased plasma FSH (A), LH (B) and E_2 (C).

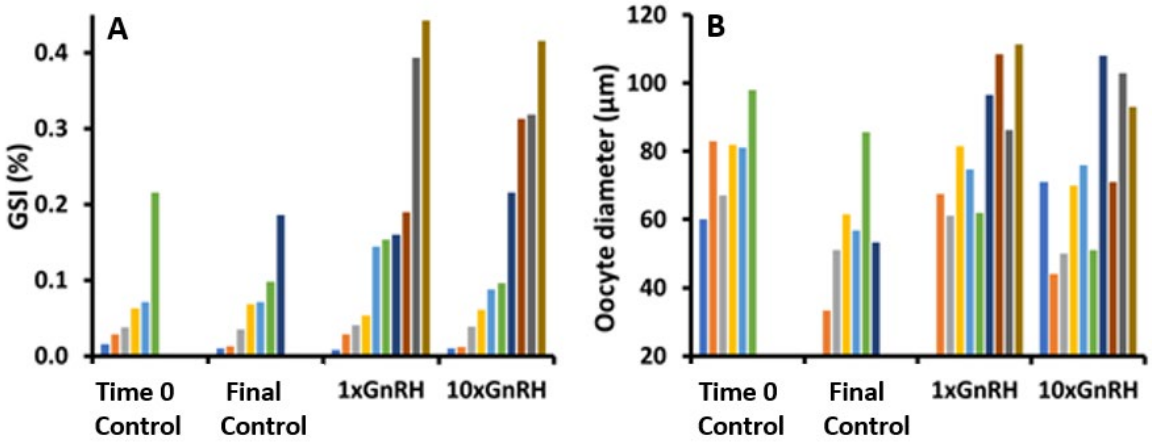


Fig 19. Gonadosomatic index (GSI; A) and oocyte diameter (B) data from Control and rggGnRH-fed immature orange spotted grouper.

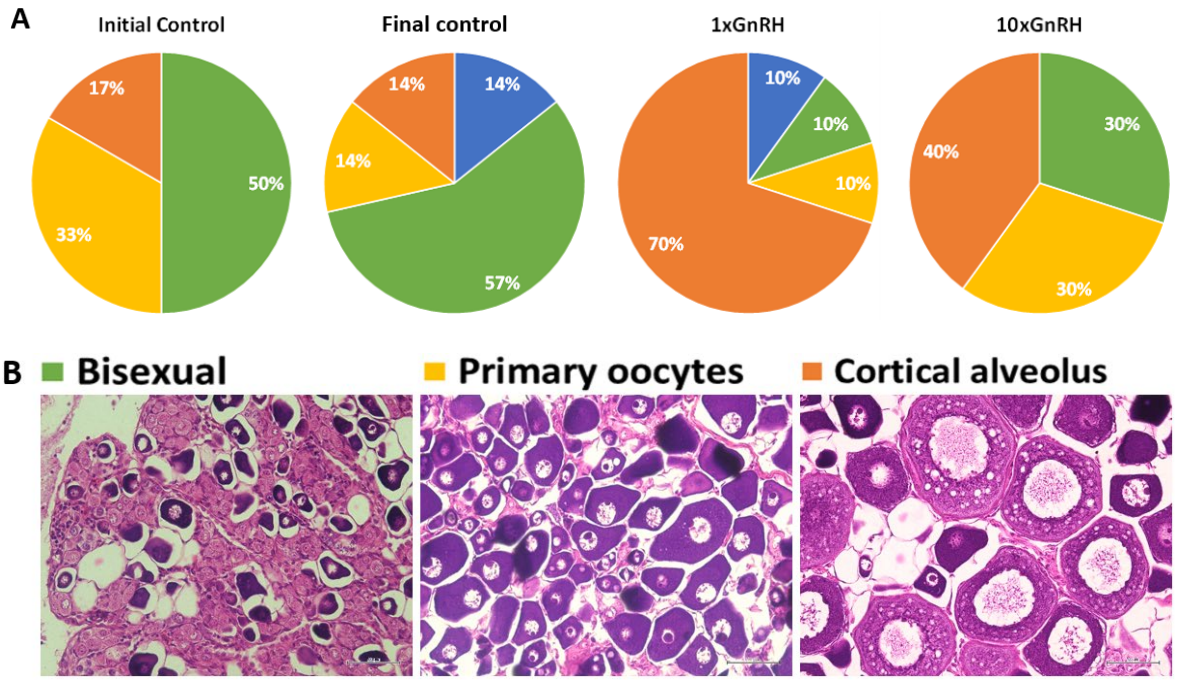


Fig 20. (A) Proportion of bisexual (green), primary oocyte (yellow) and cortical alveolus (orange) stages in Control and rggGnRH-fed immature orange spotted grouper. Blue indicates fish where gonad tissues could not be dissected or could not be clearly identified due to minute size. (B)

shows the histological characteristics corresponding to the different gonadal stages. (Images: P Palma)

Altogether the data proved rggGnRH's capacity to stimulate gonadal development in immature grouper. It is remarkable that the hormonal treatment was delivered together with the feed, thus eliminating handling stress. Nevertheless, optimisation of the protocol is still required as data variability was observed, which may have resulted from uneven quantity of the hormone ingested by the fish in the treated group.

The GnRH system in the brain integrates the environmental and metabolic cues for gonadal development and spawning (Zohar et al. 2010). In some species, the action of GnRH on the pituitary is inhibited by dopamine, a brain neuropeptide (Dufour et al. 2005; Levavi-Sivan et al. 2010). In groupers, it is not clear whether dopamine inhibition of GnRH and its downstream effect on the pituitary gland exists. Using the yeast expression system, we generated a synthetic recombinant FSH (rggFSH) in protein form as another strategy for advancing gonadal maturation in giant grouper (Palma et al. 2019b). We also generated a rggFSH in DNA form, which, when encapsulated, was orally delivered (Palma et al 2019c). The pituitary FSH regulates the early stages of gonadal development (Yaron et al. 2003) and, as shown in other species of fish, is essential for the timely onset of puberty (Zhang et al. 2014). Administration of the synthetic protein FSH in immature tiger grouper (*E. fuscoguttatus*) by injection and of DNA FSH by the oral route accelerated gonadal development as well as sex reversal (Palma et al. 2019b, 2019c) (Fig 21). The results of these two studies identified the crucial role of FSH in sex change and point to factors other than male sex steroids that may be responsible for testicular development during sex change (Palma et al. 2019b,c). The rggFSH will be an invaluable tool in future studies to understand the mechanism behind sex reversal in a protogynous hermaphrodite.

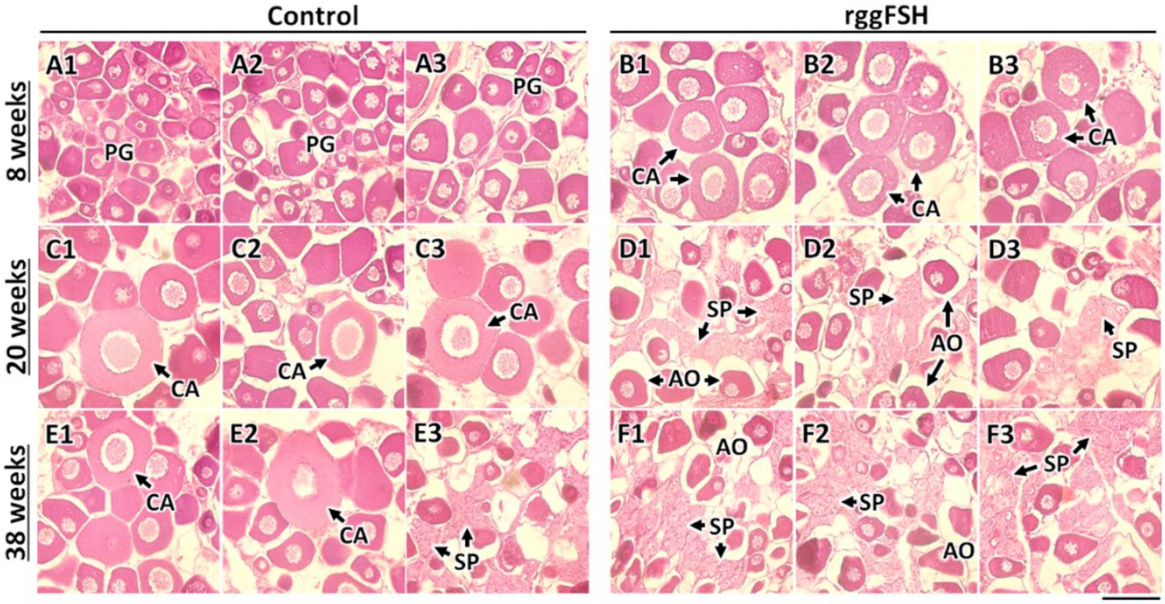


Fig. 21. Histological evidence at the gonad level showing stimulation of sexual maturation and sex reversal in immature tiger grouper treated with rggFSH. Each photo represents a replicate fish. PG – primary growth oocytes; CA – cortical alveolar oocytes; AO – atretic oocytes; SP – spermatogonial cells; Scale bar = 50 µm. This figure appears in Palma et al. (2019b) and copyright belongs to the journal (Biology of Reproduction, Oxford University Press).

Surrogate technology as an alternative breeding strategy to generate "smaller and faster maturing giant grouper broodstock" (Australia, Philippines)

Germ cell transplantation (GCT) is a technology that enables the production of offspring of a target species (donor) in a surrogate (host) with desirable traits (Yoshizaki et al. 2003, 2010). In case of giant grouper, which is late maturing and large in size at maturity, an ideal surrogate would have a short generation time and mature at a much smaller in size. The success of GCT depends on several factors. Donor germ cells must be transplanted during a specific window of time when the host's endogenous primordial germ cells migrate towards the future gonad site (Takeuchi et al. 2003; Yazawa et al. 2010). The ideal type of germs cells to be transplanted are primordial germ cells and spermatogonia A, both of which are able to differentiate into functional sperm or eggs depending on the host's sex (Okutsu et al. 2006). Host must be sterile or devoid of its endogenous germ cells to promote the exclusive production of the donor germ cells. Sterility can be achieved by triploidy (Okutsu et al. 2007), knockout of *deadend*, a germline-specific gene (Weidinger et al. 2003) and hybridisation. The donor and host must be evolutionarily close, less than 30 million years apart, otherwise the donor germ cells are unable to proliferate in the host's gonad (Bar et al. 2016).

Microscopic examination of serially sectioned orange spotted grouper larvae (3-15 days post-hatch; mean total length) revealed primordial germ cells in the abdominal cavity in larvae 10-12 days post-hatch (Fig 22A,B). Growth rate was also fastest at this stage (Table 4). These observations indicate that 10-12 days post-hatch is the window when transplantation is suitable in this species.

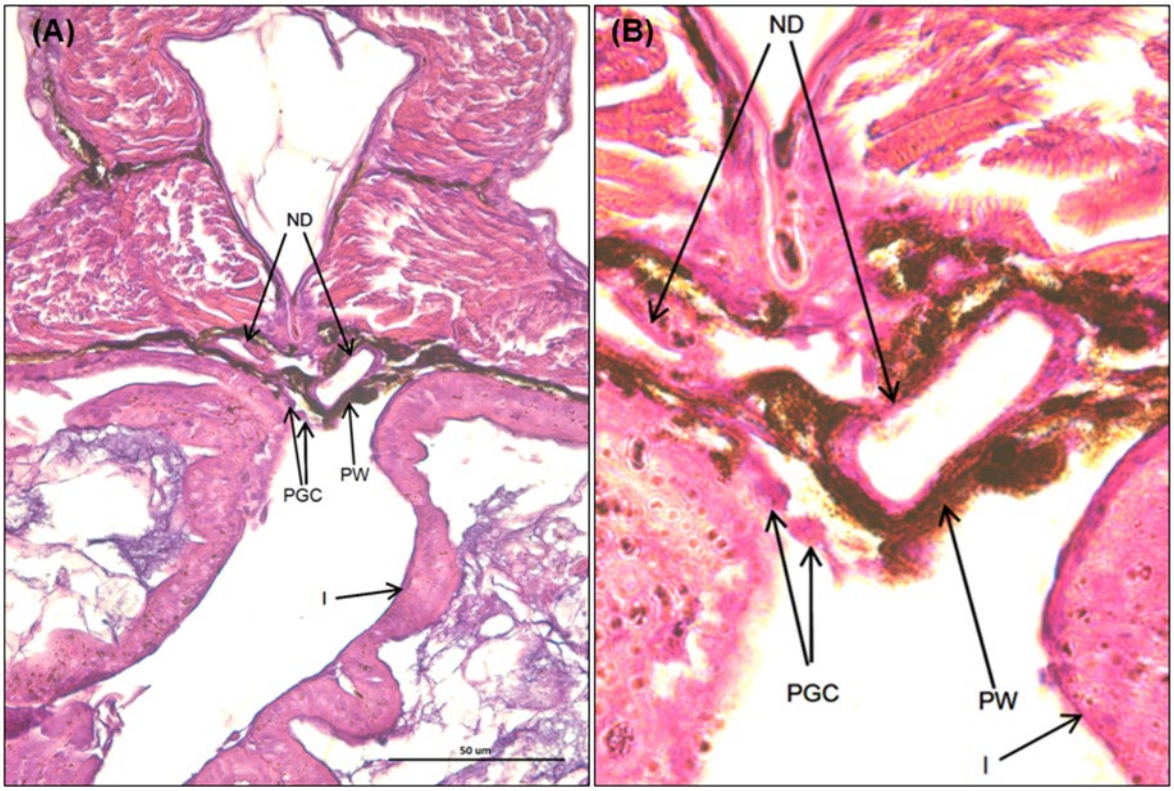


Fig 22. Transverse sections of orange spotted grouper larvae at 10 DPH showing primordial germ cells migrating towards the genital ridge. PCG= primordial germ cell, PW= peritoneal wall, ND= nephritic duct, I= intestine, A, 40x magnification; B, 100 x magnification. (Images: D. Bright)

Table 4. Length increment of orange-spotted grouper larvae from 3-15 days post hatch. Growth rates were highest at 9 to 10 DPH. The shaded area shows the days in which PGCs are migrating and are most suitable for GCT.

	Larval length (mm)							
DPH	**1**	**2**	**3**	**4**	**5**	**Average**	**Increment**	**% increase**
3	2.3	2.3	2.01	2.21	2.32	2.228	-	-
4	2.51	2.33	2.34	2.4	2.4	2.396	0.17	7.54%
5	2.69	2.51	2.45	2.15	2.29	2.418	0.022	0.92%
7	2.66	2.48	2.18	2.33	2.51	2.432	0.014	0.58%
8	2.89	2.96	2.21	2.57	2.69	2.664	0.232	9.54%
9	3.43	3.41	3.38	2.76	3.21	3.238	0.574	21.55%
10	3.77	4.54	4.5	3.74	4.34	4.178	0.94	29.03%
11	4.88	5.15	5.11	4.24	5.12	4.90	0.722	17.28%
12	4.39	4.27	5.55	5.7	5.57	5.096	0.196	4.00%
13	4.54	4.91	6.59	5.61	7.04	5.738	0.642	12.60%
14	6.83	6.66	7.38	6.2	7.36	6.886	1.148	20.01%
15	7.36	8.17	7.75	8.17	8.26	7.942	1.056	15.34%

Unlike primordial germ cells that are mostly abundant in gonads of fish at larval stages, spermatogonia A (SpgA) are still abundant even in mature testis (Bar et al. 2016). SpgA cells also have the capacity to form functional sperm or eggs, when transplanted into a male or female host, respectively. Trials using tiger grouper testis showed the applicability in a grouper species of the dissociation protocol developed for Southern bluefin tuna (Bar et al. 2016). SpgA-enriched fraction was obtained (Fig 22A) and the viable cells were labelled with the fluorescent dye PKH26 (Fig 22B). The quality of the donor testis is important as poor quality tissue does not completely dissociate, yielding dead cells (Fig 22C). GCT success was demonstrated in transplanted orange spotted grouper larvae where PKH26-labelled cells were observed in 8 out of 15 recipients 22 days post-transplantation (Fig 23A-C

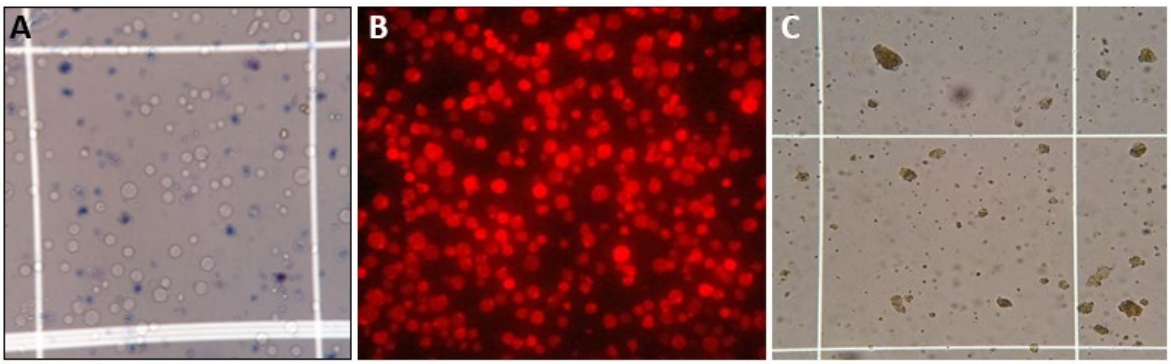


Fig 22. (A) Viable (transparent) and dead (blue) SpgA cells. (B) Fluorescent germ cells labelled with PKH26. (C) Dead cells and undissociated cyst clusters derived from poor quality testis. (Images: D. Bright)

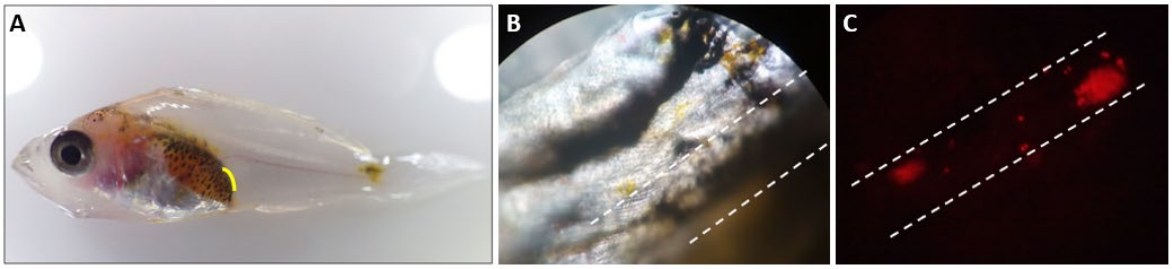


Fig 23. (A) Orange spotted grouper 22 days post-transplantation (34 days post-hatch) showing the region (coloured yellow) where photos in B and C were taken from. (B) Body cavity of dissected larvae viewed on bright field and with fluorescence (C). Magnification of B and C is 20x. Dotted lines represent genital ridge location. (Images: X. Libunao)

Sterility of the host is essential to ensure that only the donor gametes will proliferate in the host's gonads, subsequently producing donor-derived offspring alone (Okutsu et al. 2007). Grouper hybrids were examined by dissection and histology to assess gonadal development. In 2.5-3.0 kg orange spotted x tiger grouper hybrids, gonads were tiny and undeveloped compared with the gonads of pure line orange spotted grouper of the same age although smaller in size (0.5-1.0 kg) (Fig 24A-C). Histological section of the gonad from a 4-year old giant grouper x tiger grouper hybrid weighing 7 kg showed non-developing oocytes (Fig 25A,B). Dissection of further 35 giant x tiger grouper hybrids (2-4 years old; 3-15 kg) in RIA3 confirmed the lack of developing gonads. There have been reports that some hybrid groupers are not totally sterile (Luan et al. 2016) and this requires further verification. However, production solely of donor-derived gametes has been demonstrated in hybrids of four marine fishes belonging to the Sciaenid family (Yoshikawa et al. 2018).

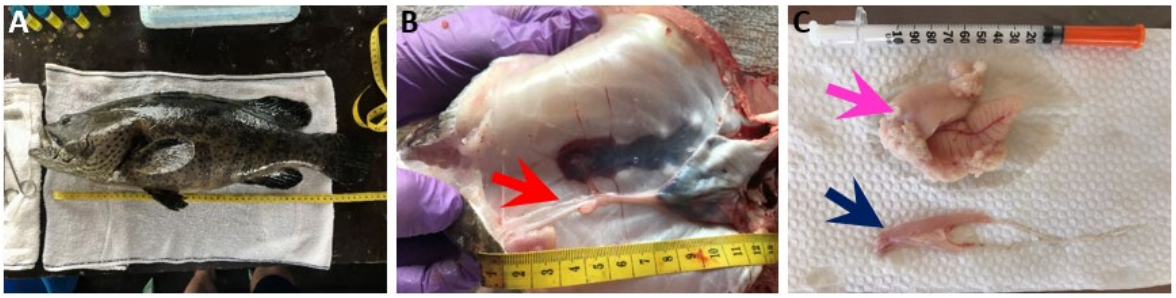


Fig 24. Hybrid orange spotted and tiger grouper (A) showing undeveloped gonad (B). Gonads of pure line orange spotted grouper of similar size developing as female (C, pink arrow) and primary male (C, blue arrow). (Images: P. Palma)

Donor-derived gametes have also been successfully produced in triploid hosts. For instance, tiger puffer (*Takifugu rubripes*) was produced in triploid grass puffer (Hamasaki et al. 2017) while trout offspring was produced in triploid salmon (Okutsu et al. 2007). In this project, triploid honeycomb grouper is being explored as potential surrogate for giant grouper, in addition to tiger grouper x orange spotted grouper hybrids. Evolutionary analysis of groupers showed that the distance between giant grouper and the three potential surrogates (tiger, orange spotted and honeycomb grouper) are closely related, indicating the likelihood of GCT success in these species.

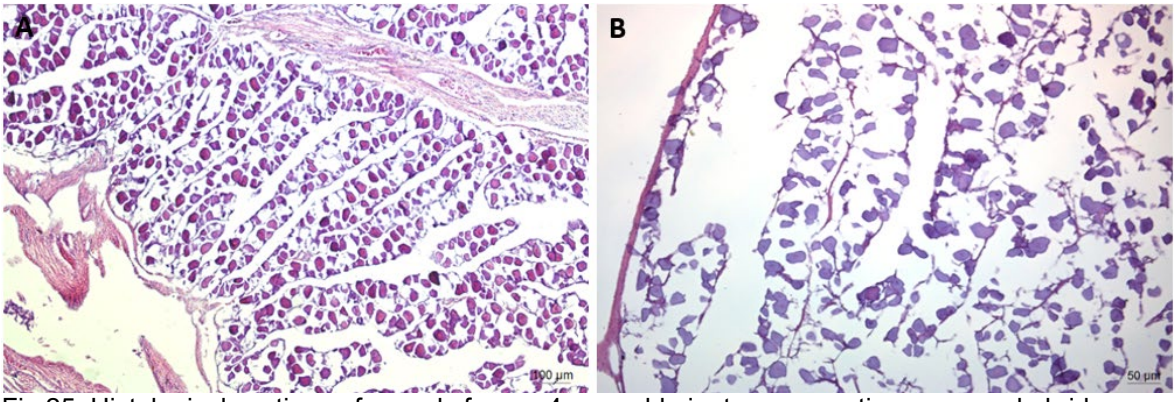


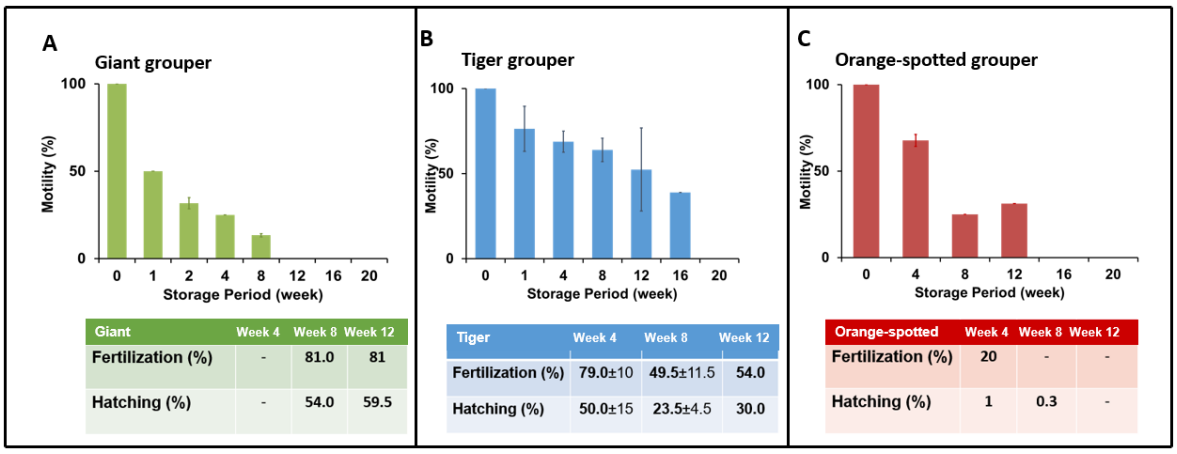
Fig 25. Histological sections of gonads from a 4-year old giant grouper x tiger grouper hybrid weighing 7 kg exhibiting non-developing oocytes. (Images: D. Bright)

We succeeded in conducting germ cell transplantation in the delicate grouper larvae hosts. We have generated expertise and tools that can take the current achievements to the next level. Specifically, from the giant grouper gonad transcriptome, we have sequences for germ cell markers, including Vasa, Nanos½, Notch1, CxCR4, WT1a/b and LY75. We also have the sequence of the *dnd* gene, a spermatogonia A marker. These molecular tools are useful in confirming the identity of germ cells to be transplanted. The identity of the donor-derived offspring and their parentage can be ascertained using the microsatellite and Cox1 markers also validated in this project (Bright et al. 2016a). Maturation and sex reversal can be advanced, and spawning induced, using the recombinant giant grouper GnRH, FSH (Palma et al. 2019b, 2019c) and LH. The developed assays (ELISAs for VTG, FSH and LH) can be used to monitor the developmental changes of the surrogate. Furthermore, fundamental knowledge on reproductive biology was advanced using the antibodies against grouper FSH and LH. These advances and tools can be applied to generate “smaller and faster maturing” giant grouper broodstock.

Gamete and sperm cryopreservation (Philippines)

The asynchronous maturation in captivity and protogynous nature of giant grouper and other grouper species restricts the availability of gametes for germ cell transplantation as well as hybrid production. Cryopreservation, which utilises extremely low temperatures to preserve the viability of biological materials for indefinite period of time (Pegg, 2007), can be applied to address the limited availability of male giant grouper broodstock and the unsynchronized spawning of different grouper species. We successfully cryopreserved the sperm of giant grouper, tiger grouper and orange-spotted grouper (Fig 26) in liquid nitrogen by modifying the methods described by Fan et al (2014). In giant grouper, sperm motility was retained until 8 weeks of storage, but sperm remained viable until 12 weeks, based on fertilisation and hatching rates (Fig 26A). Tiger grouper was the most robust, with sperm being motile after 16 weeks of storage in liquid nitrogen and also remaining viable (Fig 26B). In contrast, whilst orange-spotted grouper sperm were still motile 12 weeks after cryopreservation in liquid nitrogen, viability was limited only to 4 weeks of storage (Fig 26C). As different grouper species spawn at different times relative to the moon phase, these periods of storage times are significant for hybrid grouper production.

We further determined whether viability of grouper sperm can be maintained in a bio-freezer only, or at -80°C. Liquid nitrogen is proven effective for cryopreservation purposes, but it is costly, hazardous to handle and transport, and not widely or readily available. We validated a protocol that utilised Ficoll 70 as a cryoprotectant (Yuan et al. 2016). Tiger grouper sperm were observed to be viable after 3 months of storage in a bio-freezer even without Ficoll 70 (Fig 27A, B). Giant grouper sperm were motile one week after storage in bio-freezer (Fig 27C) although viability could not be confirmed due to very low numbers.

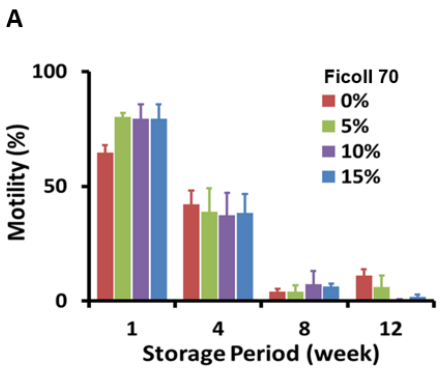


Giant	Week 4	Week 8	Week 12
Fertilization (%)	-	81.0	81
Hatching (%)	-	54.0	59.5

Tiger	Week 4	Week 8	Week 12
Fertilization (%)	79.0±10	49.5±11.5	54.0
Hatching (%)	50.0±15	23.5±4.5	30.0

Orange-spotted	Week 4	Week 8	Week 12
Fertilization (%)	20	-	-
Hatching (%)	1	0.3	-

Fig 26. Percentage motility and corresponding fertilisation and hatching rates obtained from grouper sperm cryopreserved in liquid nitrogen.



Ficoll 70	%FR	%V	%HR
0% (LN)	69	80	35 ±9
0%	70	78	32 ±3
5%	76	77	32 ±9
10%	72	72	39±10
15%	78	78	32 ±7

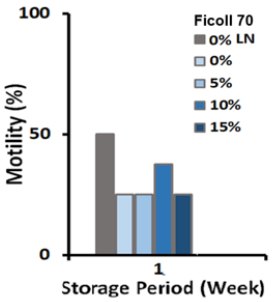


Fig 27. Percentage motility (A) and indicators of viability (B) of tiger grouper sperm cryopreserved in bio-freezer with or without Ficoll 70. (C) Percentage motility giant grouper sperm cryopreserved in the same conditions as tiger grouper sperm.

In order to improve the quality of cryopreserved sperm, different cryopreservation media parameters were evaluated. Osmolality was best at 450 milliosmoles (Fig 28). A pH of 7 was best for giant grouper sperm (Fig 29A) while a pH range of 6 to 8 was optimal for tiger grouper (Fig 29B) and orange-spotted grouper (Fig 29C). Sperm could be diluted with the diluent up to 4 times without affecting motility (Fig 30). The experiments evaluating different sperm to diluent ratio were conducted at 4°C and results showed that sperm motility can be maintained at this temperature for up to 10 days. Although there are aspects of the cryopreservation protocol that still need further optimisation, results obtained so far show that grouper sperm can be stored at 4°C and at -80°C without compromising viability. While this is a short term alternative, it is a cost-effective and accessible option for farmers, especially for hybrid production.

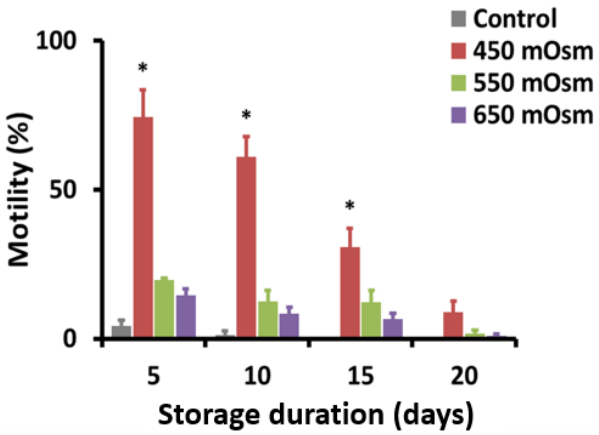


Fig 28. Percentage motility of cryopreserved orange spotted grouper sperm in media at different levels of osmolality. Asterisk indicates significant difference ($p<0.05$).

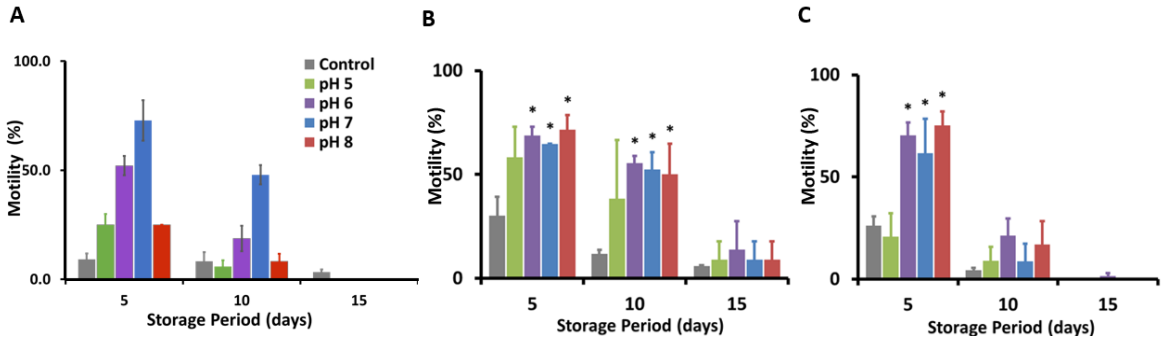


Fig 29. Percentage motility of giant grouper (A), tiger grouper (B), and orange spotted grouper (C) sperm cryopreserved in media with different pH levels. Asterisk indicates significant difference ($p<0.05$).

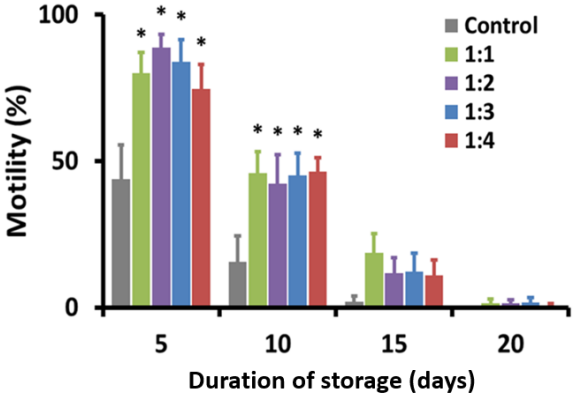


Fig 30. Percentage motility of tiger grouper sperm cryopreserved at different sperm to diluent ratio. Asterisk indicates significant difference ($p<0.05$).

Optimal sex ratio, spawning synchronisation, fertilisation and hatching rates (Vietnam, Philippines)

In captive conditions, the environmental, social and nutritional cues for final gonadal maturation and spawning are greatly diminished or altogether absent (Mylonas and Zohar, 2007), hence broodstock have to be conditioned to complete the seasonal maturation and spawn cycles. The ratio between male and female is a critical social cue for groupers that are known to be aggregate spawners in the wild (Sadovy et al. 1994). Few combinations were tested in partner countries due to the limited number of available mature broodstock. In Vietnam, a maturation rate of 80-90% in males and 40-60% in females was observed in broodstock stocked at 2:1 ratio (6 males and 3 females) either in a 50 m^3 cage or 90 m^3 tank. In Nha Trang area, central Vietnam, mature males were observed from March to November while mature females were observed from July to October. At SEAFDEC/AQD's Igang Marine Station in the Philippines, spawning of mature fish were not noted throughout the year (Fig 31), however these were fish that just matured and were on their first cycles of spawning.

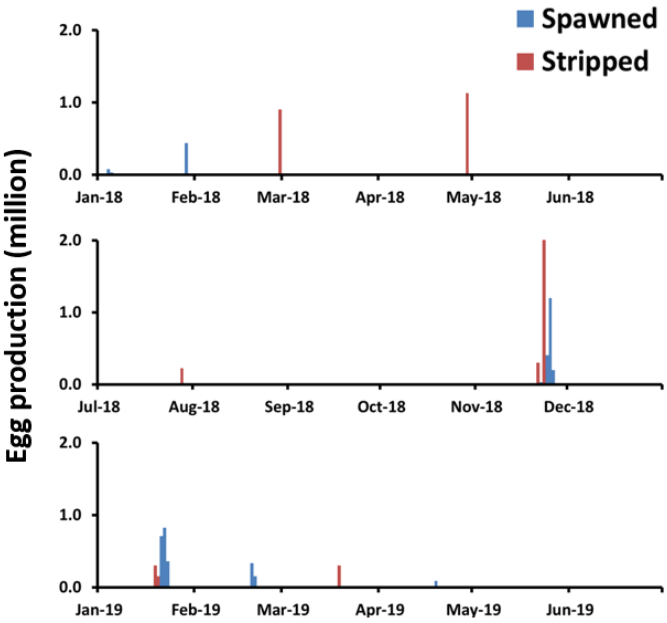


Fig 31. Spawning record at Igang Marine Station. Broodstock were sampled every month, about 4 days before the full moon, to determine their readiness for spawning induction.

In the absence of spawning cues, final gonadal maturation was stimulated with GnRHa, either in the form of slow-release implants prepared at USC, or its commercially available version (Ovaplant by Syndel, Ferndale, WA). Spawning was induced with hCG. There were 16 spawning batches in Vietnam yielding 35 kg of fertilised eggs produced. Fertilisation rate ranged from 0%-75%.

In the Philippines, giant grouper were successfully produced using a combination of GnRHa and hCG treatments to induce spawning; however, fertilisation and hatching rates were very low and larval mortalities were very high (>90%). Nevertheless, a few giant groupers survived from three spawnings (Fig 32A-C), with the first spawn in July 2018 now more than one year old (Fig 32A).

Fig 32. Batches of giant groupers spawned at Igang Marine Station, SEAFDEC/AQD. Spawning were in July 2018 (A), November 2018 (B) and February 2019 (C). (Images: A&C-P. Palma; B-J Chan)

Development of larval rearing technologies

Transcriptomic analysis of early developing giant grouper larvae (Australia)

One of the reasons giant grouper larvae is difficult to rear in aquaculture is the challenge of providing appropriate prey at first feeding due to the larvae's tiny mouth gape. Mass production of high quality first prey has not been established (Rimmer and Glamuzina, 2019). Furthermore, there is a lack of understanding regarding the larvae's digestive physiology and appetite regulation throughout larval development. We employed transcriptomics to understand the molecular changes in larvae from day 1 to day 14 post-hatch, with particular emphasis on the development of the digestive system (Anderson et al. 2018a). Expression of appetite regulating genes was detected as early as day 1 post-hatch. Transcripts that are involved in protein, carbohydrate and lipid digestion were differentially expressed and showed specific pattern of expression with larval development (Fig 33). Upregulation of digestive genes were noted at day 3 post-hatch (mouth opening/first feeding) and at day 6 post-hatch (swim bladder inflation), with the latter likely representing an increased ability to hunt for prey. The upregulation of genes for chitin digestion from day 3 to day 6 post-hatch implies the ability to digest chitin in a way that makes the exoskeleton available as a nutritional resource rather than just digesting it to access the soft internal tissues (Anderson et al. 2018al). This is consistent with the observation that copepods provided at first feeding improve larval survival and growth rate (Fig 34).

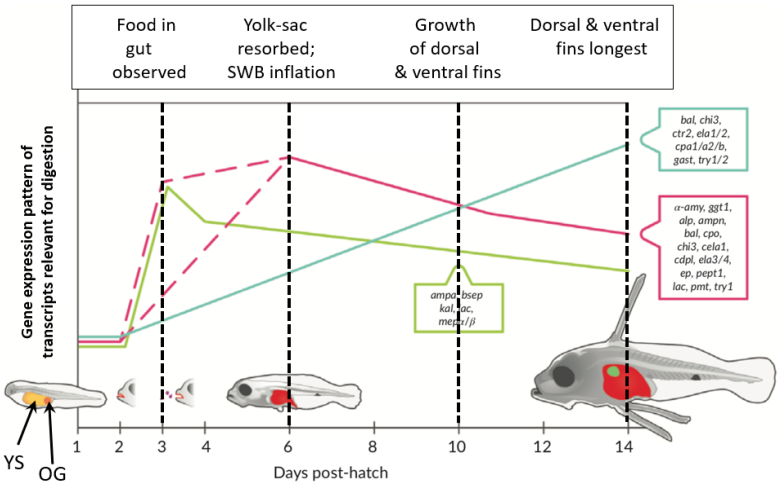


Fig 33. Diagrammatic representation of global expression of digestion-relevant genes in early developing giant grouper larvae (modified from Anderson et al. 2018a). SWB – swim bladder; YS-yolk sac; OG – oil globule

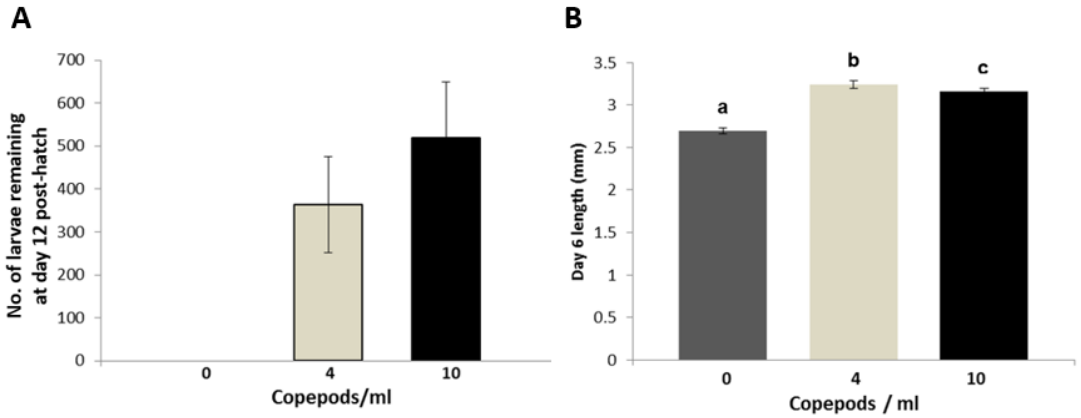


Fig 34. Impact of copepod feeding on growth and survival of giant grouper larvae. Number of surviving larvae from 9000 fertilised giant grouper eggs stocked in 300 L tanks (A). Average total length of larvae samples collected at day 6 post-hatch (B). Different letters denote significant difference ($p<0.05$).

With the transcriptomic data, we have established a significant resource for giant grouper larvae that were raised in optimal conditions, and therefore can be used to assess alternative diets.

Copepod culture and usage in grouper larval rearing in Vietnam

Copepod nauplii are fed to hybrid grouper larvae from day 8 to day 20 post-hatch (Fig 35). The most common copepod species found in pond culture are *Calanus sinicus*, *Oithona rigida* and *Paracalanus parvus* (Fig 36). Copepods at nauplii and copepodite stages are of the suitable sizes for grouper larvae (Table 5). Copepod feeding helps to stabilise larval survival rate from hatching to 6 cm fingerlings (around 3% for hybrid, orange spotted and tiger grouper). Copepods are also a much cheaper live food than Artemia nauplii. The production cost of a kilo of copepods is US$5 while the market price of Artemia is US$35-50 per kg.

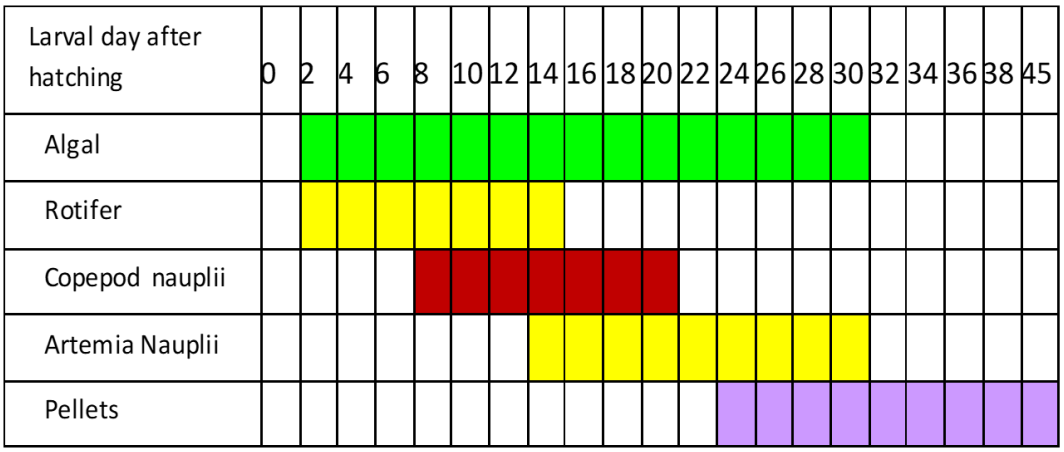


Fig 35. Feeding schedule of hybrid grouper larvae practised in RIA1 and RIA3.

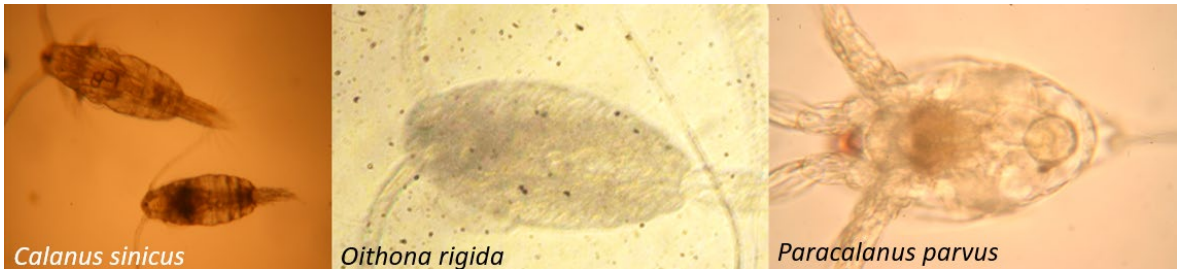


Fig 36. Most common copepod species cultured or found in rearing water of groupers in Vietnam. Species were identified following Støttrup and McEvoy (2003). (Images: Cao Van Hanh)

Table 5. Body length of copepods at different stages of development

Species	Body length (µm)		
	Nauplius	Copepodite	Adult
Calanus sinicus	68.7-122.4	142.5-206.4	214.6-242.7
Oithona rigida	64.3-112.6	130.8-217.8	223.6-357.4
Paracalanus parvus	82.5-116.5	165.7-226.5	250.8-515.7

Proales similis culture and feeding trials in the Philippines

P. similis is a minute rotifer measuring 83±11 µm in length and 40±6 µm wide. It is 38% smaller and 60% narrower than the super small type of rotifer, *Brachionus rotundiformis*, and is therefore a good live food candidate to feed marine fish larvae with very small mouth openings (Wullur et al. 2009). Experimental results at SEAFDEC/AQD showed that feeding *P. similis* twice daily significantly enhanced survival rate in orange spotted grouper at 35 day post-hatch (Fig 37A) as well as improved growth rate from day 12 post-hatch (Fig 37B). Higher survival rate was also obtained in tiger grouper at 35 days post-hatch, even if it was fed only once a day (Fig 37C). The feeding protocol has been applied to giant grouper larvae resulting in successful rearing beyond metamorphosis (Fig 32).

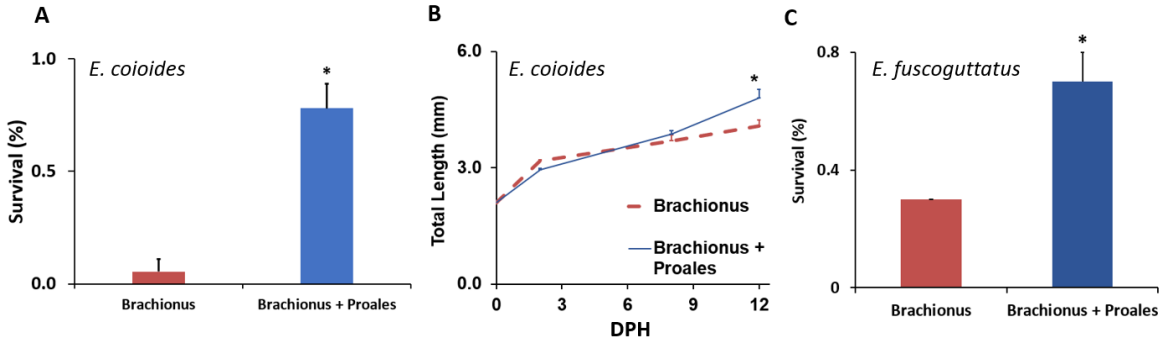


Fig. 37. Survival (A) and growth rate (B) of orange spotted grouper (*E coioides*) fed with *P. similis*. The survival rate of tiger grouper (*E. fuscoguttatus*) in a similar experiment is shown in (C). Asterisk denotes significant difference between the two groups ($p<0.05$).

Identification of feeding habits by DNA barcoding (Australia)

To replace the use of stable isotopes, DNA barcoding was utilised to gain insight regarding the food preference of giant grouper larvae when reared in a green water system. The sequence of the mitochondrial gene cytochrome c oxidase subunit I (Cox1) has been previously used successfully to discriminate species in a complex community that included copepods (Watson et al., 2015, Cheng et al., 2013, Bucklin et al., 2010). Cox1 gene was better able to discriminate between closely related *Neocalanus* copepod species compared to other commonly used barcoding genes such as 12S, nuclear ITS and 28S (Machida and Tsuda, 2010). Barcoding technology will allow for the identification of whole or partially digested copepods from the guts of giant grouper larvae. The species-level identification of gut contents will shed light on the feeding preferences of GG larvae, and this information will serve as a guide for future rearing protocols that aim to improve larval growth and survival.

Highly degenerate primers were designed to PCR amplify a short fragment (~300 bp) of the COX1 gene, which is used to identify micro-organisms present in the stomach of giant grouper larvae. Products obtained indicated the primers are useful to identify different types of live food (Fig 38A), however grouper DNA is also amplified (Fig 38B). Following the technique by Vestheim and Jarman (2008) and Leray et al. (2013), blocking primers were designed to inhibit the amplification of grouper DNA whilst enhances amplification of DNA from ingested food organisms (Fig 39).

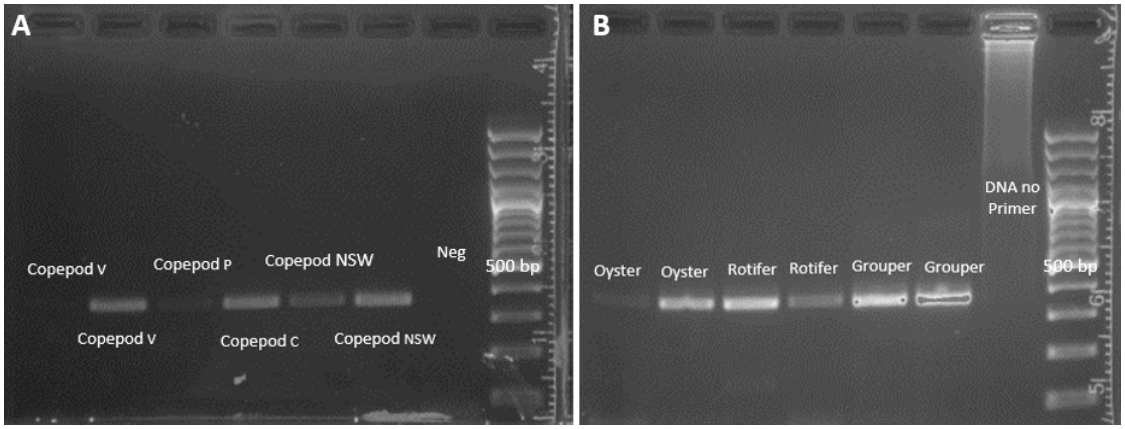


Fig 38. PCR amplification of a fragment of COX1 gene using universal primers. Whilst amplification was successful using DNA template from several larval food organisms, such as copepods, oyster trochophore and rotifers (A and B), the primers can also amplify COX1 from grouper templates (shown in B only). Samples were from Vietnam (V), Philippines (P) and Cairns, Australia (C). (Images: L. Dennis)

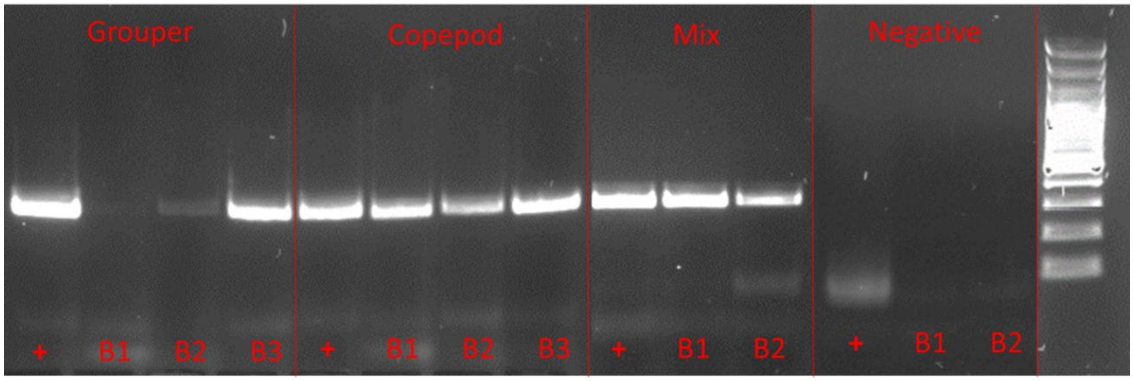


Fig 39. Selective amplification of copepod DNA versus DNA from grouper larvae. Blocking primers were designed and PCR reaction was optimised to suppress amplification of COX1 from grouper DNA template but not from copepod DNA. Primers used were: + = universal primers only; B1, B2, B3 = blocking primers paired with a universal primer. Templates were grouper, copepod and mix (grouper + copepod) DNA. Sequencing of PCR product from Mix template using B2 blocking primer revealed 93% similarity with copepod, confirming the effectiveness of the blocking primer in allowing selective amplification of the copepod DNA only. (Images: L. Dennis)

Genetic approaches to broodstock management

Apply genetic markers to prevent inbreeding and enable genetic selection

Establishing parentage of offspring is essential in the management of giant grouper broodstock in order to prevent inbreeding in the succeeding generations. There have been previous reports that most giant groupers in the market are derived from inbred stock (Kuo, 2014). According to data from this study, about 2/3 of giant groupers mature first as functional females (Palma et al. 2019a). By the time they change to functional males, their offspring are already maturing as females, hence there is a chance that the offspring's mother will eventually become the father of the generation that follows. We validated microsatellite markers, which together with mitochondrial DNA markers, provided full confidence in genotyping giant grouper broodstock and assigning parentage. Using these markers, we found that in a series of spawning events, the dominant male is the predominant sire on the first two days of spawning, while as the series of spawning events progressed, the other males also participated, contributing to successful fertilisation (Fig 40A, 40B). In contrast, hierarchy was not observed among female broodstock (Fig 40C, 40D). These observations indicate that genetic diversity in F1 broodstock can be maximised by taking offspring from spawns later in the series rather than just from the first couple of spawns (Bright et al. 2016a, Appendix 4). This is an important finding that should be considered when implementing a genetic management program for groupers as well as routine husbandry. In addition, the microsatellite markers are especially important when fish are selected for colouration, a trait that can command high price.

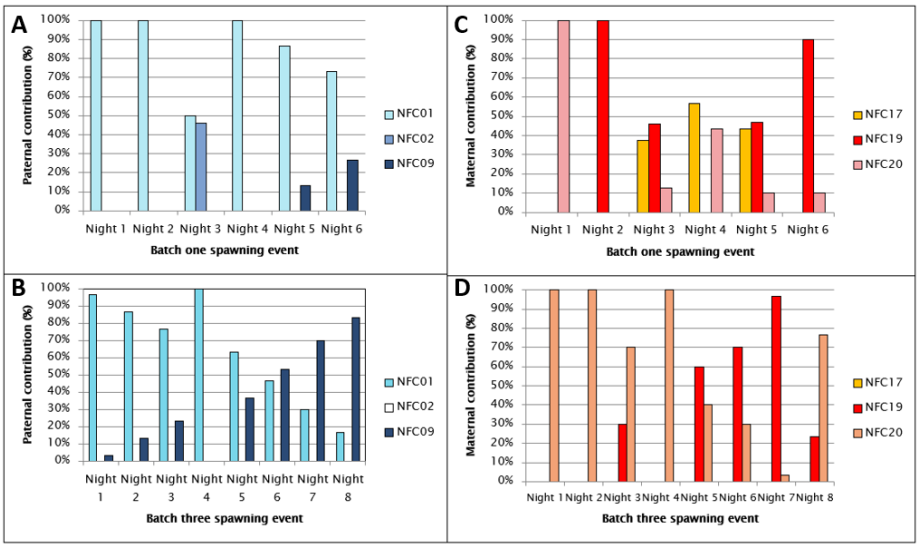


Fig 40. Paternal (A and B) and maternal (C and D) assignment rate of giant grouper offspring for three consecutive batches of spawns. Modified from Bright et al. (2016).

In addition to pedigree assignment, the DNA markers were also applied to confirming grouper species and identifying their geographic source. During the SRA phase of this project (FIS/2012/037), we found previously incorrectly identified species among the giant grouper broodstock in Vietnam. In a genotyping study conducted in the Philippines, we identified private alleles among the DNA markers that can distinguish giant grouper populations (Fig 41). Since importation of groupers, either of broodstock or eggs and fingerlings, is a common industry practice, the DNA markers validated in this project would be extremely useful as a management tool in confirming the species and tracking the geographic source of imports, especially those intended for broodstock.

Fig 41. Results of genotyping study of giant grouper broodstock in SEAFDEC/AQD, Philippines. Fish were sourced from two provinces, Iloilo and Palawan. Encircled are private alleles indicating specific geographic source of the broodstock.

Viral nervous necrosis (VNN) is a devastating disease in groupers and therefore VNN resistance is a highly desirable trait for selection (Knibb et al. 2017). In this project, a study on the viral load of hybrid groupers affected by a VNN outbreak showed that dead larvae had 8 times log VNN than those that survived (Fig 42A, B). Parentage assignment of the samples with species-specific alleles successfully determined 23 putative full-sib families for 121 offspring in the 161 samples analysed. Analysis of these families showed a significant correlation between family mean values for mortality and for VNN levels (Pearson correlation = 0.916, $p<0.01$) (Fig 43A, B).

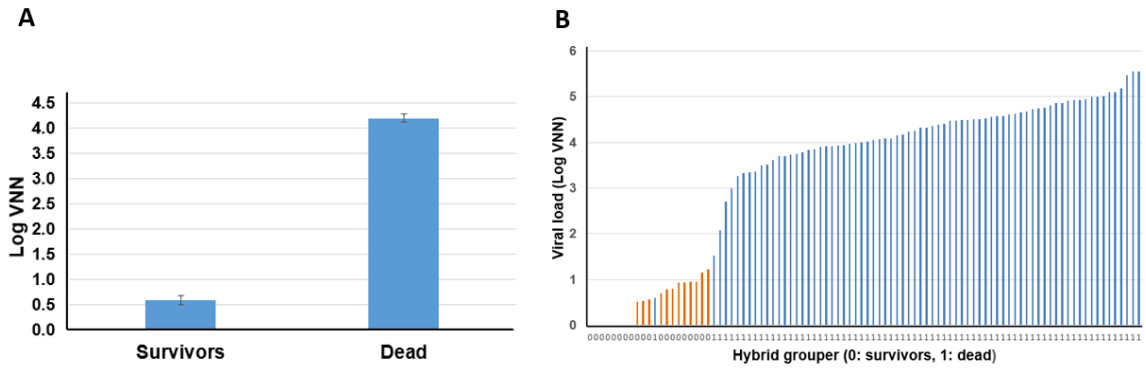


Fig 42. Viral load of hybrid groupers affected by a VNN outbreak. Individual family values averaged in A are shown in B.

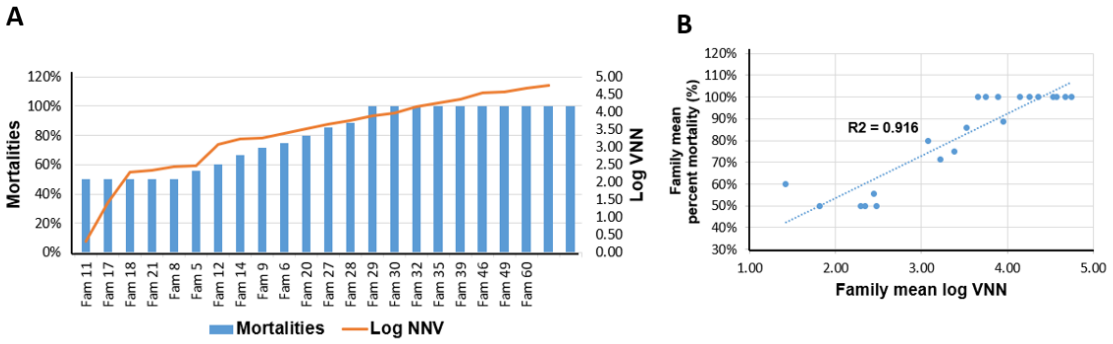


Fig 43. Significant correlation between family mean values for mortality and for VNN levels (Pearson correlation = 0.916, p<0.01).

Analysis of the nucleotide sequences encoding for the capsid protein of the nervous necrosis virus (NNV) revealed unique markers of the virus from different geographic regions (Fig 44). However, analysis of the deduced protein sequences from the nucleotide sequences showed a high level of conservation, indicating that the different viral strains can survive regardless of geographic location. Results imply that repeat infections are likely if biosecurity measures are not exercised when moving groupers from one region to another or within a region (Knibb et al. 2017).

Region	Species	Year	Sample Type	Cull†	Geno-type	Accession no.	12	18	60	69	99	120	132	135	144	192	222	234	336	405	417	480	492	564	579	594	597	627	630	648	654	663	679	700	721	702	738	741	747	766	792	795	825	828	834	837	866	868	869	915	918	925	939	945	960	969
Indon	Grouper H	2008J	F	0	18	HQ859924	.	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	A	T	.	.	.
Indon	Grouper H	2008J	F	3	18	HQ859925	.	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	A	T	.	.	.
Indon	Grouper H	2008J	F	3	18	HQ859926	.	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	C	.	.	.	.	A	T	.	.	.
Indon	Grouper T	2008J	F	1	17	HQ859927	.	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	T	A	T	.	.	.	.	.	.	.	.	.	.	A	T	T	C	T
Indon	Grouper T	2008J	F	3	17	HQ859928	.	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	T	A	T	.	.	.	.	.	.	.	.	.	.	A	T	T	C	T
Indon	Grouper T	2008J	F	3	17	HQ859929	.	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	T	A	T	.	.	.	.	.	.	.	.	.	.	A	T	T	C	T
Mal 1b	Grouper T	2008D	F	1	46	HQ859938	.	.	.	T	C	A	A	.	.	.	.	.	.	.	.	A	.	.	C	G	.	C	G	.	T	T	.	G	.	T	.	.	.	.	T	.	.	.	C	C	T	A	C	.	A	A	T	.	.	T
Mal 1b	Grouper T	2008D	F	3	46	HQ859939	.	.	.	T	C	A	A	.	.	.	.	.	.	.	.	A	.	.	C	G	.	C	G	.	T	T	.	G	.	T	.	.	.	.	T	.	.	.	C	C	T	A	C	.	A	A	T	.	.	T
Mal 1b	Grouper T	2009A	F	1	14	HQ859945	.	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	G	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	A	T	.	.	.
Mal 1b	Grouper T	2009A	F	1	14	HQ859946	.	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	G	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	A	T	.	.	.
Mal 1b	Grouper T	2009A	F	3	14	HQ859947	.	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	G	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	A	T	.	.	.
Mal 2	Grouper T	2009J	F	0	56	HQ859940	.	T	.	.	C	A	.	G	.	.	.	.	.	C	.	.	.	.	.	.	A	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	T	A	C	.	.	.	.	A	.	.	C	.
Mal 2	Grouper T	2009J	F	3	56	HQ859941	.	T	.	.	C	A	.	G	.	.	.	.	.	C	.	.	.	.	.	.	A	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	T	A	C	.	.	.	.	A	.	.	C	.
Mal 2	Grouper T	2009J	F	3	56	HQ859942	.	T	.	.	C	A	.	G	.	.	.	.	.	C	.	.	.	.	.	.	A	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.	.	T	A	C	.	.	.	.	A	.	.	C	.
Singap	Grouper G	1991	C	0	28	AF281657	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.	C	.
Singap	Grouper G	2001	C	0	29	AF318942	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	T	.	C	.
Vietna	Grouper H	2015	F	0	36	USC Vie1	.	.	.	.	.	.	.	G	.	.	T	C	A	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	C	T	.	.	.	.	.	T	.	A	.	.	C	.
Vietna	Grouper H	2015	F	3	36	USC Vie2	.	.	.	.	.	.	.	G	.	.	T	C	A	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	C	T	.	.	.	.	.	T	.	A	.	.	C	.
Vietna	Grouper H	2015	F	3	36	USC Vie3	.	.	.	.	.	.	.	G	.	.	T	C	A	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	C	T	.	.	.	.	.	T	.	A	.	.	C	.
Vietna	Grouper H	2015	F	3	36	USC Vie4	.	.	.	.	.	.	.	G	.	.	T	C	A	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	C	T	.	.	.	.	.	T	.	A	.	.	C	.
Vietna	Grouper OS	2007	F	0	40	EF492143	.	.	.	.	.	.	.	G	.	.	T	C	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	.	A	.	.	C	.
Taiwan	Grouper G	2000	C	0	2	AF245004	.	A	.	.	.	.	.	G	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	A	.	.	C	.
Taiwan	Grouper G	2013	C	0	38	KM588181	.	.	.	.	.	.	.	G	.	.	T	C	.	C	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	.	.	.	.	T	.	A	.	.	C	.
Taiwan	Grouper GT	2015	F	0	11	USC Tai1	.	.	C	.	.	.	.	G	.	T	T	C	.	C	.	.	T	T	.	.	.	.	.	.	.	.	.	.	G	.	.	T	.	.	.	T	.	.	.	.	.	T	.	A	.	.	C	.
Taiwan	Grouper GT	2015	F	3	11	USC Tai2	.	.	C	.	.	.	.	G	.	T	T	C	.	C	.	.	T	T	.	.	.	.	.	.	.	.	.	.	G	.	.	T	.	.	.	T	.	.	.	.	.	T	.	A	.	.	C	.
Taiwan	Grouper GT	2015	F	3	11	USC Tai3	.	.	C	.	.	.	.	G	.	T	T	C	.	C	.	.	T	T	.	.	.	.	.	.	.	.	.	.	G	.	.	T	.	.	.	T	.	.	.	.	.	T	.	A	.	.	C	.
Taiwan	Grouper G	2000	C	1	3	AF245003	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	A	.	.	C	.
Taiwan	Grouper OS	2011	C	0	6	KT071606	.	.	.	.	.	.	.	G	.	.	T	C	.	C	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	G	T	.	.	.	.	C	.	.	.	T	.	A	.	.	C	.
Taiwan	Grouper RS	2003	C	0	27	EU391590	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.	A	T	.	.	.
Taiwan	Grouper sp	1995	C	0	34	AY690596	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.

Fig 44. Example of a region of the NNV RNA2 sequence showing nucleotides specific for the geographic source of the viral strain (modified from Knibb et al. 2017).

Hybridisations to achieve desirable traits (Vietnam, Philippines)

The availability of mature male giant grouper broodstock enabled the rapid expansion of the hybrid grouper industry in Vietnam. The cross between male giant grouper and female tiger grouper is the most popular as the offspring retains the fast growth and delicate flesh quality of giant grouper and disease resistance of tiger grouper. Fertilisation rates of hybrids range from 47% to 75% while hatching rate ranged from 56% to 80%. Survival rate from larvae to fry (1.5-2 cm) is 14.5% while from fry to fingerling (5-7 cm) is 70%. In grow-out, the survival rate from fingerling to harvest ranges from 65-70%. RIA3 has supplied either fertilised eggs (Table 6) or fingerlings (Table 7) to many farms (Fig 45-47). Hybrid grouper fingerling production in Vietnam now supplies 2/3 of the domestic demand greatly reducing imports mainly from Taiwan and China.

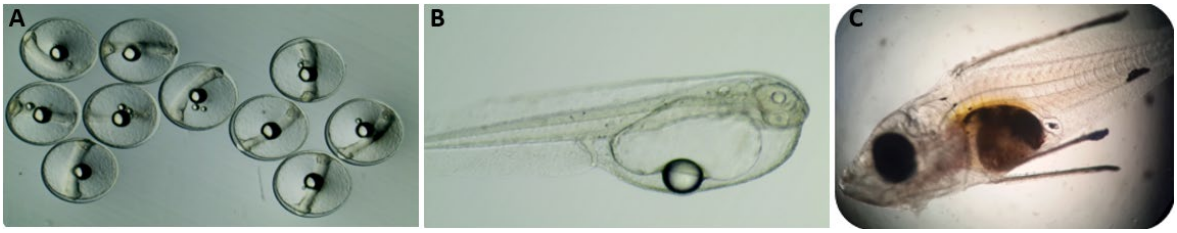


Fig 45. Fertilised hybrid grouper eggs (A), newly hatched larvae (B), 12 day post-hatch fry (C). (Images: T.Q. Thai)

Table 6. Fertilised hybrid grouper eggs supplied to Vietnamese farmers.

Province	Total/year (kg)	Nursing type	Year
Khan Hoa	5-6	Tank and pond	2018-2019
Binh Thuan	4-6	Pond	2018-2019
Vung Tau	1-2	Pond	2018-2019
Quang Ninh	5-6	Tank and pond	2017-2019

Fig 46. Fish farms in Vietnam receiving fertilised eggs. (A) Ms Nga, Binh Thuan province; (B) Mr Hoa, Khan Hoa province; (C) Mr Manh, Khan Hoa province (Images: T.Q. Thai)

Table 7. Hybrid grouper fingerlings supplied to farmers

Year	2015	2016	2017	2018	2019 (up to June)
No. of fingerlings	4,000	12,000	12,400	28,000	30,000
Grow-out farm area in Vietnam	North	North and Central	North, Central and South	North, Central and South	North, Central and South
Type of culture	Cage	Cage	Cage and pond	Cage and pond	Cage and pond
Feed	Trash fish	Trash fish	Trash fish and pellet feed	Trash fish and pellet feed	Trash fish and pellet feed

Fig 47. Fish farms in Khanh Hoa province, Vietnam, receiving fingerlings for grow-out. (A) Mr Quyen; (B) Mr Chuong; (C) Mr Hong. (Images: T.Q. Thai)

In addition to male giant grouper and female tiger grouper crosses, a cross between male giant grouper and orange spotted grouper has also shown promise as another grouper hybrid for aquaculture. Table 8 below summarises the fingerling production conducted at RIA3. In 2018, 8,000 fingerlings (5-7 cm) were supplied to grow-out farms in Kien Giang province while 10,000 fingerlings were supplied in the area early in 2019.

Table 8. Fingerling production of hybrid orange spotted grouper (female) with giant grouper (male).

Date	Activity description	Production data
October 2018	- 2 mature female orange-spotted groupers (average egg diameter 550 µm) were induced with hCG hormone similar to the treatment for the production of hybrids with tiger grouper. - 0.5 kg of fertilized eggs were used for fingerling production. The eggs were reared in 4 tanks (V=8m^3). The environmental parameters: salinity 30-33 ppt, temperature 27- 29°C, DO>5.2 mg/l, pH 7.5-8.0.	- Fertilization rate: 57% - Hatching rate: 60% - Survival rate: ~ 2% Produced a total of 8,000 hybrid fingerlings (5-7 cm).
February 2019	- 2 mature female orange-spotted groupers (average egg diameter 500 µm) were induced to spawn as above. 0.7 kg of fertilized eggs were obtained. The eggs were reared in 4 tanks (V=8m^3) and 1 tanks (V=12m^3). The environmental parameters: salinity 32-33 ppt, temperature 27-28°C, DO>5.0 mg/l, pH 7.8-8.2.	- Fertilization rate: 68% - Hatching rate: 75% - Survival rate: > 3% Total hybrid grouper fingerlings produced - 10.000 (5-7 cm).

At SEAFDEC/AQD in the Philippines, the hybrid between tiger grouper and orange-spotted grouper showed significantly higher growth rate compared to pure line orange-spotted grouper (Fig 48). In addition to having the advantages of fast growth and disease resistance, the grouper hybrids add versatility to the industry, making it more competitive and consumer-responsive. The apparent sterility of the hybrids make them also suitable as surrogate for giant grouper.

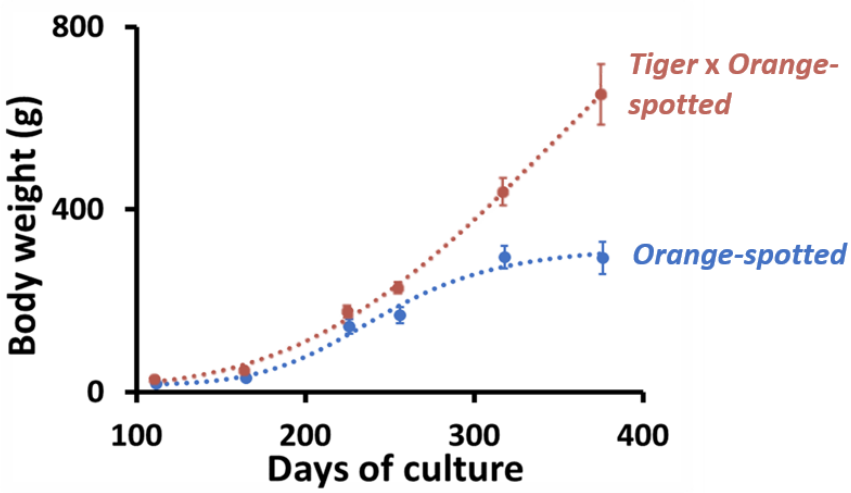


Fig 48. Growth rate of tiger grouper and orange-spotted grouper hybrid compared with pure line orange-spotted grouper.

Socio-economic impacts analysis of the hybrid grouper industry in Vietnam

The bio-economic study of the hybrid grouper industry in Vietnam revealed a rapidly expanding industry, which is segmented into three distinct phases of production: spawning or production of fertilised eggs, larval rearing and grow-out (Dennis et al. under revision; Appendix 6). Most farms are involved in only one production phase. Growing to market size is carried out either in sea cages (Fig 49A) or earthen ponds (Fig 49B), while the nursery phase is done in indoor tanks (Fig 49C).

Fig 49. Hybrid groupers are grown to market size in sea cages (A) and earthen ponds (B). The nursery phase is conducted in concrete indoor tanks (C). (Images: L. Dennis)

Hybrid grouper is desirable due to strong market demand, high sale price, fast growth and high survival rate. The 12 farms surveyed in Hai Phong in Northern Vietnam and Khan

Hoa in Central Vietnam generated profit in 2018 (Fig 50A), with the highest profits coming from earthen pond farms in Central Vietnam (Fig 50B). Compared with the different marine species for aquaculture, hybrid grouper ranked highest in terms of profits (Fig 51).

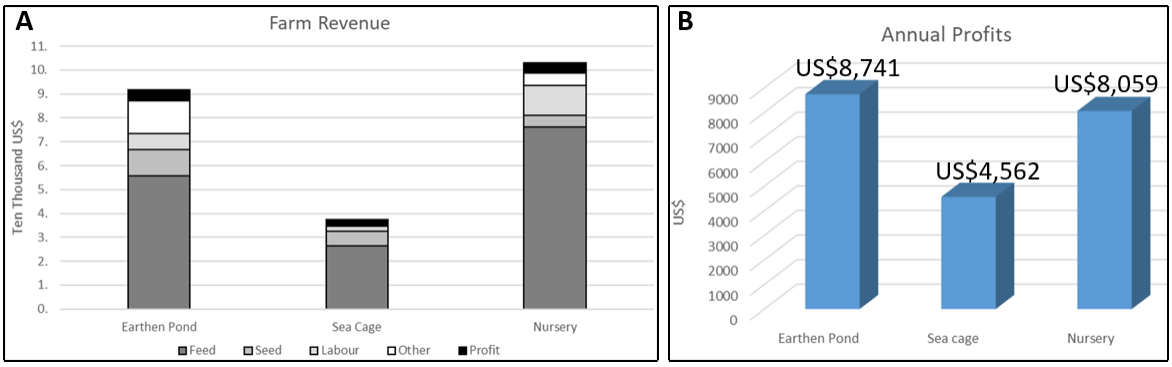


Fig 50. Farm revenue (A) and annual profits (B) profits among the surveyed farms involved in hybrid grouper culture (Dennis et al, under revision).

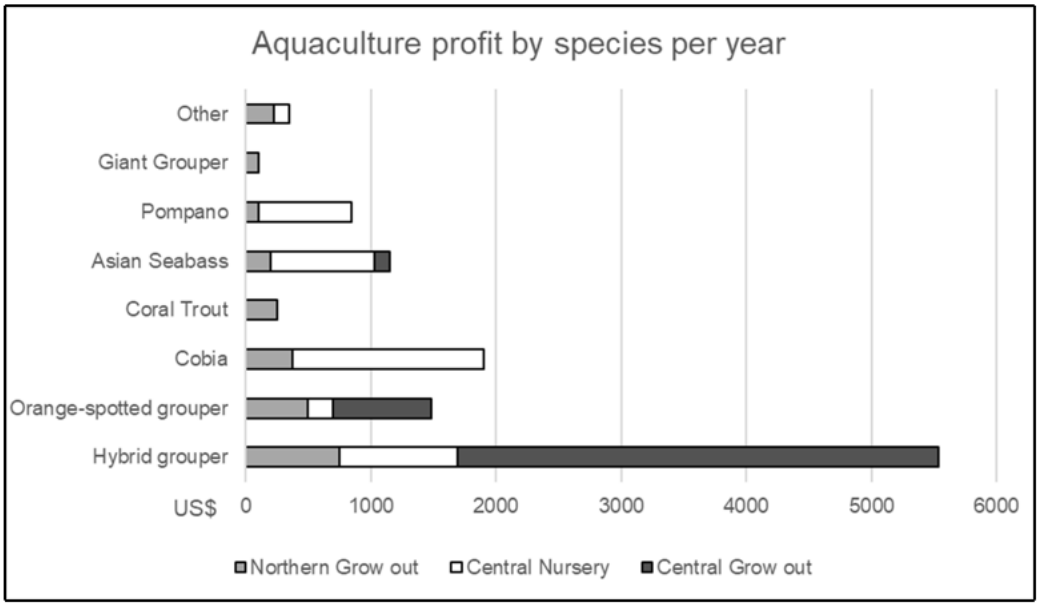


Fig 51. Profit by species among the surveyed farms involved in hybrid grouper culture (Dennis et al, under revision).

Farmers have identified constraints to the expansion of the hybrid grouper industry. The availability of hybrid fingerlings is still limited due to the difficulties in spawning giant grouper and challenges in larval rearing. Feed costs are increasing while domestic suppliers of feeds are lacking, and hybrid grouper diets not optimised. Nevertheless, results of surveys indicate that the industry will continue to expand based on the hybrid grouper as a new crop. Furthermore, the industry will continue to move towards a full life-cycle aquaculture to meet market demand (detailed in Dennis et al. under revision; Appendix 6).

The social sustainability benefits derived from the economic impacts of the industry were highlighted in a photovoice study which involved 10 randomly selected grouper farmers in Central Vietnam Respondents confirmed that grouper farming has provided positive outcomes for them, improving lives across dimensions of income and jobs, and is providing equitable opportunities for men, women and the young (Fig 52). The income

obtained provided for increased educational opportunities, learning new and sustainable skills for family members. Farmers also consider grouper farming as a viable long-term family venture that will benefit even the next generation. One of the downstream socio-economic benefits of grouper farming is being felt in the tourism industry, where demand for groupers is high among the domestic and international tourists, both of which are increasing in numbers especially in regions where groupers are farmed. During the survey, farmers have expressed issues that negatively impact the hybrid grouper industry. Among the biggest concerns are scarcity and high prices of feed, including trash fish, and problems due to climate change, such as increasing water temperatures and recurrent typhoons. Water pollution from a nearby sugar factory is also a worry for the farmers.

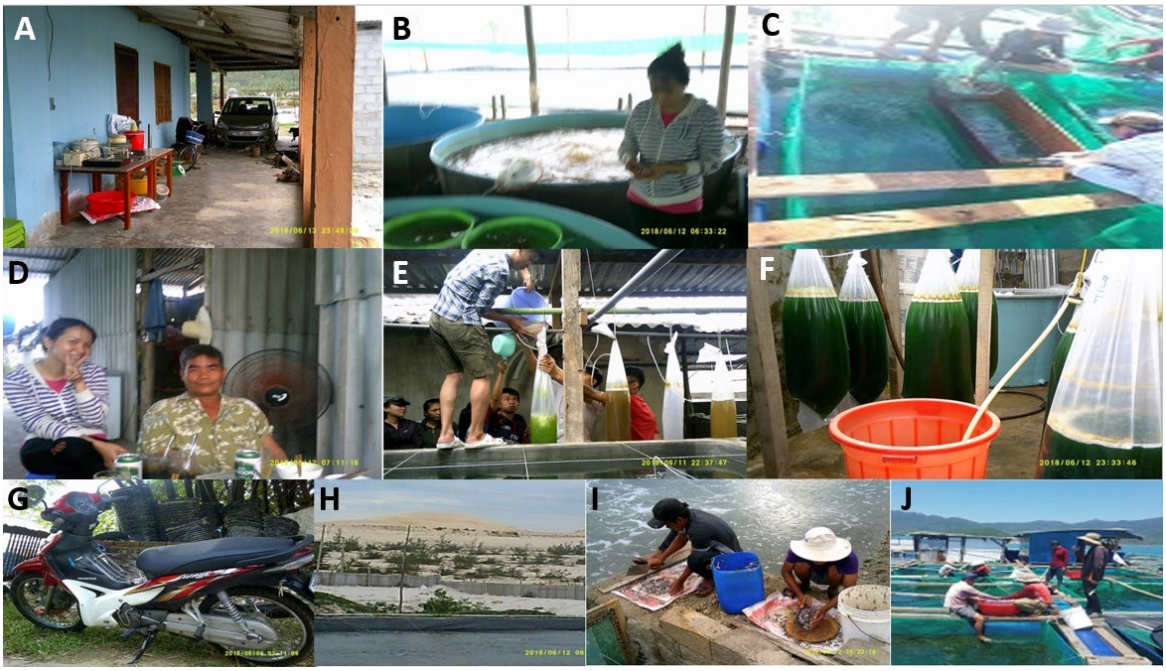


Fig 52. A collection of photos from the photovoice study showing the various aspects of grouper farming (except for A and G showing home and family transport upgrade resulting from income from grouper farming). (Images: J. Pierce)

From the sustainability perspective, grouper farming has given hope, purpose, income, jobs for men and women, better and more prosperous lives, promoted families working together, and has provided a wider positive impact on the communities within which grouper farms are located (Pierce, 2019).

8 Impacts

8.1 Scientific impacts – now and in 5 years

Scientific impacts are originating from novel knowledge, cutting edge tools and technologies we generated in this project. These were published in 7 peer reviewed journals, one of which under Nature Publishing. Further advances contained in 3 manuscripts currently in preparation are also contributing to these impacts. Among the key advances and tools are the discovery of diandry in giant grouper, synthetic recombinant hormones that stimulate maturation and sex change, immunoassays to measure reproductive hormones, germ cell transplantation techniques, oral delivery method of hormonal treatment and transcriptomic data. The scientific impacts of this project relate to grouper reproductive biology and aquaculture. They facilitate greater control over the life cycle of both giant grouper and other grouper species and generate a major shift away from wild-caught to aquaculture-reared farming in Australia, Vietnam and the Philippines. This already led to a paradigm shift in broodstock management and extends to the uptake of grouper hybrids in partner countries.

In the Philippines, recombinant hormones and oral delivery technologies developed in the project (Palma et al. 2019b,c) are being applied beyond groupers to accelerate the reproductive development of milkfish broodstock. Milkfish is one of the country's most valuable protein sources, particularly among the disadvantaged communities. The milkfish aquaculture industry in the Philippines is experiencing a severe shortage of milkfish fry, importing up to 50% of their requirement from Indonesia, which exposes the industry to biosecurity issues and poor genetics. The project is being carried out by our former John Allwright fellow and project collaborator, Mr Peter Palma. In addition to milkfish, the same technologies and scientific approach are also being applied in pompano, another important aquaculture species that does not spawn under culture conditions without hormonal stimulation.

The vitellogenin (Palma et al. 2019a) and FSH immunoassays (Nocillado et al. 2019) developed in this project were utilised to study the reproductive biology of Atlantic bluefin tuna, an endangered species according to the IUCN red list, by a collaborator in Italy, which culminated in a joint publication (Carnevali et al. 2019).

The optimised protocol for efficient production of synthetic recombinant proteins from groupers (Palma et al. 2019b) using the yeast expression system was also applied to produce a recombinant sea cucumber spawning hormone. The biological activity of the recombinant hormone was demonstrated by the successful stimulation of spawning of sea cucumber and is described in the published paper (*Frontiers in Genetics,* vol 10, Feb 2019), which is co-authored by three key USC participants of this project. Sea cucumbers worldwide are endangered due to overexploitation and its aquaculture is threatened by poor reproductive performance of captive broodstock. An Aus4innovation grant was awarded to Prof Abigail Elizur, this project's Australian project leader, together with Dr Nguyen Huu Ninh, the RIA3 project leader, to address the issue in sea cucumber aquaculture utilising the successful technology developed in this project.

In addition to country partners, we have established collaborations with groups in Japan (Institute for East China Sea Research, Nagasaki University and Tokyo University of Marine Science and Technology), Italy (Universitá Politecnica delle Marche), Canada (Department of Biology, University of Ottawa), Israel (Faculty of Agriculture, Food and Environment, Hebrew University), Taiwan (National Taiwan Ocean University) and New Zealand (New Zealand Institute for Plant and Food Research Ltd), attesting to the recognition of our achievements in the field. These new partners are adopting our technologies to develop culture technologies for the relevant species.

In the next 5 years, we anticipate further expansion of the scientific impacts of this project through enhancing the aquaculture of groupers and alleviation of their declining wild stocks, further development of aquaculture technologies in a variety of species, made possible as a result of the application of the novel knowledge obtained and tools developed in this project.

8.2 Capacity impacts – now and in 5 years

Substantial capacity impacts from this project are manifested in two ways: (1) Human resource capacity building- application and dissemination of the new knowledge and expertise obtained by John Allwright Fellows, USC trainees, HDR students and project staff in partner countries to various stakeholders both locally and internationally; and (2) Infrastructure capacity building- utilisation of the procured giant grouper broodstock as well as facilities and equipment in partner countries towards a rapidly growing grouper aquaculture industry.

Mr. Peter Palma, a John Allwright Fellow from the Philippines is now a Research Scientist at SEAFDEC/AQD, with his promotion granted exclusively thanks to the high quality of his publications during this project. He has now received funding to conduct independent research and is applying the expertise he gained during his post-graduate research towards the improvement of culture techniques of other economically important species in the Philippines such as milkfish and pompano. He also contributes to further capacity building of staff at SEAFDEC/AQD by sharing his expertise with other researchers. He participates as a lecturer in the international marine finfish training courses conducted by SEAFDEC/AQD, as well as the Center's internship program for fisheries and biology undergraduate students, high school students and technical staff from industry. From 2014-2019, SEAFDEC/AQD trained a total of 74 university students, 30 high school students and 48 staff from private farms on grouper aquaculture and seed and live food production. There were 16 training courses on marine finfish and grouper culture with a total of 181 participants from 18 countries (70% from the Philippines, 12% from other Southeast Asian countries and 13% from the Middle East, Africa and the Pacific Islands), 43% of which were women. Mr Joshua Superio and Ms Josielou Chan both underwent training in molecular and immunoassay technologies at USC and also contribute to training programs at SEAFDEC/AQD (Fig 53). The country's project leader, Dr. Evelyn Ayson, also led training courses on groupers and marine fish culture in the towns of Concepcion and Ajuy in Iloilo province, which were devastated by typhoon Haiyan in 2017 (Fig. 54). Former research assistants of the project have been awarded scholarships towards Masters' degree in European universities (2) and in the Philippines (1).

Fig 53. Ms Josielou Chan and Mr Joshua Superio, former USC trainees, demonstrating sampling techniques to students (A). High school and university student trainees with John Allwright Fellow Mr. Peter Palma (B), who also lectures and demonstrates in marine finfish training courses conducted by SEAFDEC/AQD (C, D). (Images: A&B-P. Palma; C&D-SEAFDEC/AQD)

In Vietnam, JAF Fellow Mrs. Luu Thi Giang was promoted upon her return from her MSc studies at USC to the position of Laboratory Manager at the Centre of Aquaculture Biotechnology, RIA1, and is conducting projects on the culture of giant catfish and pompano. Two staff from RIA1, Mrs Thao and Mr Hai, attended a training course on marine finfish hatchery at SEAFDEC/AQD supported by this project and are applying the techniques they learned in their current positions. Mrs Nga, who finished a short training course on genetic technologies at USC, is applying her skills in aquaculture projects at RIA3. Mr Tran The Muu, completed his PhD in Vietnam under this project. He is now a Vice Director at RIA1 Hanoi. Dr Truong Ha Phuong participated in a workshop on Marine Hatchery Techniques at the National Taiwan Ocean University. The skills and expertise obtained by key staff from RIA3 and RIA1 are disseminated to fish farmers, who consider their collaborative relationship with the two Institutes crucial in the growth of the hybrid grouper aquaculture industry (discussed further in economic and social impacts sections). In addition, RIA1 and RIA3 also trained a total of 45 Vietnamese university students through practical training courses on seed production technologies.

The infrastructure provided by the project to partner countries underpinned the scientific advances achieved in this project as well as the rapid growth of the hybrid grouper industry in Vietnam. Broodstock tanks (90 m^3; n=2), nursing tanks (400 m^3; n=4), nursing ponds (200 m^2), cages and nets (100 m^3; n=5) at RIA3 and RIA1 were constructed or upgraded. Equipment such as microscope, DO meter, scales, refractometer, chemicals, micro-rotifers and copepod stocks altogether facilitated the seed production and larval rearing activities. RIA3 acquired 16 new giant grouper breeders. RIA1 maintained a total of 50 breeders (18 males and 32 females). SEAFDEC/AQD procured 25 giant grouper (1-25 kg) and constructed 4 units of 10mx10m cages.

In the next 5 years, the downstream impacts of the dissemination of new knowledge and skills will become even stronger and widespread as the trainees continue to implement their acquired skills in their respective places of work. The broodstock procured by the project together with the improved facilities will provide long-term capacity for Vietnam and the Philippines to sustain the new aquaculture industry of hybrid groupers (giant grouper x tiger grouper and giant grouper x orange spotted grouper), which is expected to continue its expansion.

8.3 Community impacts – now and in 5 years

The project contributed to the positive socio-economic and environmental changes gained by stakeholders not directly identified with the project, for instance the tourism industry in Vietnam and the cyclone-stricken communities in Iloilo province in the Philippines.

8.3.1 Economic impacts

Farmers of hybrid groupers in Vietnam have higher disposable income as a consequence of profits resulting from the “mini-boom” of the hybrid grouper industry as shown in a study by Dennis et al. (accepted pending revision, *Aquaculture*) of 12 grouper farms in Northern and Central Vietnam. Consumers pay high market price for plate sized fish, and the short culture time of hybrids and the high survival during grow-out yield high profits for farmers. The average sale price for hybrid grouper has increased from US$7-8.8/kg to US$9-15/kg. All farms interviewed generated annual profit (Fig 50). Greatest profits came from earthen pond farms with a median profit of US$8,741 per year. Profits from Central region nurseries was US$8,059 per year while from Northern sea cage farms median profit was US$4,562.

A separate study by Dr. Janine Pierce that utilised photovoice revealed that the tourism industry is also deriving benefits from the availability of live groupers, which are popular among local consumers and international tourists that have been increasing in number in recent years. In the next 5 years, Vietnamese grouper farmers will continue to have additional income and enjoy better lives resulting from the booming hybrid grouper industry, which is not yet meeting demand.

In the Philippines, there is an opportunity for the grouper aquaculture industry to grow, particularly production of hybrids for the domestic market, as a result of the availability of mature male giant grouper broodstock, and knowledge in its culture. During this project, SEAFDEC/AQD has already demonstrated their capacity to produce hybrids as well as pure line giant grouper. Grouper aquaculture is currently limited, but SEAFDEC/AQD reports that all available grouper fingerlings are sold and waiting list exists for all species cultured indicating that strong demand is likely to continue and therefore grouper aquaculture in the Philippines will grow.

8.3.2 Social impacts

This project had a female project leader, a female country leader in the Philippines, two female research fellows in Australia and a female JAF fellow, showcasing females play strong leadership roles in aquaculture research and development both in Australia and internationally, and providing role models for ECR and MCR females.

There are already social impacts in partner countries from the project’s outputs. The photovoice study of grouper farming in Ninh Thuan, Khanh Hoa and Nha Trang provinces in Vietnam revealed that grouper farming is providing positive contributions to the livelihood and social wellbeing of the farmers, benefitting men, women and the young, increasing opportunities for education and to learn new skills. In addition, positive impacts are being derived by the tourism industry due to the popularity of hybrid groupers by local and international tourists who visit regions where grouper farms are located. During an extreme weather event in central Vietnam, RIA3 assisted grow-out farmers who lost their

sea cages by providing hybrid grouper fingerlings produced from the grouper broodstock at the Institute to establish new cage farms.

In the Philippines, SEAFDEC/AQD has been raising awareness about giant grouper biology, farming and the status of their wild population through their regular marine finfish hatchery training for international and local students. Protocols which have been developed at SEAFDEC/AQD for grouper broodstock management, spawning induction and larval rearing have been shared with partners in the industry, academia, and government, among others. These training and information dissemination programmes enhance the technical and leadership skills of the participants, enabling them to have better jobs as well as become effective leaders in their area of work.

The financial capacity of farmers and those along the supply chain, and the dissemination of enhanced skills and expertise by project partners to other sectors such as academia, government, and industry guarantee that the social impacts of this project will continue for years to come.

8.3.3 Environmental impacts

Giant groupers were listed as vulnerable in the most recent evaluation of the IUCN Redlist (now revised to Data Deficient), and it is a no-take species in Australia due to environmental concerns. The IUCN Redlist also notes that many species of groupers are vulnerable in parts of their range. The new knowledge gained on giant grouper reproductive biology together with the reproductive and genetic technologies developed in the project will contribute towards both a shift toward aquaculture rather than wild capture and may aid in more effective management of giant grouper stocks. The success of hybrid grouper as a new crop should relieve pressure on wild grouper stocks which are currently captured both as adults (for food) and as fingerlings that are caught for grow-out (often with very low survival rates). It is also reducing the risk of pathogens from imported fingerlings. New biological information regarding giant grouper reproductive biology that we obtained is available to advise policy and resource management guidelines.

8.4 Communication and dissemination activities

Activities were undertaken to disseminate the project outputs in as wide an audience as possible. There were nine publications in scientific journals, seven resulting directly from the project and two from collaborations. One publication was with Nature Publishing. As the review panel have noted, “many of the publications were “first-authored” by Vietnamese or Filipino staff, while almost all publications included them as co-authors”. There were seven international conference presentations, five of which were presented by partner country participants, and three of the conferences were hosted by a partner country. There were two completed Master theses (one from each partner country) and two PhD theses in progress (Australia). There is one submitted manuscript (accepted pending revision) and three manuscripts in preparation. As detailed in Section 8.2 (Capacity Impacts), numerous local high school and university students benefited from the training programs undertaken by partner institutions, as well as trainees from other Southeast Asian countries and from as far as the Middle East and Africa. SEAFDEC/AQD conducted training courses on grouper and marine fish culture to victims of typhoon Haiyan in Iloilo Province to provide alternative means of livelihood (Fig 54).

Fig 54. Dr Evelyn Ayson, country Project Leader for the Philippines, conducted training courses on grouper and marine fish culture to participants from the towns of Concepcion (A) and Ajuy (B) in 2017 (August and September, respectively). These towns in Iloilo province were worst hit by typhoon Haiyan. (Images: SEAFDEC/AQD)

In an episode of the Good Cooks on SBS Food Network, giant grouper in the Philippines was featured as a delectable fish suitable both for the home cooks and chefs alike. The program provided a promotional platform for giant grouper as an important aquaculture species which can benefit grouper farmers in marketing their produce. Similarly, in the September 2018 issue of ACIAR Vietnam newsletter, an article in Vietnamese featured the delightful dishes (Fig 55) prepared from Tran Chau Ca Mu, the hybrid grouper.

Fig 55. Gourmet feast - sashimi (A) and steamed (B) hybrid grouper. Yum! (Images: L.V. Chi)

9 Conclusions and recommendations

9.1 Conclusions

We achieved the project aim and objectives with scientific, capacity and community impacts already occurring during the life of the project, within and beyond the partner countries. One of the bottlenecks in giant grouper aquaculture is the late and large size at maturity of the species, more so with the males, which are mostly mature females that undergo sex reversal. The discovery of primary males, which are individuals that mature directly as males bypassing the female phase, is an important advance for aquaculture as smaller males can be sourced as broodstock. Together with the technologies developed in the project for accelerating male and female maturation through hormonal manipulation, and the potential of surrogate technology, for which an optimised germ cell transplantation protocol was shown to be feasible, it is possible to obtain mature male giant grouper less than 10 kg in size, which would be a huge advantage for grouper farmers. In parallel with the successful breeding of groupers, genetic tools were developed to prevent inbreeding and species misidentification, and biosecurity measures highlighted, which are essential in broodstock management of a sex-changing species.

Innovative approaches were employed to understand the feeding requirements of giant grouper larvae, another bottleneck in giant grouper aquaculture. Genes regulating appetite and digestion in early developing giant grouper larvae were identified using transcriptomics. This is complemented with DNA barcoding, to identify the food organisms ingested by larvae reared in a green water system, essentially revealing their food preference. In addition, feeding trials were conducted to identify alternative live foods, such as a strain of super small rotifers, and these were found to improve larval growth and survival rate.

Impacts at the community level in Vietnam are compelling as a result of the rapid growth of the hybrid grouper industry, which was underpinned by the capacity building efforts of the project. Capacity building in the Philippines is producing high calibre scientists and technical staff who are instrumental in disseminating the new knowledge and techniques generated by the project to various stakeholders in Southeast Asia and other parts of the world.

The project produced cutting edge tools and technologies, including, but not limited to, recombinant hormones in protein and DNA forms, specific antibodies, germ cell transplantation, immunoassay and molecular protocols, oral delivery method of hormonal treatment, transcriptomic data, sperm cryopreservation techniques, and culture methods for live foods. These have contributed to the enormous scientific impacts of the project that extend not only to the culture of other important species of marine fish but also to other species such as endangered sea cucumbers and frogs.

In conclusion, the ongoing scientific, capacity and community impacts are expected to continue in the next five years resulting from the wider application of the knowledge generated from the project. The impacts altogether will ensure grouper aquaculture is able to flourish, meeting both market demand and the need to alleviate the declining stocks of the species in the wild, and resulting in improved livelihoods for multiple communities as well as development of future enterprises, such as feed and seed production.

9.2 Recommendations

As already reported by the review panel, there are opportunities justifying continuation of the project to ensure impacts are strengthened in the long term.

There is a need to more reliably generate male giant grouper broodstock at a younger age and smaller size. This would ensure sufficient male broodstock are available for ongoing

commercial production and that adequate genetic diversity in stocks is available. This would have the dual advantage of underpinning any future enhancement of wild grouper populations that may arise from the overharvesting of this species. The heavy reliance on male giant grouper for hybrid production makes the discovery of “primary” males extremely valuable to future development and is a topic that warrants further research to determine what is underpinning this process and how it can be used for future production.

One of the major constraints to hybrid production is giant grouper milt supply. The misalignment of the reproductive seasons of the various grouper species and the relatively small number of giant grouper males in captivity suggests that new approaches should be developed, including consideration of the risks that could be posed by gamete distribution (genetics and diseases).

Hybrid fingerling production success is highly variable and generally stands at < 3%, which can be improved. Live feed production during early larval rearing was considered to be one area in which gains could be achieved with the “barcoding” approach used in this project warranting further work.

It is essential to develop grow-out diets for hybrid groupers. No grouper specific diet was developed, and certainly none directly designed for the growing hybrid grouper needs. The available formulated diets (pellets) have very poor feed conversion ratios. Trash fish is still widely used as feed however are less efficient than pellets as well as promoting adverse environmental outcomes. There are no mills in Vietnam that currently produce grouper feed, and trials to optimise growth would help the growth of the industry and the shift away from environmentally damaging trash fish. To address this important matter, while also addressing the growing concern regarding over reliance on fishmeal in fish diets, we propose to assess diets formulated from sustainable alternative protein source (including single cell proteins, insect meal and agriculture by-products), as well as innovative feeding strategy such as nutritional programming. We are already working with feed companies (Mavin, Cargill, Ocialia, CJ) who are interested in the uptake of formulated diets for the manufacturing for this species in Vietnam. The availability of hybrid grouper specific diet would greatly assist in the expansion of this industry. In Australia there is a great interest in the development of a grouper specific diet, with the sector growing through the increased supply of grouper fingerlings by The Company One to growers in Qld. Some of the prawn farms that were closed due to white spot virus are now growing groupers. The combination of classical assessment of different feed formulations including sustainable protein sources together with the exploration of dietary biomarkers using nutrigenomics and developing feeding management through nutritional programming would ensure sustainable growth of the industry and boast additional industries such as feed manufacturing and production of alternative sustainable protein sources.

There is also a need to comprehensively understand the reproductive biology of hybrid groupers to confirm whether they are sterile or not, as there are mixed reports on this matter. Escapees may pose a threat to grouper populations in the wild and could be a significant environmental liability, which could be managed if the age, time of reproduction and reproductive capacity of the hybrids are determined. This knowledge is essential to ensure a responsible and sustainable expansion of the industry.

A thorough socio-economic impact study of the hybrid grouper industry in Vietnam capturing multiple seasons should be conducted to ensure its sustainability. This study should include environmental and risk factors to ensure sustainable growth. Such a study would highlight other areas of constraint and could also serve as a model for the Philippines where the hybrid grouper industry is yet to take off.

These recommendations would expand the impacts from this project in the long term, enable further capacity building in partner countries, and examine in detail the socio-economic and environmental impacts of these endeavours, ensuring that benefits are equitable and sustainable.

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10.2 List of publications produced by the project

There were nine publications in peer reviewed journals, seven resulting directly from the project and two from collaborations developed during this project. One publication was with Nature Publishing. Editors of three journals featured our work on the cover of the journal (Fig 56). Many (71%) of the publications were first-authored by Vietnamese or Filipino staff, while almost all publications included Australian and partner country staff as co-authors. There were seven international conference presentations, five of which were presented by partner country participants, and three of the conferences were hosted by a partner country. There were two completed Master's theses (one from each partner country) and two PhD theses in progress (Australia). There is one submitted manuscript currently being revised and three manuscripts in preparation.

Fig 56. Cover feature of journals publishing our key findings on the (A) acceleration of female maturation and sex change in giant grouper (Palma et al. 2019b), (B) genes regulating appetite and digestion in giant grouper larvae (Anderson et al. 2018), and (C) non-invasive approach in administering hormonal treatment in tiger grouper (Palma et al. 2019c).

Published in refereed journals (directly from this project)

Palma P, Nocillado J, Superio J, Ayson EG, Ayson F, Lu MW, Elizur A. 2019. Orally delivered plasmid of follicle stimulating hormone induced gonadal development in tiger grouper (*Epinephelus fuscoguttatus*). **Marine Biotechnology** 21:697-706.

Palma P, Nocillado J, Superio J, Ayson EG, Ayson F, Bar I, Elizur A. 2019. Gonadal response of sexually immature protogynous grouper (*Epinephelus fuscoguttatus*) to long-term recombinant follicle-stimulating hormone administration. **Biology of Reproduction** 100 (3): 798-809.

Palma P, Takemura A, Libunao GX, Superio J, DeJesus-Ayson E, Ayson F, Nocillado J, Dennis L, Chan J, Thai TQ, Ninh NH, Elizur A. 2019. Reproductive development of the threatened giant grouper *Epinephelus lanceolatus*. **Aquaculture** 509: 1-7.

Anderson K, Knuckey R, Canepa M, Elizur A. 2018. A transcriptomic investigation of appetite regulation and digestive processes in giant grouper, *Epinephelus lanceolatus*, during early larval development. **Journal of Fish Biology** 93(4): 694-710.

Anderson K, Kuo CY, Lu MW, Elizur A. 2018. A transcriptomic investigation of digestive processes in orange-spotted grouper, *Epinephelus coioides*, before, during, and after metamorphic development. **Gene** 661: 95-108.

Knibb W, Luu G, Premachandra HKA, Lu MW, Nguyen HN. 2017. Regional genetic diversity for NNV grouper virus across the Indo-Asian region – implications for selecting virus resistance in farmed groupers. **Scientific Reports** 7: 10658.

Bright D, Reynolds A, Nguyen NH, Knuckey R, Knibb W, Elizur A. 2016. A study into parental assignment of the communal spawning protogynous hermaphrodite, giant grouper (*Epinephelus lanceolatus*). **Aquaculture** 459: 19-25.

Published in refereed journals (arising from collaborations)

Carnevali O, Maradonna F, Sagrati A, Candelma M, Lombardo F, Pignalosa P, Bonfanti E, **Nocillado J**, **Palma P**, Gioacchini G, **Elizur A**. 2019. Insights on the seasonal variations of reproductive features in the Eastern Bluefin tuna. **General and Comparative Endocrinology** 282: 113216.

Chieu HD, Turner L, Smith MK, Wang T, **Nocillado J**, **Palma P**, Suwansa-ard S, **Elizur A**, Cummins SF. 2019. Aquaculture breeding enahancement: maturation and spawning in sea cucumbers using a recombinant relaxin-like gonad-stimulating peptide. **Frontiers in Genetics** 10, 77.

Nocillado J, Palma P, Fielder S, Zanardini M, **Dennis L, Elizur** A. 2019. Development of specific enzyme-linked immunosorbent assay for yellowtail kingfish (*Seriola lalandi*) follicle stimulating hormone using recombinant gonadotropins. **General and Comparative Endocrinology** 282: 113208.

Manuscript accepted pending revision

Dennis LP, Ashford G, Thai TQ, Vu VI, Ninh NH, Elizur A. Hybrid grouper in Vietnamese aquaculture: production approaches and profitability of a promising new crop. Submitted to **Aquaculture; under revision**.

Manuscripts in preparation

Palma P, Chan J, Superio J, Libunao GX, de Jesus-Ayson EG, Ayson F. Preservation of economically important grouper (*Epinephelus* spp.) spermatozoa at low and ultra-low temperatures. To be submitted to Theriogenology.

Palma P, Nocillado J, de Jesus-Ayson EG, Wang TF, Brooks P, Levavi-Sivan B, Shpilman M, Elizur A. Feeding of recombinant GnRH1 expressed intracellularly in yeast stimulated gonadal development in immature *Epinephelus coioides*. To be submitted to Journal of Biotechnology.

Dennis LP, Nocillado J, Palma P, Takafumi A, Soyano K, Elizur A. Development of a giant grouper luteinizing hormone enzyme-linked immunosorbent assay (LH ELISA) and its use in following sexual development in grouper. To be submitted to General and Comparative Endocrinology.

Conference presentations

Palma P, Nocillado J, Superio J, Ayson EG, Ayson F, Bar I, Elizur A. Studies towards advancing reproductive development in giant grouper (*Epinephelus lanceolatus*) using recombinant hormone manipulation. Oral presentation at the 12th Asian Fisheries and Aquaculture Forum, Iloilo Convention Center, Iloilo, Philippines. April 2019. **Awarded as best student research presentation (postgraduate level).**

Palma P, Takemura A, Libunao GX, Superio J, de Jesus-Ayson EG, Ayson F, Nocillado J, Dennis L, **Chan J**, Thai TQ, Ninh NH, Elizur A. Reproductive development of the threatened giant grouper *Epinephelus lanceolatus*. Oral presentation at the 12th Asian Fisheries and Aquaculture Forum, Iloilo Convention Center, Iloilo, Philippines. April 2019.

Palma P, **Chan J**, Superio J, Libunao GX, de Jesus-Ayson EG, Ayson F. Preservation of economically important grouper (*Epinephelus* spp.) spermatozoa at low and ultra-low temperatures. Oral presentation at the International Science, Technology, and Engineering Conference, Bohol, Philippines. May 27-30, 2019.

Palma P, Nocillado J, Superio J, Ayson EG, Ayson F, Bright D, Bar I, Elizur A. Recombinant follicle stimulating hormone-induced testicular development in immature protogynous grouper (*Epinephelus sp*.). Poster presented at the **18th International Congress on Comparative Endocrinology**, Chateau Lake Louise, Canada. June 2017.

Palma P, Nocillado J, Superio J, Ayson EG, Ayson F, Bright D, Bar I, Elizur A. Smaller and younger broodstock. Poster presented at **2017 USC Research Showcase**. November 2017. (Poster awarded runner-up)

Bright D, Nocillado J, Canepa M, Reynolds A, Knuckey R, Elizur A. 2016. Social structure affects reproductive development and sex change in giant grouper (*Epinephelus lanceolatus*). Poster presented at the 8th International Symposium on Fish Endocrinology, Gothenburg, Sweden. 28 June – 2 July 2016.

Nocillado J, Palma P, Bright D, Elizur A. 2016 Application of recombinant follicle stimulating hormone to manipulate reproduction in giant grouper, *Epinephelus lanceolatus*. Poster presented at the 8th International Symposium on Fish Endocrinology, Gothenburg, Sweden. 28 June – 2 July 2016.

Conference presentation arising from collaboration

Carnevali O, Maradonna F, Bovari G, Lombardo F, Pignalosa P, Bonfanti E, **Nocillado J**, **Palma P**, Gioacchini G, **Elizur A**. 2018. Variations of steroid hormones and FSH levels in Atlantic bluefin tuna caught in the Mediterranean Basin during spawning and post-spawning season. Poster presented at the 11th Symposium on Reproductive Physiology of Fish. Manaus, Brazil. June 2018.

MSc Theses (JAF Fellows)

Palma P. 2017. Studies towards advancing reproductive development in giant grouper (*Epinephelus lanceolatus*) using recombinant hormone manipulations. University of the Sunshine Coast.

Luu THG. 2017. Molecular approaches to better understand the epidemiology and host resistance to nervous necrosis virus in Asian groupers. University of the Sunshine Coast.

11 Appendices

11.1 Appendix List:

Below is the list of Appendices submitted as separate files for those without copyright restrictions. For published materials with copyright, files can be obtained online through the digital object identifier (DOI) link.

Number	Content
1	Supplementary Tables
2	Anderson K, Knuckey R, Canepa M, Elizur A. 2018. A transcriptomic investigation of appetite regulation and digestive processes in giant grouper, *Epinephelus lanceolatus*, during early larval development. **Journal of Fish Biology** 93(4): 694-710. DOI:10.1111/jfb.13798
3	Anderson K, Kuo CY, Lu MW, Elizur A. 2018. A transcriptomic investigation of digestive processes in orange-spotted grouper, *Epinephelus coioides*, before, during, and after metamorphic development. **Gene** 661: 95-108. DOI.org/10.1016/j.gene.2018.03.073
4	Bright D, Reynolds A, Nguyen NH, Knuckey R, Knibb W, Elizur A. 2016. A study into parental assignment of the communal spawning protogynous hermaphrodite, giant grouper (*Epinephelus lanceolatus*). **Aquaculture** 459: 19-25. DOI.org/10.1016/j.aquaculture.2016.03.013
5	Knibb W, Luu G, Premachandra HKA, Lu MW, Nguyen HN. 2017. Regional genetic diversity for NNV grouper virus across the Indo-Asian region – implications for selecting virus resistance in farmed groupers. **Scientific Reports** 7: 10658. DOI:10.1038/s41598-017-11263-4
6	Dennis LP, Ashford G, Thai TQ, In VV, Ninh NH, Elizur A. Hybrid grouper in Vietnamese aquaculture: production approaches and profitability of a promising new crop. Submitted to **Aquaculture***, under revision
7	Pierce J. 2018. Impact of grouper farming in Vietnam through the grouper farmer lens, Photovoice Report*
8	Palma P, Takemura A, Libunao GX, Superio J, DeJesus-Ayson E, Ayson F, Nocillado J, Dennis L, Chan J, Thai TQ, Ninh NH, Elizur A. 2019. Reproductive development of the threatened giant grouper *Epinephelus lanceolatus*. **Aquaculture** 509: 1-7. DOI:org/10.1016/j.aquaculture.2019.05.001
9	Palma P, Nocillado J, Superio J, Ayson EG, Ayson F, Bar I, Elizur A. 2019. Gonadal response of sexually immature protogynous grouper (*Epinephelus fuscoguttatus*) to long-term recombinant follicle-stimulating hormone administration. **Biology of Reproduction** 100 (3): 798-809. DOI:10.1093/biolre/ioy228
10	Palma P, Nocillado J, Superio J, Ayson EG, Ayson F, Lu MW, Elizur A. 2019. Orally delivered plasmid of follicle stimulating hormone induced gonadal development in tiger grouper (*Epinephelus fuscoguttatus*). **Marine Biotechnology** 21:697-706. DOI.org/10.1007/s10126-019-09914-w
11	Carnevali O, Maradonna F, Sagrati A, Candelma M, Lombardo F, Pignalosa P, Bonfanti E, Nocillado J, Palma P, Gioacchini G, Elizur A. 2019. Insights on the seasonal variations of reproductive features in the Eastern Bluefin tuna. **General and Comparative Endocrinology** 282: 113216. DOI.org/10.1016/j.ygcen.2019.113216

12	Nocillado J, Palma P, Fielder S, Zanardini M, Dennis L, Elizur A. 2019. Development of specific enzyme-linked immunosorbent assay for yellowtail kingfish (*Seriola lalandi*) follicle stimulating hormone using recombinant gonadotropins. **General and Comparative Endocrinology** 282: 113208. DOI.org/10.1016/j.ygcen.2019.113208
13	Video: Giant grouper showing territorial behavior which is characteristic of males and indicator of diandry
14	Bright et al. 2016 ISFE8 Gothenburg, Sweden
15	Nocillado et al. 2016 ISFE8 Gothenburg, Sweden
16	Palma et al. 2017 18th ICCE Alberta, Canada
17	Palma et a. 2017 USC Research Showcase
18	Dennis LP, Nocillado J, Palma P, Takafumi A, Soyano K, Elizur A. Development of a giant grouper luteinizing hormone enzyme-linked immunosorbent assay (LH ELISA) and its use in following sexual development in grouper. To be submitted to General and Comparative Endocrinology*.
*	The documents with an * cannot be published with the report on the web as they are currently under review or not submitted as yet.