Development of broodstock conditioning-, induced spawning-, and hatchery methods for the South African spotted grunter (*Pomadasys commersonnii*).

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ABSTRACT

The domestication of candidate marine finfish aquaculture species is an indispensable program to procure undisturbed supply of larvae for research or commercial purposes. The current study focused on a widely distributed haemulid candidate species locally called the spotted grunter (*Pomadasys commersonnii*). The spotted grunter is a high valued species, described as premier fish with fleshy meat and taste. It is an ideal aquaculture species because it grows fast, can tolerate salinity of less than 1 to 90 %, the females spawn more than once during the spawning period, juveniles of this species can be reared in high stocking density, and accept a supplementary diet (economic pond culture). The current study is a first to experimentally develop efficient procedures for controlled reproduction of captive reared spotted grunter broodstock, and with tested repetitiveness.

Broodstock females and males of two years and older were include. Spotted grunter did not spawn spontaneously in captivity, though pre-spawn conditioning of F1 and F2 breeders could be achieved by applying the following developed procedures which include spawning induction: (1) feeding breeders with squid and/or pilchards (3% body mass/day) and rearing them in a fully equipped marine recirculating aquaculture system at the marine research aquarium of the Department of Forestry, Fisheries and the Environment (DFFE) at Sea Point, Cape Town, (2) maintaining a stocking density of about 4 - 7 kg/m³, (3) applying a photothermal program with photoperiod exposure set consistently at 12 Dark: 12 Light (12D: 12L) (250 lux) – with thermal program split in a 3 week consistent exposure setting (24 ± 0.05 ºC), followed by 2 day cycles (x3) with temperature fluctuation of ± 5 °C per cycle. Histological examination of the ovaries indicated that final ovarian maturation status was achieved in conditioning programs, but ovulation and spawning needed be induced artificially in captive breeders. Spawning induction was facilitated by finally increasing water temperature from 19 to 24 °C, and finally using Ovaprim® (gonadotropin-releasing hormone analogue [GnRHa]) as decapeptide (spawning inducer (0.25 mℓ/kg). The Ovaprim® was applied intramuscularly

(IM) at only 50 % of the prescribe dose (0.5 mℓ/kg). The physical handling, however, resulted in a stress response of non-feeding for about one week. This unavoidable period of prolonged stress in spotted grunter broodstock following artificial induction of spawning, must be considered in broodstock management strategies.

The reproductive capacity of spotted grunter broodstock is a function of size and age, since GnRHa-induced broodstock older than four years were predominantly non-responsive to spawning induction interventions (reproductively dysfunctional). Females in the two to three years age range performed reliably with predictable and highly fecund maximized spawning output success. Young breeders in this age group spawned eggs with gradually increased quantities and consistently high fertilization rates when exposed to the conditioning programs without interruptions. The histology of their ovaries showed cohorts of oocytes at different stages of maturity: perinucleolar stage of the primary growth of the oocyte, primary oocyte, cortical alveoli secondary oocyte, late vitellogenic oocyte and hydrated oocytes. This, together with the spawning frequency data, confirms that spotted grunters are group asynchronized multi-batch spawners. Where synchronized spawners release all their eggs of similar maturity stage and deplete their ovaries fully, asynchronized spawners will not release all their eggs but only those batches that are fully developed to pre-spawn stage. The unreleased premature intraovarian oocytes will develop further in batches and be available as mature eggs following subsequent conditioning programs.

The latency time from spawning induction to first spawning activity was about 32 h at 24 ± 0.05 ºC. Broodstock conditioning success was marked by a relatively high collective fertilized egg yield (> 97 %) which was indicative that high quality gametes were produced with trial repeated consistency. The collective quantity of spawned eggs was substantively increased when the same breeders were exposed to the mentioned thermal fluctuation program, as compared to a constant temperature exposure only (24 ± 0.05 ºC). Fertilized eggs were positively buoyant (floating/pelagic), whilst non-fertilized eggs were negatively buoyant (sinking).

The diameter of the fertilized eggs was 1.52 -1.60 mm (mean ± SE = 1.54 ± 0.01 mm, *n* = 10) and the eggs hatched within about 24 - 26 h after fertilization at 21 ± 0.05 ºC. The length of the larvae 5 h after hatching was 2.44 - 2.51 mm (*n* =10). Larval growth and survival were monitored for the first seven days post-hatch (DPH) under three distinct feeding set-ups with approximately 8000 hatchlings each: (i) clear water (filtered sea water) that served as the control treatment; (ii) algae rich *Nannochloropsis oculata* water at a final concentration of 500 x 10^3 -1 x 10^6 cells/mℓ , and (iii) 0. 125 g/ℓ of bentonite clay. These feeding set-ups were exposed to an overhead illumination of 750 lux and maintained at 21 ± 0.05 °C. Mortality rates were high between one and three DPH, and the survival steeply declined from four to six DPH, irrespective of larval density, until ≤ 20 larvae remained at three DPH in all the treatments. At three DPH, the clear water treatment still demonstrated high larval survival of 5198 ± 458.31 compared to 2843 ± 564.33 larvae in tanks of *N. oculata* and 3092 ± 407.41 larvae from the bentonite clay treatment. At six DPH, the larval survival was approximately 5.1 % for both the clear water and *Nannochloropsis* treatment groups, whilst survival of the bentonite treated group was slightly better at 8.5 %.

The growth rate of the *P*. *commersonnii* larvae were not significantly different ($p > 0.05$) across the treatment groups from hatching to two DPH. Clear water treatment demonstrated superior growth from three DPH until termination (seven DPH) obtaining a final averagelength of 3285 ± 41.32 µm (mean ± SE). While the growth rate was not significantly different ($p > 0.05$) at three DPH between *N*. *oculata* and bentonite treatments, the growth of larvae reared in bentonite clay suspension was significantly more ($p < 0.5$) than *N*. *oculata* at trial termination, achieving respective final lengths of 3106 ± 25.35 µm and 2908 ± 9.20 µm.

The development of larval eye pigmentation was first observed three DPH and feeding commenced four DPH in all the treatments. Throughout the rearing trials, the larvae fed close to the surface area, whilst no cannibalism was observed. Inflation of the swim bladder was observed at five DPH, until termination of the rearing trial (seven DPH). The survival of larvae was low in all treatments, and this was attributed to culture

contamination with an opportunistic and pathogenic microbial species introduced with the live rotifers into the hatchery, as well as with introduced diluted algae cultures. Microbial contamination persisted in all larval rearing trial. It is very likely that the microbes proliferated in the hatchery tanks as a result of the unavoidable increase in organic material (uneaten food and larval metabolic waste). These aspects deserve intensive investigation in future research.

In conclusion, the current study successfully developed procedures for all aspects of controlled and programmable spotted grunter reproduction under captive conditions. Weaned juveniles (21 DPH) could only be produced under high algae density conditions, which act as a probiotic against pathogenic microbes introduced by zooplankton such as rotifers (first feed). The developed hatchery procedures can now be applied in presumptive commercial spotted grunter hatcheries, which can also be utilized for stock recruitment in stock replenishment programs such as for estuaries or species conservation areas.

TABLE OF CONTENTS

DECLARATION i

ACKNOWLEDGEMENTS ii

ABSTRACT iv

TABLE OF CONTENT viii

LIST OF FIGURES x

LIST OF TABLES xii

CHAPTER 1: GENERAL INTRODUCTION **1**

1.1 Global overview of aquaculture 2

1.2 Aquaculture and finfish production status in South Africa 4

1.3 Natural reproduction and spawning induction of finfish 5

1.3.1. The hormonally regulated natural reproductive cycle 5

1.3.2. Reproductive challenges in captivity 7

1.4 Larval rearing and pseudogreen water technique 10

1.5 The spotted grunter, *Pomadasys commersonnii* 18

1.5.1 Species selection and the taxonomy of *P*. *commersonnii* 18

1.5.2 External morphology of *P*. *commersonnii* 20

1.5.3 Origin and distribution of *P*. *commersonnii* 21

1.5.4 Feeding biology of *P*. *commersonnii* 21

1.5.5 Natural reproduction of *P*. *commersonnii* 22

1.6 Aims and objectives of the study 25

1.6.1 Goal 25

1.6.2 Objectives 25

1.7 Null hypothesis 26

1.8 Research questions 26

CHAPTER 2: **BROODSTOCK CONDITIONING** **28**

2.1 Introduction 28

2.2 Objectives 32

2.3 Material and methods 32

2.3.1 Study animals 32

2.3.2 Place of study 32

2.3.3 Ethical clearance 32

2.3.4 System design 33

2.3.5 Broodstock conditioning and experimental treatments 36

2.3.6 Gonadosomatic index and the histology of the ovaries 37

2.3.6.1 Paraffin wax sections 37

2.4 Results 38

2.5 Discussion 41

CHAPTER 3: INDUCED SPAWNING **47**

3.1 Introduction 47

3.2 Objectives 52

3.3 Material and methods 52

3.3.1 System design 52

3.3.2 Assessment of spawning readiness and distribution of fish over experimental tanks 52

3.3.3 Spawning induction 53
3.3.3.1 Hormonal preparation and administration 53
3.3.3.2 Hormonal induction of fish at constant temperature exposure 54
3.3.3.3 Hormonal induction of fish exposed to fluctuating temperature 55
3.3.3.4 Assessment of spawning success 55
3.3.4 Statistical analysis 56
3.4 Results 57
3.5 Discussion 60

CHAPTER 4: LARVAL REARING TECHNIQUES 69
4.1 Introduction 69
4.2 Objectives 70
4.3 Material and methods 71
4.3.1 Hatchery design 71
4.3.2 Algae and Rotifer production 72
4.3.2.1 Algae production 72
4.3.2.2 Mass production of rotifers 75
4.3.3 Experimental design 76
4.3.4 Statistical analysis 77
4.3.5 Water parameter testing and monitoring 77
4.4 Results 78
4.5 Discussion 85

CHAPTER 5: CONCLUDING DISCUSSION **91**

REFERENCES **96**

LIST OF FIGURES

Figure		Page
1.	Schematic representation of the reproductive axis of fish.	6
2.	Classification of larviculture methods for gilthead sea bream.	17
3.	The spotted grunter, *Pomadasys commersonnii*.	21
4.	A 12 kℓ broodstock rearing tank at the Marine Research Aquarium, Sea Point, Cape Town.	34
5.	Broodstock tank with filter system components at the Marine Research Aquarium in Sea Point, Cape Town.	35
6.	Microphotographs of histological examination from the ovaries of the spotted grunter, *Pomadasys commersonnii*.	40
7.	The reproductive parameters of the *P. commersonnii* broodstock spawned with induction agent (0.25 mℓ/kg Ovaprim®).	59
8.	The hatchery system used for the rearing of the *P. commersonnii* larvae.	72
9.	Biomass production of *Nannochloropsis oculata.*	73
10.	The number of larvae of the *P. commersonnii* larvae amongst the treatments over the six days of rearing.	81
11.	The size of *P. commersonii* larvae over the seven days culture period.	83

12. The early life stages of *P. commersonnii* larvae. 84

LIST OF TABLES

Table		Page
1.	The composition of the industrial fertiliser used for the mass production of the algae, *Nannochloropsis oculata*.	75
2.	The number of *P. commersonnii* larvae within individual treatments over the rearing period of six days. Data are presented as mean ± standard error, *n* =3 per treatment.	80
3.	The size of *P*. *commersonnii* larvae within the individual treatments, the data are presented as mean ± standard error (µm).	82

CHAPTER 1: GENERAL INTRODUCTION

It is estimated that 690 million people in the world live in hunger and this number is projected to exceed 890 million people (9.8 % of global population) by 2030 excluding the impact of Covid-19 (Food and Agriculture Organisation [FAO], *et al*., 2020c). At regional and continental outlook, fish consumption continues to be the lowest in Africa, increasing from 9.1 in 2012 to 10 kg per capita in 2018 (FAO, 2012; 2020a). Furthermore, malnutrition of people on the Africa continent is at concerning chronic levels. FAO *et al*. (2021) reported that 281.6 million people in Africa were malnourished, which is an increase of 89.1 million from 2014. Thus, food security and healthy nutrition cannot be overemphasised.

Consumption of fish provide more than 3.3 billion people with 20 % of their average per capita intake of animal protein (FAO, 2020a) and is an excellent source of long-chain omega-3 fatty acids (LC ω-3FA). Examples are eicosapentaenoic acid (20:5 ω-3; EPA), docosahexaenoic acid (22:6 ω-3; DHA) and arachidonic acids, which have health benefits such as anti-inflammatory properties, promotes neurological and cognitive developments, and is associated with reduced heart diseases (Hansen and Harris, 2007; Hu *et al*., 2003; Calder, 2006; Neuringer *et al*., 1988). The LC ω-3FA of fish is superior to that of plant sources such as walnuts, and animal diet; beef, chicken, and pork (Blasbalg *et al*., 2011; Ponnampalam *et al*., 2006; Brenna, 2002). It would be prudent, therefore, for nations who are facing undernourishment and malnutrition to have access to fish products as a healthy food source. This is, however, not feasible in many situations where countries are landlocked or exposed to harsh climatic conditions (such as arid regions) so that there are no options to fish from oceans, rivers, or lakes. Furthermore, pricing of seafood is linked to global demand and supply, and fisheries are under pressure because the rate of human population increase is higher than fish supply/growth (FAO, 2020b), hence, the cost of seafood from commercial suppliers are out of reach to many societies.

The farming of aquatic animals (aquaculture) may be a way to correct the supply-and-demand ratio of fish to humans, thereby making seafood more affordable and accessible to all people on a regular basis, while conserving wild stocks and aquatic resources in the process. Aquaculture, therefore, has the potential to address two of the United Nation's Sustainable Development Goals: 2. eradication of hunger and poverty; 14. conservation and sustainable use of the oceans, seas, and marine resources (FAO, 2018; FAO, 2020b).

1.1 Global overview of aquaculture

Aquaculture is defined as the farming of aquatic organisms such as crustaceans, fish, molluscs, and plants in a controlled or semi-controlled environments for commercial, research or education purposes (De Silva and Anderson, 1994 Oddsson, 2020). Aquaculture enjoys continuous faster growth rate and more economical faster food conversion ratio than other major food productions (FAO, 2018). Globally, aquaculture production is recorded at 114.5 million tonnes, valued at USD 263.6 billion (FAO, 2020a) up from 51.1 million tonnes in 2009 (aquatic plants excluded) (FAO, 2010). Several technical factors have immensely contributed to the global fish production: the development and advancement of broodstock conditioning techniques, employment of exogenous hormone therapies to regulate reproduction, environmental manipulations and larvae rearing technologies. The increased awareness of the health benefits of sea food, as well as the ecological damage brought on from overfishing of wild stocks to supply the ever- increasing needs of the rising human population, are also important driving forces for aquaculture globally.

According to FAO (2020b), farmed aquatic animals were dominated by the finfish subsector, reaching 54.3 million tonnes production, and valued at USD 139.7 billion, indicating its global demand. Of the finfish subsector, carp species and salmonids are relatively well-established finfish in culture and are the leading commercial aquaculture species (De Silva *et al*., 2008; FAO, 2020b)). There is, however, a need for diversification of aquaculture to make the industry better equipped to handle future challenges, such as

the effects of climate change, consumer behaviour and market changes, declining fish stocks and health of wild stocks, as well as resource changes. Diversification strategies in aquaculture usually means a shift to alternative species (Thilsted *et al*., 2016).

There are 622 commercially farmed aquaculture species, excluding species produced from aquaculture research experiments, cultivated as live feed in hatchery operations, or as ornamental aquatic animals (FAO, 2020b). Of the farmed aquaculture species, 27 belong to the finfish and species groups and 20 of these finfish species accounted for 83.6 % of total finfish production, making finfish farming the most diverse subsector (FAO, 2020b). The diversification of aquaculture species requires the domestication of new species (Milla *et al*., 2020). These candidate species that are of commercial interest experiences reproductive dysfunctions in un-natural captive environments as a result of the failure to stimulate the hypothalamic-pituitary-gonadal pathway in the artificial environments. Of the species that readily undergo gametogenesis in captivity, final oocyte maturation, ovulation and spawning may not be predictable; the manipulation and control of the hormonal reproductive pathway of fishes is, therefore, a fundamental process towards the development of the aquaculture industry (De Silva *et al*., 2008), and the application of hormone therapies is common practice to realise spawning in fish that experience reproductive dysfunction in captivity.

Human chorionic gonadotropins, pituitary extracts, gonadotropin analogues (luteinizing-release hormone analogues) are common spawning aids used to maximise the procurement of eggs, and address gametogenic failure in captive fish (Mylonas and Zohar, 2001). These hormones are either applied alone or in collaboration with dopaminergic inhibitors (pimozide, metoclopramide, dopamine) to block the effects of the internal gonadotropin-releasing regulating mechanism.

Hypophysation (a technique where fish are injected with an extract from pituitary glands) and synthetic chemical hormones therapies are methods used in fish breeding to induce spawning of candidate species in unnatural culture environments (Mylonas and

Zohar, 2001). The application of such techniques has economized breeding significantly and led to security of large quantities of larvae, regardless of the spawning season and environments - a landmark process for food security and poverty alleviation. Asian countries are key players in the production of fingerlings from aquaculture facilities, hence their dominant, high-ranking position on global aquaculture production. In the early 2000s, China for example, had bred at least 52 marine fish and reached sustainable fry production (Hong and Zhang, 2003). They reported more than ten million *Plectorhynchus cinctus* fry (Order: Perciformes, Family: Haemulidae), one hundred thousand to a million *Parapristipoma trilineatus* (Family: Haemulidae), one to ten million of individual grunt species *Hapalogenys nitens* (Family: Hapalogenyidae) and *Pomadasys hasta* (Family: Haemulidae) (Hong and Zhang, 2003). In Japan, Taiwan, and Vietnam as well, hatchery-derived fingerlings have been intensively driven by hormone propagation and their implants. In these countries, species such as *H. nitens* and *P. hasta* had produced over a million of fingerlings per species.

1.2 Aquaculture and finfish production status in South Africa

The South African aquaculture industry is still in its infancy stages with a total production of 6365.8 tons valued at R1115772300 (Department of Environment, Forestry and Fisheries [DEFF], 2021). The total production represents both the marine and freshwater industries, contributing 4244.4 and 2121.4 tons, respectively (DEFF, 2021). In 2010, it was reported that the marine aquaculture production was 1992.20 tons while the freshwater culture industry 1140.8 tons, doubling its total production from 2010 to 2019 (Department of Agriculture, Forestry and Fisheries [DAFF], 2012; DEFF, 2021). The industry is now estimated to have created a total of 3468 jobs and 6500 jobs in total across the industries value chain. The freshwater culture industry is represented by species of trout (*Oncorhynchus mykiss* and *Salmo trutta*); tilapia (*Oreochromis mossambicus* and *Oreochromis niloticus*); catfish (*Clarias gariepinus*); carp (*Cyprinus carpio* and *Ctenopharygodon idella)* marron crayfish (*Cherax tenuimanus),* and ornamentals (DEFF, 2021). The marine industry is dominated by the culture of marine molluscs: mussels'

(Mytilus galloprovincialis and *Choromytilus meridionalis)*; abalone *(Haliotis midae)* and oysters (*Crassostrea gigas)* contributed 51, 36 and 11 %, in collective mass respectively, for 2018 total marine production (DEFF, 2021).

The culturing of marine finfish is an emerging industry subsector in the country and has reported production from 2.7 tons in 2008 to 115. 52 tons in 2017 (DEFF, 2020). Production declined to 73.8 tons in 2018, because of a farm closure (DEFF, 2021). The dusky kob (*Argyrosomus japonicus*; Family: Sciaenidae*)* is the only commercially farmed marine finfish in the country, from four farm operations: cage culture and semi recirculating aquaculture system (RAS), located in the Western Cape, a pond facility from KwaZulu-Natal, and a RAS in the Eastern Cape.

1.3 Natural reproduction and spawning induction of finfish

1.3.1. The hormonally regulated natural reproductive cycle

Environmental conditions, such as temperature, photoperiod, and availability of feed are external ques that impacts on natural hormone control processes in fish (Figure 1). These cues specifically modulate reproduction via endocrine mechanisms that involve the pineal gland-hypothalamus-pituitary-gonadal axis. The reproductive cycle of fish is divided into spermatogenesis and vitellogenesis (the first phase), and spermiation and oocyte maturation (the second phase) (Figure 1; Mylonas *et al*., 2010). The pineal gland in the brain receives and convey information about the light-dark cycle and via melatonin, the hypothalamus in the brain is activated to release gonadotropin-releasing hormone (GnRH); this neuropeptide hormone acts on the pituitary in the brain to release peptide hormones into the circulatory system, the so-called gonadotropins – follicle stimulating hormone (FSH) and luteinizing hormone (LH) act on the gonads to increase the production of steroid hormones (sex hormones, specifically) and stimulate production of either sperm or ova (Figure 1) (Bentley,1998; Van Der Kraak, 2009; Mylonas et al., 2010). In males, the gonadotropins cause the testes to make testosterone, while in females, they

cause the ovaries to make estrogen and progesterone. A detailed description of the production of sperm can be found in Schulz and Miura (2005), Mañanós *et al*. (2008) and Mylonas et al. (2010). FSH induces the production of female sex hormone estrogen which, in turn, binds to estrogen receptors on the liver to produce vitellogenin (VT) (Pankhurst, 2008). VT is an egg yolk precursor protein that incorporates phosphate and sugar lipid molecules, i.e. a phospholipoglycoprotein that is the precursor of the lipoproteins and phosphoproteins that make up most of the protein content of egg yolk. Vitellogenesis is the process through which maturing oocytes in the ovary accumulate yolk for the eventual nourishment of a developing embryo in a fertilised egg. Detailed coverage on how the egg yolk protein is endocytosed by VT-mediated receptors.

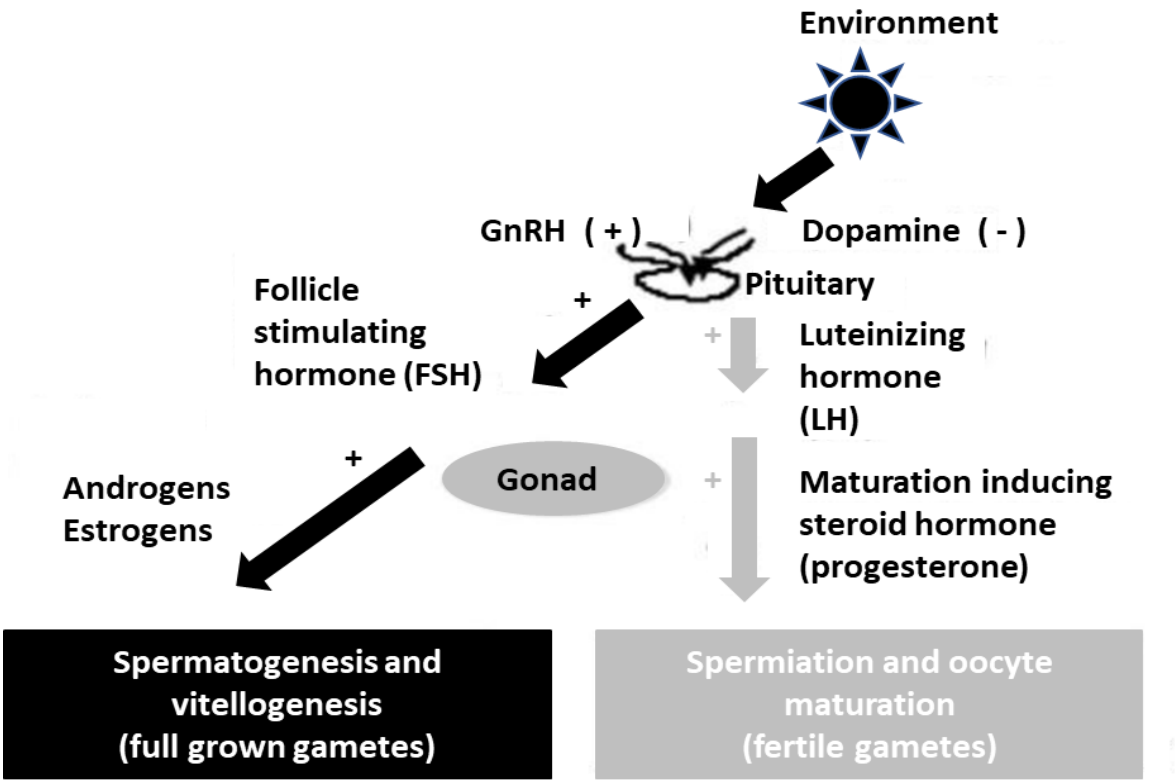

Figure 1: The schematic representation of the reproductive axis in fish from Mylonas *et al*. (2010) depicting the two phases of the reproductive cycle and the endocrine control of gametogenesis and final maturation.

Gonadotropin-releasing hormone (GnRH) is a neuropeptide hormone released from the hypothalamus in response to environmental signals and is the master switch in activating the illustrated hormone cascade. The neurotransmitter dopamine may have an inhibitory effect on the release of gonadotropins from the pituitary (Peter *et al*., 1986). Not shown: the pineal gland and the hypothalamus. oocytes can be found in Carnevali *et al*. (1999a), Mastubara *et al.* (2003), Hiramatsu *et al*. (2006) and Reading *et al*. (2018). LH predominantly induces the final maturation and ovulation of gametes (Izquierdo *et al*., 2001).

There are different types of ovarian development strategies in fish: fish that spawn once during the reproductive season (synchronous and single-batch group synchronous spawners) and those that spawn multiple times (multiple-batch group synchronous and asynchronous spawners) (Tyler and Sumpter, 1996). Asynchronous fish are those with cohorts of oocytes at different stages of development while fish with synchronous ovarian development have their oocytes at the same stage of development (Tyler and Sumpter, 1996). Atlantic blue tuna (*Thunnus thynnus*), and sea bream are examples of multiple batch asynchronous spawners: these fish spawn multiple times within a short period of time (daily or weekly) (Zohar *et al*., 1995; Papadaki *et al*., 2008) while sea bass, red porgy (*Pagrus pagrus*) (multiple-batch synchronous) spawns multiple times within their spawning period (Carrillo *et al*., 1995; Mylonas *et al*., 2004). On the other hand, freshwater eel (*Aguilla sp*.) and Pacific salmon (*Oncorhynchus sp*.) are examples of synchronous species: they spawn once in their lifetime (Mylonas *et al*., 2010) while striped bass (*Morone saxatilis*) and rainbow trout (*Oncorhynchus mykiss*) (single batch synchronous species) spawn once during their respective reproductive season (Mañanós *et al*., 2008).

1.3.2 Reproductive challenges in captivity

Changes in environmental conditions can disrupt the endocrine processes of gametogenesis and final reproductive maturation of fish (Mylonas *et al*., 2010). Zohar and

Mylonas (2001) classified reproductive dysfunction in captive enclosures into (i) failure to undergo vitellogenesis and spermatogenesis, (ii) post-vitellogenin oocytes fail to undergo final oocyte maturation and (iii) absence of spawning at the end of the reproductive cycle of both males and females. Artificial broodstock conditioning cues (photo-thermal and salinity), captive-induced stress and the lack of spawning substrate have been reported to disrupt reproductive success (Zohar and Mylonas, 2001). Moreover, fish holding capacity (design, size, water volume, depth) and stocking densities are factors considered to promote successful spawning. For instance, Mozambique tilapia (*Oreochromis mossambicus*) prefer low stocking density while the African catfish (*Clarius gariepinus)* is reproductively more successful at high breeder keeping (Shubha and Reddy, 2011; Shoko *et al*., 2016). Additional cues like flow rate of water are an important determinant for spontaneous spawning and quality of eggs such as for Nile tilapia (*Oreochromis niloticus*) (Tsadik and Bart, 2007), therefore when these culture conditions are not met, reproduction is suppressed.

Female fish exhibit serious reproductive challenges in captivity, more so than male broodstock fish (Mañanos *et al*., 2008; Milla *et al*., 2021). Species such *S*. *senegalensis*, *Lutjanus gutattus*, *M*. *saxatilis* and *Morone chrysops* are a few examples of fish that undergo final oocyte maturation in captivity but fail to ovulate and spawn (Agulleiro *et al*., 2006; Ibarra-Castro *et al*., 2004; Mylonas and Zohar, 2001). This was shown to be due to dysfunctional release of LH from the pituitary (Mylonas and Zohar, 2007; Mylonas *et al*., 2010). For example, blood analysis of cultured sea bass revealed low plasma levels of LH at the termination of oogenesis (Mylonas *et al*., 1998), whereas wild captured sea bass had high plasma LH levels on their spawning grounds, which was also associated with oocyte maturation and ovulation (Mylonas *et al*., 1997). It was further established that transcription levels, synthesis and stores of LH in the pituitary did not differ significantly between the captive and wild species, thus suggesting failure of LH release in captured fish (Steven, 2000). Correspondingly, the levels of LH in the pituitary of female gilthead sea bream in captivity were maximal, but its plasma levels were undetectable, resulting in atresia of oocytes. Thus, under normal reproductive conditioning

of fish, final ovarian maturation and ovulation are marked by a plasma surge in LH (Zohar *et al*., 1988).

Captive male broodstock fish undergo complete spermatogenesis and spermiation in captivity, however, they produce diminished and lower quality of milt (Mylonas and Zohar, 2007; Mañanos *et al*., 2008; Mylonas *et al*., 2010), thus negatively affecting fertilisation of eggs. Mylonas *et al*. (1998) reported reduced milt production when spermiating striped bass were transferred from a large circular tank (50 m^3) to a smaller circular tank (1.5 m^3) and implicated stress induced by smaller confinements or even handling, which in turn, led to poor stimulation of the pituitary to release gonadotropins. Cabrita *et al*. (2006) exposed wild *S*. *senegalensis* to the same conditions as laboratory reared F1 generation of the same species and found that the sperm volume and milt production of F1 individuals were relatively poor compared to wild caught individuals.

In culture conditions, final oocyte maturation and spermiation are generally validated by swollen belly females, running milt from male gonads, and changes in the colouration of the gonopore in both sexes. Social interaction, such as spawning behaviour, are also observed prior to breeding and these indicators are considered together with stripping of fish to examine the development of gametogenesis and maturation (measuring oocyte diameter and evaluating the quality of sperm) prior to breeding in culture conditions (Shiraishi *et al*., 2005; Dou *et al*., 2007). Spontaneous gamete release and hand strip may, however, not be possible in certain fish species (Viveiros *et al*., 2002) and since confirmation of final vitellogenesis is required prior to the induction of spawning in breeder fish, a useful tool is required to determine vitellogenesis status without using invasive techniques. A generally used practice is to remove oocyte(s) from the ovarian lumen with the use of a catheter and negative pressure (suction) for examination. This invasive procedure may damage ovarian stromal tissue and cause bleeding and tissue plugs in immature ovaries (Ohs *et al*., 2011; Dr Chris Fouche, personal communication). In addition, it is not always possible to discriminate between the genders of sexually mature fish in certain fish species. This lack of sexual dimorphism in large populations of

broodstock is problematic for hatchery managers which require selection of females and males for spawning induction (Rottmann *et al*., 1991; Mylonas *et al*., 2007b, 2010; DiMaggio *et al*., 2013).

Reproductive dysfunction of fish species in unnatural captive settings is addressed via the selective employment of hormonal therapies and the manipulation of rearing environments (Bromage, 2001). Temperature and salinity can significantly influence the output and quality of gametes. Following spawning induction, cold water species such as white sturgeon, when exposed to higher stress incurring temperatures of 15 and 18 °C, 20 to 40 % of the female broodstock ovulated and resulted in 0 to 30000 hatched larvae and ovarian regression (follicular atresia), while lower temperatures of 10 to 15 °C resulted in 100 % ovulation from all the female broodstock and led to 200000 hatched larvae (Webb *et al*., 1999). Spawning induced common carp, exposed to varying salinities yielded eggs with improved hatching rates and larval survival more than two-fold in salinities of 0, 5, 10 ppt (parts per thousand) compared to 15 and 20 ppt treatments. Contrarily, euryhaline species have shown spawning success in a wide range of salinity differences (Haddy and Pankhurst, 2000). It is evident that optimal environmental conditions are required in hatchery practices.

1.4 Larval rearing and pseudogreen water technique

Marine finfish larval rearing is challenged by low survival rates (Conides *et al.*, 2000), which is directly related to the rearing technique applied (Divanach *et al*., 1996). Freshwater fish larvae are generally large and readily accepts formulated feeds, whilst marine larvae are small and respond only positively to specific live zooplankton (Tocher, 2010). Formulated diets are inert and not digestible in most post hatch marine finfish larvae especially during the transition from feeding on live prey to formulated feed (weaning) The feed concentrates on the water surface or sink to the bottom of the tank (Conceição *et al*., 2010) if they are not consumed immediately by weaned larvae, therefore losing essential nutrients via leaching into the water, whilst it also become a

pollutant (Hamre *et al*., 2013).

The ontogenetic and dietary shift from depletion of oil globule to exogenous feeding is a sensitive and risky process due to major morphological developments of the alimentary canal (Rønnestad *et al*., 2013), limiting the digestion of dry feeds and effective assimilation of its nutrients (Hamre *et al*., 2013). For instance, the gastric enzyme activities of pigfish and southern flounder larvae were low or undetectable at three days post hatch (DPH) but rapidly increased later (Faulk and Holt, 2009; Thompson *et al*., 2019;). Therefore, live prey items such as *Artemia*, copepods, and rotifers are employed in hatcheries, introducing exogenously derived enzymes of the live prey to the primordial gastrointestinal tract of the larvae, thus aiding in digestion during the early life stages of the larvae (Das *et al*., 2012). Protein and amino acids are also less bioavailable in formulated feeds than in live prey due to leaching into the water, poor stability in water and microbial degradation especially if the feed is not eaten (Hamre *et al*., 2013). In addition, the foraging ability of the larvae is limited due to the small gape during the first feeding incidences, poor movement of the larvae due to lack of or the inflation of the swim bladder of or the inflation of the swim bladder, availability, and mobility of the prey items (Hunter, 1981; Rønnestad *et al*., 2013).

Co-feeding regimes have been developed to ensure the delivery of a balanced nutritional diet to the larvae, which may somewhat lack when one feeding item is fed to the larvae. For example, Lazo *et al*. (2000) reported improved growth and survival of larvae red drum (*Sciaenops ocellatus*) fed with zooplankton or combination of zooplankton with extremely small sized formulated diet (micro-diets) compared to those fed on micro-diet alone. Thompson *et al*. (2008) fed pigfish larvae a co-diet of rotifers enriched with *Isochrysis galbana* (a unicellular algae) and micro-diet (control group) while other treatments were of gradual replacement of rotifer with micro-diet from 28 DPH. They found that larval growth was not significantly different between the treatments and the control group. On the other hand, co-feeding of French grunt with live rotifers and copepods improved survival significantly, while there was no growth difference between

the co-feeding regime and both single feeding treatments with either rotifer or copepods (Hauville *et al*., 2017). Elsewhere, Hamre *et al*. (2008) found a significantly higher survival rate when Atlantic cod larvae were maintained on rotifers enriched with a combination of iodine and selenium, although growth was suppressed.

Euryhaline rotifers, *Branchionus plicatis* and *Branchionus rotundiformis*, are widely used in marine and crustacean larviculture (Dhert, 1996). Rotifers are important during the early stages of larvae due to their small size and are slow swimmers that are distributed throughout the entire water column. Moreover, rotifers have poor escape response when threatened (Buskey *et al.,* 1993), therefore the larval would conserve energy preying on rotifers than when hunting and locating other mobile prey items. Hirata (1979) and Yúfera and Pascual (1980) reported rearing these planktonic animals at high densities of 530 animals/mℓ in algae enriched water (*Nannochloropsis* or *Chlorella sp*.) at 36 % salinity. In addition, rotifers can ingest yeasts and varieties of microalgae in culture conditions (Conceição *et al*., 2010) and serve as important live food item to feed fast-growing larval species (Hamre *et al*., 2013), hence the mass culture of rotifers (Treece and Davis, 2000; Conceição *et al*., 2010). Despite this, rotifers are nutritionally incomplete, they lack or have low levels of essential eicosapentaenoic acid, docosahexaenoic acid, and arachidonic acids whereas copepods can synthesize these highly unsaturated fatty acids (Chen *et al*., 2019). Unfortunately, the intensive mass production of copepods remains a bottleneck (Chen *et al*., 2019). Nonetheless, nutrient bio-encapsulation, and enrichment processes are necessary remedies to improve the nutritional deficiencies of rotifers and Artemia.

Büke *et al*. (2005) suggested that the death of red porgy (*Pagrus pagrus*) larvae observed during the larval rearing experiment was due to starvation caused by fact that the small sparid larvae could not ingest rotifers. Smaller prey like calanoid copepod nauplii would be more suitable and with the required nutrient profile to nourish small fish larvae. Fouche and Goodman (2017) recommended the use of rotifers alone in rearing spotted grunter larvae since cannibalistic behaviour was observed when *Artemia* was also

included as live prey. Kentouri *et al*. (1995) reported that a rapid diet shift from rotifers to *Artemia* and increased *Artemia* feeding caused some digestive problems, resulting in higher mortalities. It is therefore prudent to provide larvae with a variety of prey species and adding algae as feed for these prey items (Sirakov *et al*., 2015).

Many genera of microalgae; *Chlorella, Tetraselmis, Isochrysis, Pavlova, Phaeodactylum, Chaetoceros, Nannochloropsis, Skeletonema, Scenedesmus,* and *Thalassiosira* are of economic importance owing to their comprehensive utilization in human and animal feed manufacturing; cosmetic, pharmaceutical, and human-wellness industries and aquaculture hatcheries (Borowitzka, 1997; Yamaguchi, 1997; Apt and Behrens, 1999; Muller-Feuga, 2000). There is also an emerging interest to exploit their high oil content for biofuel usage (Velichkova *et al*., 2012; Sharma *et al*., 2013; Hattab and Ghaly, 2014).

Microalgae predominantly support the nutritional requirements of marine invertebrates: mussels, oyster, clams, larval and juvenile stages of abalone, as well as zooplankton – a first feed required during the early life stages of fish (Volkman *et al*., 2006; Hemaiswarya *et al*., 2011). The algal nutritional requirements for zooplankters are a necessity in both laboratory/aquaculture conditions and marine environments. *Haematococcus pluvialis, Dunaliella salina, and Arthrospira platensis* for example, are natural sources of bioactive compounds such as astaxanthin and beta-carotene, responsible for the pigmentation of salmon, trout, ornamental fish, and prawn (Sirakov *et al*., 2015).

Algal species, such as *I. galbana* and *Nannochloropsis oculata* are commonly applied to set up green water culture environments for larvae rearing and are excellent sources of DHA, AA and EPA (Reitan *et al*., 1993; Barden *et al*., 2014; Broach et al., 2015b; Camacho-Rodríguez *et al*., 2020). Essential fatty acids are necessary for marine fish larval diets; arachidonic acid (AA) for example, has been positively correlated with improved survival of European sea bass after handling stress (Atalah *et al*., 2011) while DHA was associated with maximal growth and survival of yellowtail flounder, black sea

bass (*Centropristis striata*) (Copeman *et al*., 2002; Rezek *et al*., 2010). In general, microalgae should exhibit key attributes for use as nutritional enricher of rotifer or introduction into larvae rearing tanks to generate green culture conditions; ease to culture, appropriate size ingestion and digestion, digestible cell wall, high nutritional profile, faster growth rate, and easy to culture (and mass production), and is none-toxic (Guedes and Malcata, 2012).

The larvae of some marine cultured fish species such as European sea bass, add seabream, halibut *(Hippoglossus hippoglossus)*, cod (Naas *et al*., 1992; Reitan *et al*., 1994; Cahu *et al*., 1998) and freshwater species such as prawns (Anderson *et al*., 1990), tilapia, catfish, and carp species (Neori 2011; Islam *et al*., 2017) to name a few, have benefitted greatly from the addition of live or dead (frozen or dried) microalgae into larval rearing tanks. Performance indices such as growth, survival and digestive activities of larvae are generally enhanced under “green” water culture conditions. These are conditions where larvae are reared in the same tank with cultures of fertilized microalgae (Neori, 2011). Green water cultures offer several benefits; Støttrup *et al*. (1995) mentioned feeding stimulants contained in algae, thus increasing the appetite of larvae. Reitan *et al*. (1994) and Van der Meeren *et al*. (1991) evidenced the consumption and assimilation of algae by larvae (some in yolk sac stages) and the presence of algae in the gut triggered an increase in pancreatic enzymes and gastrointestinal hormones in European sea bass (Cahu *et al*., 1998). Since marine fish larvae are visual feeders (Hunter, 1981), visual is improved in green water culture conditions, enabling the larvae to locate and capture the prey. The foraging ability of halibut larvae for example, was enhanced when the microalgae was added into the larval rearing tanks (Naas *et al*., 1992).

Bengtson *et al*. (1999) reported that summer flounder larvae maintained on *Tetraselmis suecica* green water resulted in more than 40 % survival, considering that live feed organisms will also feed on these algae. In another study, the survival of greenback flounder *Rhombosolea tapirina* was 38.1 ± 15.1 % and 20.0 ± 1.9 % in *T. suecica*

greenwater and clear water, respectively (Shaw *et al*., 2006). However, larval growth was not significant between the culture conditions in both studies. The daily addition of *N. oculata* increased the growth rate of miiuy croaker demonstrated to enhance tolerance to starvation (Shan and Li, 2014). Similarly, cod larvae grown in *I*. *galbana* green water led to significant survival of larvae and increased feeding incidents than in clear water (Naas and Harboe, 1992). Habibut larvae of 33 days old exposed to outdoor conditions commenced feeding at day three post hatch while none started feeding in clear water. At the termination of the experiment, survival was poor in clear water tanks (live zooplankton feeds only) when compared to microalgae enriched treatments, 1.2 and 30 % respectively (Naas and Harboe, 1992).

Stuart and Drawbridge (2011) generated green culture conditions for yellowtail, *Seriola lalandi* through daily addition of algae paste and illuminated the treatments with low, medium, and high light intensities. The investigation demonstrated the obligatory requirement of green water for the rearing of yellowtail since over 99 % mortalities were reported in clear water. Nonetheless, larval growth increased with the increase of light intensity in the tanks, regardless of whether algae was present or not. However, notochord length of the larvae, including survival and swim bladder inflation were significantly enhanced under combined high turbidity and highest light regime (Stuart and Drawbridge, 2011).

Navarro and Saraquete (1998) used freeze-dried *Nannochloropsis oculata* to enrich rotifers and added the freeze-dried algae into the gilthead seabream larval rearing tanks. The study found that the survival of the larvae raised on rotifers enriched with the freeze-dried algae and the freeze-dried algae enriched larval rearing water was not significantly different to larvae raised on live *N*. *oculata* enriched rotifers and larvae rearing water. However, when the larval tanks received no algae (but the rotifers enriched with the freeze-dried algae), the survival rate was 50 % less and the intestinal structures appeared less pronounced. Furthermore, the growth of the larvae raised with no addition of algae into the rearing tanks was inferior as well (Navarro and Saraquete, 1998). Similarly,

gilthead seabream larvae reared in freeze-dried *N*. *gaditana* and *I*. *galbana* suspensions produced similar growth and survival trends, however enrichment with rotifers contributed to the larval growth (Cañavate and Fernández-Díaz, 2001). Faulk and Holt (2005) successfully fed cobia larvae with *I*. *galbana* enriched rotifers and artemia as compared to feeding with *N*. *oculata* commercial enrichment product. Growth and survival between the treatments were not statistically significantly different. Furthermore, the performance indices of larvae exposed to *N*. *oculata* were inferior to *I*. *galbana* fed larvae (Faulk and Holt, 2005).

Stable algal blooms, in systems with no or little water volume exchange, are sustained by dose formulated fertilizers added to the water during the culture period. Water exchange is not recommended for the first ten days of *Sparus aurata* in green water culture since this was demonstrated to reduce larval growth (Tandler and Helps, 1985). It was suggested that the superior growth rates observed in system with no exchange was due to the improved availability of dissolved organic matter (used by the larvae) than in system where water changes were applied. However, poor growth rates were later observed when the larvae were twenty days older, in systems with no water exchanges as a result of the accumulation of unidentified metabolites. The addition of *Nannochloropsis sp*. into the larvae rearing system (flow through system) improved the survival rate of the *Sparus auratus* larvae than those raised in recirculating system (Hernández- Cruz *et al*., 1994), and attributed this observation to improved water quality in the flow through system. Cañavate and Fernández-Díaz, (2001) reported increased levels of ammonia and nitrite when no water exchanges were applied in larvae rearing experiments of *S*. *aurata*.

Pseudo-green water may offer better control of water quality parameters such as ammonia and pH and serves to suppress microbial proliferation as a function of the probiotic properties of green water (Palmer *et al*., 2007; Sharifah and Eguchi, 2011). This technique is characterized by daily replacement of microalgae and zooplankton into the larval-rearing tanks (Figure 2) at preferred calculated densities (Divanach *et al*., 1999;

Papandroulakis *et al*., 2001). Source microalgae and live prey are produced in separate facilities and introduced into the larval rearing tanks to facilitate the green water technique, in this way the maintenance and the quality of the food chain can be guaranteed (Neori, 2011). The green water technique is marked by the presence of algae and live feed not utilized in previous days within larval rearing tanks and is also significantly less efficient than the pseudo-green water culture technique.

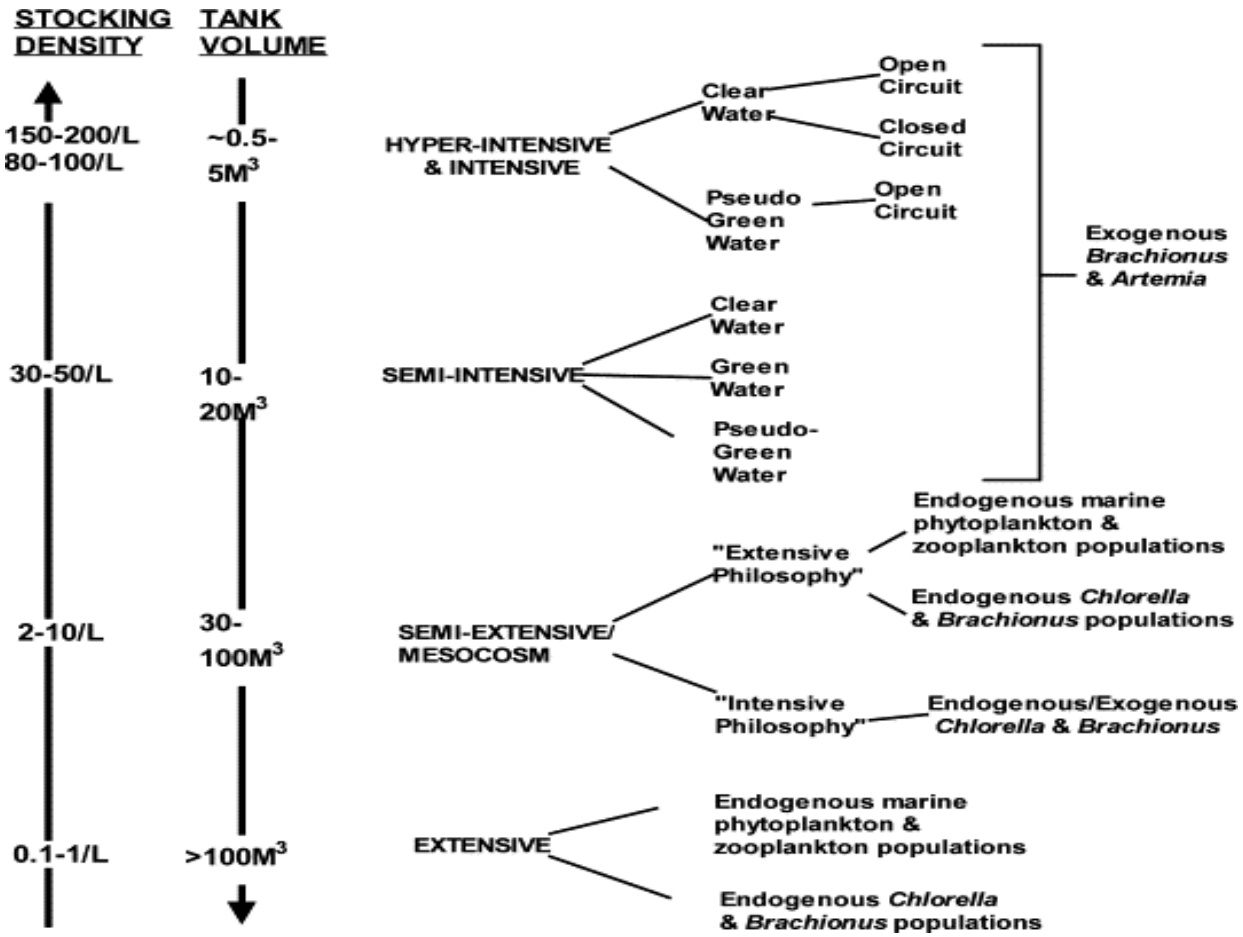

Figure 2: Classification of larviculture methods for gilthead sea bream (Divanach *et al*., 1999).

The exact mechanisms explaining the superior success of pseudo-green water culture remains unclear (Chen *et al*., 2019), however it has been hypothesized that the daily

addition of the microalgae suppress the uncontrollable algal and microbial blooms in the larvae rearing tank. This further prevents the deterioration of the water quality in the larvae rearing tank since the algae is cultured and fertilized elsewhere (Chen *et al*., 2019). The algae also contribute as an autotrophic biofilter by stripping nitrogenous waste excreted by fish larvae and live feed, whilst also serving as an antibacterial agent (Palmer *et al*., 2007). The probiotic effects of *Raseobacter* bacteria isolated from *N. oculata* suppressed *Vibrio anguillarum* (Sharifah and Eguchi, 2011) and *Chlorella sp*. as well, reduced the incidences of *Vibrio harvey* (Huervana *et al*., 2006).

1.5 The spotted grunter, *Pomadasys commersonnii*

1.5.1 Species selection and taxonomy of *P. commersonnii*

The Department of Agriculture, Forestry and Fisheries (DAFF, 2014; DAFF, 2015) expressed aquaculture interest in dusky kob; mangrove snapper (*Lutjanusargenti maculatus*, Family: Lutjanidae); spotted grunter (*Pomadasys commersonnii*, Family: Haemulidae); yellowbelly rock cod *(Epinephelus marginatus*, Family: Serranidae*)* and yellowtail (*Seriola lalandi*, Family: Carangidae). *A. japonicus* is piloted for commercialisation in Hamburg, Eastern Cape (DEFF 2021), and the yellow tail kingfish is also produced in small numbers in the East London IDZ (Dr Chris Fouche, personal communication).

Although spotted grunter was identified as a marine aquaculture species more than 20 years ago (Deacon and Hecht, 1995, 1999; Mperdempes and Hecht, 2002), its aquaculture potential has been overlooked. According to Childs (2005), *P. commersonnii* is an ideal aquaculture species because they grow fast, juveniles can be reared at high stocking densities, females spawn multiple times through the year, this is a euryhaline species that can tolerate salinity of less than 1 % up to 90 %, and good performance indices were measured.

The successful commercialisation of any aquaculture species is ultimately determined by the reproductive biology of the species, its nutrition and economic viability. Nutrition, environment and understanding the reproductive biology of the fish species, knowledge of its environment and natural nutrition are key criteria for the eventual successful integration of the species to aquaculture status. The spawning of *P*. *commersonnii* in South African coastal areas and estuaries has been documented. Irish (1997) revealed that *P*. *commersonnii* accepted supplementary diets and is a high valued species that has been described as “premier fish” with fleshy meat and taste, making it an excellent table fish (Deacon and Hecht, 1995).

Deacon and Hecht (1995, 1996, 1997a, 1999) and Bacela (1998) investigated the influence of environmental conditions, temperature, salinity, and photoperiod, as well as stocking density for rearing spotted grunter in enclosed tanks, while Childs (2005) and Webb (2002) documented the biology of the species in the wild. Despite these numerous investigations that provide a knowledge and support basis to exploit the spotted grunter in captivity, the reproductive success of this species under laboratory conditions is poorly researched (Childs, 2005; Rastgoo *et al.*, 2014).

There is no information concerning the use of spawning aids to induce *Pomadasys* species in captivity. To our knowledge, there is dominating telemetry and distribution evidence of spotted grunter in South African coastal waters and their natural spawning activities, (Webb, 2002; Childs, 2005; Kerwath *et al*., 2005; Næsje *et al*., 2005; Childs *et al*., 2008), but there is a general paucity of studies of hormonal therapies to induce final oocyte maturation, ovulation and spawning in females, as well as spermatogenesis in males of spotted grunter. Furthermore, there is a lack of data of morphological features of larvae such as length at hatch and first feeding, their growth post- hatch, and larval survival. This information is vital to eventually assess diet formulation, culture environments, and hence determine the profitability of this species in aquaculture. Consequently, the study aims to establish broodstock conditioning, induced spawning, and hatchery management methods transferable for potential application in an

industrial setting.

Pomadasys commersonnii belongs to the Haemulidae family which consist of 17 genera and more than 150 species (Smith and McKay, 1986). Haemulidae fish are called “grunters” because they make grunting noises by rubbing their pharyngeal teeth together when they are irritated. There are about 36 species that belong to the genus *Pomadasys* (Myers *et al*., 2022), five of which are represented in South African waters, e.g., *P*. *kaakan*, *P*. *olivaceum*, *P*. *maculatum* (Beckley and Fennessy, 1996), *P. furcatus* and *P. commersonnii* (Mann *et al*., 2015). The scientific classification of the spotted grunter is as follows: Phylum: Chordata, Class: Osteichthyes, Order: Perciformes, Family: Haemulidae, Genus: *Pomadasys,* Species: *commersonnii*.

1.5.2 External morphology of *P. commersonnii*

Spotted grunters (Figure 3) are oblong compressed and have long, sloping foreheads with a pointed snout (Smith and McKay, 1986). The dorsal surface of the silvery body is covered by many small brown spots that extend onto the dorsal fins (Van der Elst, 1988). *P*. *commersonnii* juveniles lack spots and differ from *P. argenteus* by not forming regular wavy lines. The spotted grunter has eight to ten anal fin rays while other *Pomadasys* species have seven or eight rays on their translucent anal fin. *P. commersonnii* differs from other co- generic species like *P*. *maculatum* by the absence of spots on the head and snout and has six pores and a median pit on the chin, whereas *P*. *maculatum* has two pores and a median pit on the chin (Beckley and Fennessy, 1996).

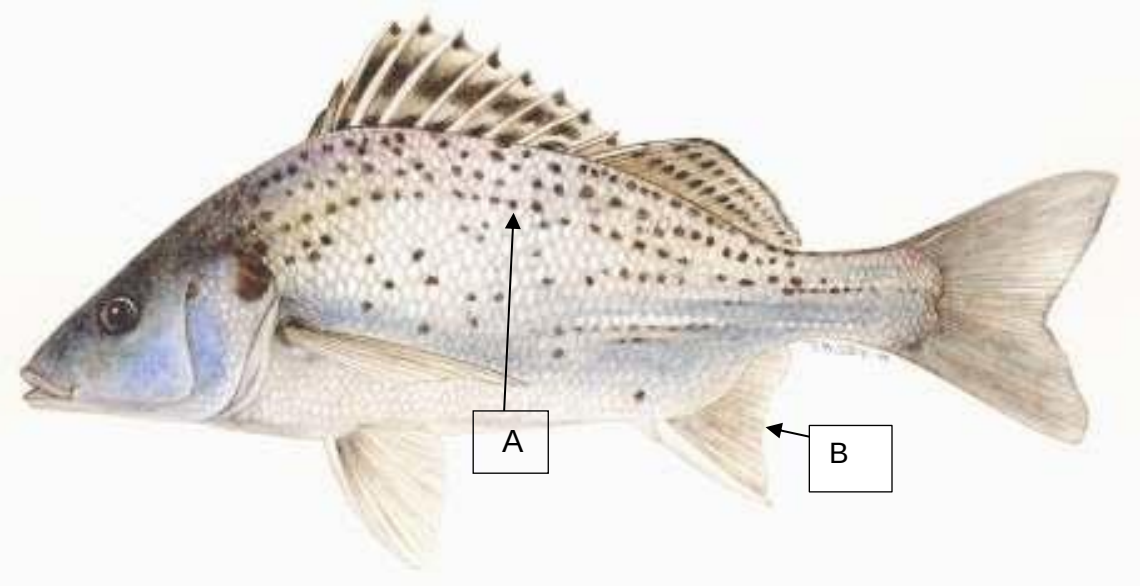

Figure 3: Spotted grunter *Pomadasys commersonnii* (Pisces: Haemulidae - Lacépède, 1802). Photography from Childs (2005). The dorsal-lateral line, (A); Eight to ten anal fin rays (B).

1.5.3 Origin and distribution of *P. commersonnii*

The natural range and origin of *P. commersonnii* is not clear. Branch *et al*. (1994) and Smith and Heemstra (2003) describe it as a Western Indian Ocean species found in inshore coastal areas and estuaries, while Blaber (1981) and Van der Elst (1988) describe it as Indo-Pacific found in temperate waters and tropical coasts. In South African waters, spotted grunters have been recorded in parts of Kwazulu-Natal, Eastern Cape, and Western Cape (Wallace, 1975a; Bacela, 1998; Childs, 2005).

1.5.4 Feeding biology of *P. commersonnii*

Pomadasys commersonnii is a macro benthos feeder, extracting prey organisms from burrows by using a gill chamber pump action: a jet of water is forced into the burrow system and the prey is blown out and consumed (Day *et al*., 1981). Several authors

confirmed that *P. commersonnii* feed on prawns, mysids and amphipods (Van der Westhuizen and Marais, 1977; Schleyer and Wallace, 1986; Hecht and Van der Lingen, 1992).

A study by Webb (2002) at the Great Fish River reported fish of small size, 70 mm total length (TL) preyed on crustaceans, such as amphipods, brachyuran crabs and shrimps: *Grandidierella lignorum* (amphipod) was the most abundant food item in the stomach of the fish, followed by crabs (*Hymerosoma orbiculare* and *Cleistostoma edwardsii*) and *Upogebia africana* (shrimp) while anomuran crabs, crabs were the least favoured prey item. Fish size of 70 - 300 mm TL preferred *G. lignorum,* followed by, *Callianassa krausii*. Conversely, adult (300 mm TL+) preyed more on *C. krausii* followed by *G. lignorum.* A study of the Gamtoos Estuary indicated that 335 - 492 mm TL spotted grunter preyed more on *U. africana*, followed by *C. krausii* (Marais, 1984). These ontogenetic dietary shifts are confirmed by Blaber (1983) and Heemstra and Heemstra (2004), where juveniles between 20 and 40 mm fed on zooplankton and small macro benthos, fish of 50- and 100- mm TL fed on small bivalves, and crustaceans while larger fish preyed organisms such as *Solen cylindriceus* (bivalve) and *C. krausii*.

Comparisons between Great Fish River and Kariega estuaries (Hecht and Van der Lingen, 1992) and Gamtoos, Sundays, and Swartkop estuaries indicated *U*. *africana* and *Clionella kraussii* (sea snail) as a popular diet for spotted grunters of different sizes. Other prey items such as anomurans frequently inhabited the surface of burrows making it simple for consumptions by the spotted grunter (Whitefield, 1998). Predator-prey interactions are, thus, influenced by different fish sizes, seasonal variations, and prey abundance.

1.5.5 Natural reproduction of *P. commersonnii*

Spotted grunter is a fast-growing species, attains a length of 12 - 15 mm in their first year (Wallace, 1975a), can grow to a maximum size of 9.5 kg in the wild, with a life span of 15

years (Wallace and Schleyer, 1979; Day *et al.*, 1981). In South African coastal marine environments, the spotted grunter reaches sexual maturity when they are 2-3 years old and the total length (TL) at which 50 % of males reached maturity was estimated at 280-320 mm and 350-380 mm TL for females (Wallace, 1975b; Day *et al.*,1981; Van der Elst and Adkin, 1991; Webb, 2002). Contrarily, the total length at which 50 % of wild female reached maturity was estimated at 42 cm and 44 cm for males in the Arabian Sea off Oman (AI-Nahdi *et al*., 2011). Furthermore, female spotted grunter matured in their second years while males attained maturity in the early part of their third year (Al- Marzouqi *et al*., 2009). Information on the maturity stage of other (none-South African) grunter species are also known: the length at first maturity of the west Atlantic *Pomadasys jubeline* females was reported to range 159.2 to 177 mm and 121.3 to 147.8 mm in males and (Bodji *et al*., 2013).

Spotted grunter spawning events have been reported to take place in shallow marine environments from August to December in South African costal environments and in the Arabian sea of Oman (Day *et al*., 1981; Al-Nahdi *et al*., 2011). Wallace (1975b) found that adult spotted grunter spawned between September and December at the St. Lucia Lake, Richard and Durban Bay. On the other hand, *P. jubeline* spawned between February and April in three Côte d'Ivoire lagoon complexes, these times constitutes a period at which temperature and salinity are high (Bodji *et al*., 2013) and spawning of *Pomadasys incisus* was from August to October in the Gulf of Tunis (Fehri-Bedoui and Gharbi, 2008).

Whitfield (1994a,b) suggested that spotted grunter is an estuaries-dependent species. Adult spotted grunters migrate from estuaries to sea, where they undergo gonadal development and spawn (Wallace and Van der Elst, 1975). Following spawning, larvae develop at sea (Whitfield, 1998). Environmental salinity conditions play an essential role to facilitate viable osmotic requirement for gonadal maturity and embryonic development in migratory fish (Chaoui *et al*., 2006). Juvenile spotted grunter (20 to 30 mm TL) and post-spawn adults enter estuaries to forage in the nutrient-rich environment (Whitfield, 1998). Juvenile spotted grunter spends one to three years in these nurseries (Wallace and

Schleyer 1979; Day *et al*., 1981; Whitfield 1998). This is further confirmed by Child (2005) who reported that small tagged spotted grunter resided in estuaries for longer periods. Furthermore, most tagged spotted grunters were recorded in the estuary during high barometric pressure, strong winds and lower temperatures in the sea (Child, 2005). Rough sea conditions influence the migration of adult to estuaries (Potter *et al*., 1990). Despite the reality that estuaries are subject to changing environmental conditions, such as turbulence, temperature, and salinity, making them harsh areas for fish growth and survival, South African estuaries present specialised environments for juvenile finfish because the murky water provide shelter, and are calm (Wallace *et al*., 1984b).

Pomadasys commersonnii tolerates salinity concentrations of 0.5 to 90 % (Whitfield *et al*., 1981) thriving between the range of 31 - 35 ppm. Blaber *et al*. (1984) reported that they can survive in salinity environments of less than 1 %, hence their survival in estuarine environments which ranges between 0.5 to 35 ppm (parts per million) salinity. The euryhaline character of the species and tolerance of wide range of turbidity (4.1 and 567 Formazin turbidity unit [FTU]) (Hecht and Van der Lingen, 1992; Whitfield, 1998; Childs 2005) present ease of culture. Furthermore, juveniles can be raised in high stocking densities rates of 6.4 kg/m^3 without affecting growth index parameters (Bacela, 1998).

Baird *et al*. (1996) reported a decline in spotted grunter catch due to over-exploitation and the increase in subsistence fisheries activities in estuaries, and stock status in the wild have collapsed (van der Bank *et al*., 2019). This led to the de-commercialisation of *P*. *commersonnii* in South Africa, although, expression of interest by the South African government to embark on the aquaculture research of spotted grunter might return the profitability of this species. The controlled use of artificially administered spawning inducers in captive grunter breeders guarantees unlimited seed supply which can, apart from aquaculture, also support recruitment practices as per conservation management requirements for habitats where populations have declined (Bell *et al*., 2008; Diana, 2009). Such a strategy can also alleviate fisheries pressure, while not disturbing the supply of

fishable stocks to the markets.

Previously, wild juveniles of *P*. *commersonnii* were experimentally reared in closed-recirculation systems (Deacon, 1995, 1996; Deacon,1997a, b; Irish, 1997; Deacon, 1999; Bacela, 1998; Mbona, 2017). Traditional aquaculture operations depended on the collection of seed stocks such as larvae, fry, juvenile, from the wild (De Silva *et al*., 2008). This may be unpredictable, leading to fluctuating and low levels of fingerling supply and rearing costs, whilst pressure is simultaneously exerted on the sustainability of natural stocks. However, *P*. *commersonnii* is mainly protected by its inshore migratory behaviour ("spotted grunters run"), which limits the regular availability of the fish to anglers.

1.6 Aim and objectives of the study

1.6.1 Goal

Developing methods that will complete the programmable control of the reproductive cycle of spotted grunter, as well as hatchery methods towards juvenile weaning.

1.6.2 Objectives

i. Condition *P*. *commersonnii* broodstock with developed photothermal program, using squid or formulated feed pellets at 1 % body mass per day.

ii. Determine the minimum dosage and efficacy of a commercially available analogue of salmon gonadotropin-releasing hormone (sGnRHa) (Ovaprim) in inducing the highest yields of viable *P. commersonnii* eggs spawned, including the latency time required between inductions and spawning at 23 °C.

iii. Record microscopic embryonic and post-embryonic ontogenetic stages until the exogenous feeding stage in larvae, to identify and monitor morphological changes

required for larval survival.

iv. Produce plankton species with predictability and as pre-requisite for larval rearing.

v. Determine the survival rate and growth of *P*. *commersonnii* larvae reared in pseudo-green water conditions as the preferred method for increased larval survival to the weaning stage.

vi. Evaluate the broodstock conditioning, spawning and hatchery methods developed as a model for effective and viable use in industry.

1.7 Null hypothesis

i. *Pomadasys commersonnii* broodstock cannot be conditioned by exposing them to programmed photothermal cues.

ii. sGnRHa does not induce spawning in spotted grunter broodstock.

iii. Monitoring embryo ontogenetic stages towards exogenous feeding is not required to raise spotted grunter larvae successfully with standard hatchery procedures.

iv. There is no significant difference between the survival and growth rate of *P*. *commersonnii* larvae reared in pseudo-green water and clear sea water.

1.8 Research questions

i. In captivity, can the spotted grunter breeder fish be conditioned and spawned, and can the offspring be raised to weaned fingerlings with industry required quantitative outputs?

ii. Is the use of pseudo-green water technology the best option to raise pre-weaned larvae at highest survival rates?

CHAPTER 2: BROODSTOCK CONDITIONING

2.1 Introduction

Broodstock nutrition is the “single most important non-genetic factor” that influences and determine the quality of eggs and viability of larvae (De Silva *et al.,* 2008). Marine, estuaries and river environments provide an unlimited and wide spectrum of nourishment to fish and other inhabiting organisms while on the other hand, domesticated broodstock is entirely dependent on the prevailing feeding protocols and culture conditions. Nutritional requirement is species-specific and often vary within the species’ life stages. Provision of adequate protein, lipids, essential amino acids, fatty acids, vitamins, and minerals in the diet is of essential importance to the gonadal development of the broodstock fish, spawning performance, and to an extent, determines the survival and growth of the larvae.

The nutritional requirements to support final oocyte maturation (FOM), ovulation and spawning performance of *P. commersonnii* broodstock fish is yet to be intensively investigated. Meanwhile, broodstock diet, artificial propagation, and larval nutrition of other haemulid species, *Orthopristis chrysoptera* and *Orthopristis ruber* (e.g., pig fish species), have been extensively evaluated (DiMaggio *et al*., 2014; Mata *et al*., 2014; Broach *et al*., 2015a,b; Faulk, 2018; Thompson *et al*., 2019). These pigfish species, as was also the case for French grunter (*Haemulon flavolineatum*) broodstock fish (Barden *et al*., 2014) were maintained on 50 % crude protein, 15 % lipid pellets and supplemented with squid meal, squid oil, and krill. Squid meal and oil, sardines and formulated balanced broodstock diets have been reported to improve egg quality and survival of other marine finfish species, such as seabass (*Dicentrarchus labrax*), gilthead seabream, (*Sparus aurata*) striped jack (*Pseudocaranx dentex*) and wild meagre (*Argyrosomus regius*) (Watanabe and Vassallo-Agius, 2003; Duncan *et al*., 2013a,b).

Incorporation of long-chain omega-3 fatty acids (LC ω-3FA) ingredients in feed formulations is essential for growth and development and improves the content of these LC ω-3FA in the flesh of fish (Watters *et al*., 2012). Elevated levels of dietary LC ω-3 FA

concentration are linked to improved egg quality (Izquierdo *et al*., 2001). LC ω-3 FA content of fish oil was proved to be superior to plant-based oil (e.g., linseed oil) in supplementation studies in both Atlantic salmon (*Salmo salar*) and red seabream (*Pagrus auratus*) (Glencross *et al*., 2003; Bell *et al*., 2003, 2004). On the other hand, excessive concentrations of dietary highly unsaturated fatty acids (HUFA) may reduce reproduction capacity of fish (Izquierdo *et al*., 2001). In terms of dietary fatty acid requirements, herbivorous and omnivorous fish have endogenous enzymes that can covert α-linolenic acid (18:3w3; ALA) to LC ω-3 FA. The latter must, however, be supplied via the diet to piscivorous or carnivorous fish because they lack such enzymes (Sargent *et al*., 1995).

Watanabe *et al*. (1984a,b; 1985, and 1991) demonstrated the importance of lipid inclusion in broodstock diets: the studies showed that krill meal, squid meal and oil in the broodstock diet improved the egg quality. Specifically, the quality of fish eggs was improved in response to the total lipid content, higher cholesterol and phospholipids present in dietary krill meal (Watanabe *et al*., 1991). Squid meal inclusion and feed supplementation boosted the reproductive success of striped jack and yellowtail fish (*Seriola quinqueradiata*) (Vassallo-Agius *et al*., 2001, 2002). Fishmeal and squid dry pellets improved egg quality, whilst squid- and fishmeal, supplemented with paprika (astaxanthin source), resulted in a superior hatching rate of eggs spawned by yellowtail broodstock (Vassallo-Agius *et al*., 2002). Since fishmeal, squid meal and oil are increasingly incorporated in the broodstock conditioning diets of marine finfish, there is a need to develop cheap, alternative diets because wild squid stocks have been optimally exploited in the South African coastal line (van der Bank *et al*., 2019) and fish prices have increased because of the competition between human consumption and production of fishmeal and fish oil (FAO 2020b).

Feed additives is a common practice in diet formulation, ranging from prebiotics, probiotics, immune stimulants, vitamins, and antioxidants. Some of the dietary additives helps economize reproductive spawning parameters. For example, the spawning performance of yellowtail broodstock improved with the dietary inclusion of the super oxidant, astaxanthin (Verakunpiriya *et al*., 1997). Astaxanthin (30 ppm) was introduced to

the yellowtail in soft dry pellets comprising of 46 % crude protein from fishmeal and 24 % crude oil, resulting in an increased number of eggs, apparent higher fertilization rate, lasting oil globule, and improved survival of larvae (Verakunpiriya *et al*., 1997). Tizkar *et al*. (2013) also reported a positive correlation with astaxanthin-supplemented feed to goldfish broodstock and an improved fertilization rate. Similar positive effects of astaxanthin are also reported for rainbow trout (*Oncorhynchus mykiss*) and cod (*Gadus morhua*) (Craik, 1985; Salze *et al*., 2005).

Eggs of the South American carp (*Catla catla*) remained immature when the broodstock diet was supplemented with vitamins only, whereas improved fecundity and successful breeding was recorded after the diet was changed to comprise fish oil, vegetable oil, and vitamin mixture (Nandi *et al.*, 2001). Whilst the dietary requirements of juvenile spotted grunter have been investigated (Deacon and Hecht, 1999; Irish, 1997; Hecht *et al*., 2003), the diet to support reproductive conditioning of the *P*. *commersonnii* broodstock is still to be established.

Several environmental parameters, such as photoperiod, temperature, salinity, rainfall, food availability and pheromones are implicated in the reproduction of wild and captive fish (Bromage *et al*., 2001 and Mylonas *et al*., 2010). One or more of these environmental parameters are necessary to stimulate natural gonadal development and is species-specific. Temperature is the primary physical factor controlling biochemical and physiological functions of fish (Beitinger and Fitzpatrick, 1979). Several authors confirmed the effect of temperature on fish oocyte development, maturation, and spawning. Webb *et al*. (1999) found that high temperature inhibited oocyte ovulation and diminished fertility of white sturgeon (*Acipenser transmontanu*s), while low temperature postponed spawning and reduced fecundity of the South American freshwater fish, *Prochilodus argenteus* (Arantes *et al*., 2011).

A thermoregulatory study with juvenile spotted grunter established the thermal preference of the species as ranging between 24 and 25 °C (Deacon and Hecht, 1995). Childs (2005) noted that adult spotted grunters avoided temperatures of 16 °C at the Great Fish River estuary. This notwithstanding, in their review on the environmental regulation of

maturation in farmed finfish, Bromage *et al*. (2001) deduce photoperiod to be the primary determinant for the timing of maturation in most finfish species that are intensively farmed, as well as in many tropical and sub-tropical species. Evidence show that photoperiod sets up endogenous rhythms that are influenced by seasonal changes to affect reproduction through the timing of pituitary hormone release, and the circulating plasma levels of luteinising hormone (LH) and follicle stimulating hormone (FSH) (Bromage *et al*., 2001). French grunter, *H*. *flavolineatum* reached final oocyte maturation and ovulation conditioned at 12D: 12L (Barden *et al*., 2014).

Several authors acknowledge the significance of photoperiod relative to temperature on both growth and on ovarian development. Photoperiod and temperature were found to have a modulating effect on the growth rate of juvenile spotted grunters (Deacon and Hecht, 1995). Thus, the physiological wellbeing of juvenile *P*. *commersonnii* was improved under 12D: 12L exposure than either 16D: 8L and 8D: 16L and was relatively high at 22.5 °C compared with temperatures of 20.5 and 28.5 °C, respectively (Deacon and Hecht, 1996).

Series of experiments were conducted to investigate the effect of photoperiod and temperature on rainbow trout broodstock: Whitehead and Bromage (1980), Bromage *et al*. (1984), Davis and Bromage (1991) showed that exposure to longer photoperiod 6D: 18L followed by a sudden change to a short light regime 18D: 6L advanced maturation, regardless of temperature variations. However, temperature was shown to have an effect: dysfunction of ovarian development was observed when temperatures reached 20 °C and poor egg quality resulted at 16 °C exposure.

The regulation of ovarian development in response to photoperiod is further evidenced by a study on the variation of light and temperature regimes and resulting effect on reproductive parameters in medaka (*Oryzias latipes*) by Koger *et al*. (1999): an absence of mature ova and moderate oocyte atresia (degeneration of ovarian follicle) in all the rainbow trout were recorded upon reduced photoperiod from 8D: 16L to 16D: 8L at a constant temperature. Additionally, when temperature was reduced from 25 to 15 °C, mature ova were few and mild oocyte atresia resulted (Koger *et al*., 1999). Similarly, the

study on the influence of water temperature on induced reproduction by hypophysation, sex steroid concentration and final oocyte maturation of the *P*. *argenteus* by Arantes *et al*. (2011) found that that when the temperature was lowered from 26 to 23 °C, 33 % of the fish failed to ovulate. Photothermal regimes can indeed be used by hatchery operators to delay or inhibit reproductive development of broodstock for effective hatchery management.

2.2 Objectives

To condition the broodstock of the spotted grunter on squid and sardine diet at 1 % body mass per day at constant and fluctuating temperature regimes regimes to promote gonadal maturation and spawning induction.

2.3 Materials and methods

2.3.1 Study animals

The F1 broodstock of *Pomadasys commersonnii* (male, n = 12 and females, n = 4) were between four to five years old while the F2 generation (males, n = 12 and females, n = 4) were two to three years old. All the broodstock were laboratory reared (with no wild experience) at the Marine Research Aquarium (MRA) of the Department of Forestry, Fisheries, and the Environment (DFFE) at Sea Point, Cape Town since 2014.

2.3.2 Place of study

The research was conducted at the Marine Research Aquarium (MRA) of the Department of Forestry, Fisheries, and the Environment (DFFE) at Sea Point, Cape Town.

2.3.3 Ethical clearance

The experimental protocols were approved by the Science Animals Ethics Committee of the University of Cape Town (Approval number 2021/V6/HM/A1) and the DFFE conditions

for research in terms of Section 20 of the Animal disease act, 1984 (Act No. 35 Of 1984).

2.3.4 System design

The population of F1 (n = 16) and F2 (n =16) broodstock grunter were separately kept in seawater in two circular tanks that were 4 m in diameter with a volume capacity of 12 kilolitre (kℓ) each (see A in Figure 4) and maintained at a stock density of 1.5 kg/m^3. LED lighting were used at 250 lux brightness and the lights were controlled by timer (10D: 14L) (Deacon and Hecht, 1996). Each of the tanks were serviced by its own recirculation filter system which were specified to maintain fish at a density of 25 kg/m^3. The filtration system (Figure 5) consisted mainly of a seven-bag sand filter, foam fractionator, degasser, biological filter (250 ℓ, Kaldnes® biomedia), sump, UV filtration (55 W Pro UV, Ultrazap, Johannesburg) and makeup water inlet. Water was abstracted via a bottom drain of the system tank by gravity flow to a settlement circular fiberglass chamber (70 cm diameter), with upper (40 cm higher than conical waste chamber) suction point of the external dirty water pump (450 W Speck Porpoise). The delivery supply of the latter firstly entered the seven-bag sand filter (Figure 5E) that was regularly serviced with pressured air supported backwash function. From the sand filter the water flow was diverted by valve control and about 40 % of the flow supplied to the foam fractionator (Figure 5C). The remaining 60 % flow was directed to the degasser that was mounted on the biofilter, allowing flow through the degasser directly into the fluidized bed biofilter (Figure 5D). T treated water from both the foam fractionator and biofilter was returned to the clean water pump. The filtered water was then abstracted from the latter with a “clean water pump” (similar type as the dirty water pump) and first treated with UV filtration, then passed through a heat pump (SF040P, 17.5 kW, Cape Town, South Africa) set at 24 ± 0.5 °C, before finally introduced into the fish tank via spray jets (2500 ℓ/min). Fresh pre-filtered (80 µm) make up seawater (5 % daily – continuous trickle) was supplied by the facility and abstracted from the nearby Queens’s Beach Sea front.

The system was connected to a control panel to maintain and monitor the functioning of the pumps and establish circulation of water. The tanks were covered with a tarpaulin canopy to maximize light exposure into the tank during broodstock conditioning, maintain

darkness during spawning and to limit visible human interferences (except during feeding and daily monitoring). Tank water was aerated with a bottom positioned leaky pipe diffuser (50 cm in length and 20 mm diameter) with valve-controlled air supply from the facility's main side channel blowers. The broodstock tanks were also equipped with an alternative side wall mounted return-water line connected to the high-density polyethylene (HDPE) cylindroconical egg-collector (Figure 4E) lined with a submersible 500 µm screen liner - to harvest floating eggs in case of planned or spontaneous spawning. The alternative return flow was only activated when spawning was anticipated. During this time, the bottom drain was closed, and the effluent line from the egg collector opened (valve control) to this sump.

Figure 4: A 12 kℓ broodstock rearing tank at the Marine Research Aquarium, Sea Point, Cape Town. HDPE broodstock tank, (A); clean water inlet with flow meter (B), side wall box connected to a horizontal egg collector gutter pipe in the inside of tank (C), and pipe connected (D), to the external egg collector tank (E), with 500 µm terylene screen liner. Tarpaulin canopy (F) above the tank can be lowered to safeguard fish against exterior disturbances, whilst also contain pre-set photoperiod requirement independent of the exterior environment.

Figure 5: Broodstock tank with filter system components at the Marine Research Aquarium in Sea Point, Cape Town. Broodstock tank (A); dirty water sump (B), foam fractionator (C), degasser top mounted on biological filter (D), sand filter (E), pre-filtered make-up water line from ocean (F) and aeration supply pipe from facility's side channel blowers (G).

2.3.5 Broodstock conditioning and experimental treatments

During broodstock selection, water was lowered in the broodstock holding tanks (35 cm deep) and the fish were anaesthetized with 200 ppm of 2-phenoxyethanol (Merck laboratories, Johannesburg) treatment prior to removal and further handling. Immediately when the fish lost balance (were inverted), soft material scoop nets were used to transfer them into1.2 kℓ water tank mixed with 2-phenoxyethanol (200 ppm), and with vigorous aeration. Broodstock selection was based on picking fish with no deformities, skin lesions, or diseases. Selected fish (n = 16 from each F1 and F2) were kept at a stocking ratio of one female to three males due to a shortage of available females that had not been subjected to prior experimentation. Sexually mature females could be identified by their conspicuously distended bellies, and with a slightly swollen gonophore, compared to males having a small papilla shaped urogenital organ The total length and weight of the fish were measured. The selected fish were then transferred to the second 12 kℓ recirculating system and exposed to a daily photoperiod of 10D: 14L for four months; the sea water temperature kept at 24 ± 0.5 °C (Deacon and Hecht, 1996) and salinity of 35% (Deacon and Hecht, 1999). During this four-month acclimatization period, the broodstock of the spotted grunter were fed 1% body weight of 1:1 combination of squid (*Todaropsis eblanae*) and pilchard (*Sardinops sagax*) over two sessions: morning at 09:00 (0.5 %) and afternoon at 17:00 (0.5 %).

The effectiveness of broodstock conditioning at a constant temperature setting was compared to a second strategy that applied a fluctuating temperature alternative to establish the most effective regime to condition the broodstock fish in preparation of spawning induction (chapter 3). As for the fluctuating temperature regimes, the spotted grunters were exposed to three pre-selected periodic lengths in which temperature fluctuations were allowed (Chapter 3). For this, the heat pumps could also be commissioned as chillers. Eight spotted grunters each from F1 and F2 broodstock were exposed to thermal fluctuation. One cycle of thermal fluctuation was achieved when the broodstock conditioning temperature was gradually dropped to 19 ± 0.5 °C at a daily rate of 1.5 °C from the holding temperature (24 ± 0.5 °C). The temperature was then raised back to 24 ± 0.5 °C at a similar daily rate a day after it reached 19 ± 0.5 °C. The constant

temperature (four months acclimatization period) and three periodic temperature fluctuations of three to four weeks were respectively tested. The behavioural interactions of the broodstock fish during the conditioning periods were visually monitored through observations.

2.3.6 Gonadosomatic index and the histology of the ovaries

For the histological examination of spotted grunter's ovaries, only two females were dissected due to the shortages of female broodstock. The two females were randomly selected during the transfer of the broodstock into the experimental tanks after the fish were exposed to the constant temperature. The females were euthanized with 1000 ppm 2-phenoxyethanol. The fish were dissected with sterilised dissecting scissors and blades to harvest the ovaries for histological examination to determine the ovarian development pattern of this species. The individual samples were labelled as A and B and were preserved in 10 % buffered formalin. The weight of fish A was 1560 kg, and measured 41.91 cm (caudal length) while fish B measured 1325 kg and 40.64 cm. The gonadosomatic index (GSI) was calculated according to Strange (1996) below and served for exploratory purposes since no scientifically valid conclusion can be drawn from two specimens only.

$$\text{GSI (\%)} = \frac{\text{gonad weight (kg)}}{\text{total body weight (kg)}} \text{ X } 100$$

2.3.6.1 Paraffin wax sections

Tissue processing and histology was conducted at the DAFF histology laboratory in the Foretrust Building, (Cape Town, South Africa) as follows: gamma radiated surgical blades were used to cut about 3 mm pieces of the fixed ovaries; the tissue pieces were inserted into labelled cassettes and subjected to dehydration and clearing in a logos microwave hybrid tissue processor (Magna Analytical, Cinda Park, South Africa). Briefly, the fixed tissues were washed in 70 % ethanol and dehydrated through a series of two increasing ethanol exchanges; rinsed in two changes of 99 % ethanol and cleared in xylene. The

tissues were then exposed to isopropanol, followed by vaporization to dry the samples, and impregnated with wax and embedded in plastic cassettes using the embedding center (AEC 380, Amos Scientific, Clayton South, Australia), and then left to solidify on the cryo console (AEC, Amos Scientific, Clayton South, Australia). The wax-embedded ovary tissue blocks were sectioned on a microtome (Microm HM 340, Thermo Fisher Scientific, Walldorf, Germany). Sections of 5 µm were floated in a 45 °C water bath (Microm SB 80, Thermo Fisher Scientific, Walldorf, Germany) to remove folds, collected onto glass slides and left-over night to dry at room temperature. The wax-embedded tissue sections were deparaffinized for five minutes in xylene, followed by rehydration in a series of descending ethanol concentrations (100, 95, 80 and 70 %) each for two to three minutes, then rinsed in distilled water for three minutes before they were stained in Mayer's haematoxylin for twenty minutes and immersed in running tap water for five minutes. The excess water was shaken off and the stained sections were then counterstained in eosin for 20 - 30 s. The slides were then dehydrated through to 100 % ethanol for two minutes and lastly cleared in xylene for two minutes. The slides were dried by blotting paper and entellan was used to mount the coverslips on the slides Images were taken with a light microscope (Axio Lab A1, Zeiss, Cape Town, South Africa) fitted with a camera (Axiocam ERc 5s, Zeiss, Cape Town, South Africa).

2.4 Results

The observations and results reported here are from both the constant and fluctuating temperature programs, except histological examination and GSI calculations that were derived from two female fish after their exposure to the constant temperature regime. Following the use of the anesthetizer (2- phenoxyethanol), capturing, and handling of the spotted grunters, the broodstock spent most of their time swimming at the bottom of the conditioning tank. The swimming activity was in a calm, coordinated school fashion except during feeding. The fish completely lost appetite for the first three to five days post handling and partial feeding response to complete recovery were observed in the following second week. When the fish resumed feeding, they raced to the surface and competed to catch the feed. This feeding behaviour and swimming activity was the same even when the broodstock fish were subjected to lower temperature of 19 °C (fluctuating

temperature program).

Physical changes in the spotted grunters served as indicators that broodstock conditioning via photothermal induction was completed. Male fish oozed milt when gentle pressure was applied from the lateral anterior mid- abdomen area towards the urogenital area (stripping process). Similar pressure to the females allowed the observer to see eggs in the ovarian sac through the pressure mediated opening of the oviduct. Also, if the abdomen towards the urogenital area was clearly distended, then the females qualified for spawning induction. No mortalities were observed throughout the thermal conditioning regimes. In a tank not forming part of this experimental setup, an unplanned thermal shock led to three ovulation episodes of small quantities of eggs (< 20) spaced by three to four days. The spotted grunters in this tank were maintained on the same conditions (feeding regime, photoperiod) as the experimental broodstock, except that the temperature rapidly dropped to 16 °C from 24 ± 0.5 °C holding temperature due to technical failure of the heat pump. When the temperature was gradually increased to 24 ± 0.5 °C , the eggs were skimmed from the external egg collector, however, they were not fertilised.

The GSI of fish A and B were 1.85 % and 0.85 %, respectively. The histological examination of the ovaries revealed cohorts of oocytes at different stages of development (Figure 6), demonstrating an asynchronous ovarian nature of the spotted grunter. In Figure 6, (A) showed cortical alveoli of primary and secondary oocyte; late vitellogenic oocyte and hydrated oocyte, indicating a mature ovum. On the other hand, (B), was dominated by perinucleolar stage of the primary growth of the oocyte and early vitellogenesis stage of development, indicating that the ovary was immature. The ovaries, A and B, however, did not show any degenerating ovarian follicles (atretic follicle).

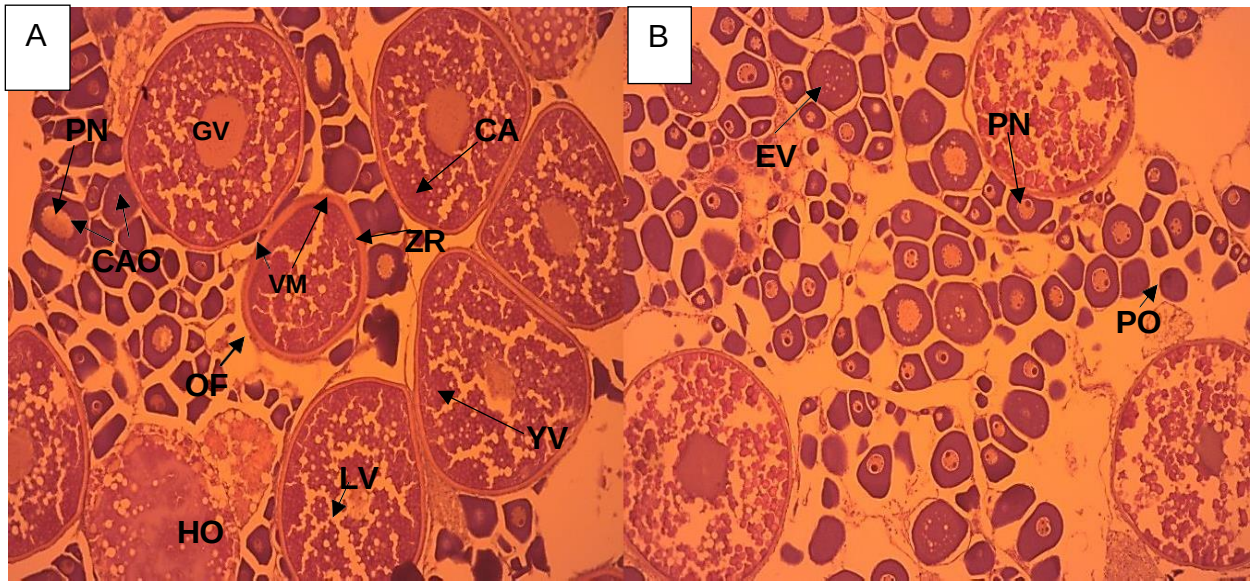

Figure 6: Microphotographs of histological examination from the ovaries of spotted grunter, *Pomadasys commersonnii*, an asynchronous multiple batch spawner fish having cohorts of oocytes at different stages of maturity. (A) Cortical alveoli, and late vitellogenesis stages (mature ovaries); Hydrated oocyte HO, Germinal vesicle GV, oocyte follicle OF, zona radiata ZR, cortical alveoli secondary oocyte CA, Yolk vesicle YV, late vitellogenesis (yolk vesicles) LV, cortical alveoli of primary oocyte CAO, vitellin membrane VM, perinucleolar stage of the primary growth of the oocyte (maturing female) PN. (B) Primary oocyte predominance during early post spawn ovarian development; Primary oocyte PO, early vitellogenesis stage EV.

2.5 Discussion

The domestication of broodstock fish is necessary and facilitates the artificial selection of desirable traits such as high growth rate, disease resistance and attractive phenotype. The conditioning environments for broodstock stimulate the gonad-hypothalamic pathways to activate gametogenesis and final maturation. Optimization of broodstock nutrition is an important criterion towards improved egg quality and subsequent better larval survival rates (Izquierdo *et al*., 2001). DiMaggio *et al*. (2014) conditioned pigfish, *O*. *chrysoptera*, on frozen squid and krill for four months preceding hormonal induction: this led to high quality spawns, indicating that nutritional and environmental conditions required for final oocyte maturation and ovulation were met (DiMaggio *et al*., 2014). The inclusion of squid meal and squid oil significantly improved the egg viability and larval survival of mangrove red snapper (*Lutjanus argentimaculatus)* (Emata *et al*., 2003), red sea bream (Watanabe *et al*., 1984a) and striped jack (Vassallo-Agius *et al*., 2001) due to high protein value and lipid sources.

The spotted grunter readily accepted the 1:1 combination of the squid and fish diet. All fully conditioned males oozed milt upon a gentle application of pressure from the abdominal area towards the urogenital area. whilst female grunter exhibited a transparent ovarian sac containing visible eggs when the oviduct was opened by antero-posterior abdominal pressure. The application of catheter biopsy is a popular technique to examine oocyte of fish that do not release eggs spontaneously (Viveiros *et al*., 2002), however, due to the high susceptibility of the grunter to injury stress, this is not a viable strategy. *Pomadasys commersonnii are* naturally benthic feeders in their native wild environments, the domesticated grunter in the present study fed throughout the entire water column but substantially on the bottom. This may have been influenced by the slow sinking diet and feed spot competition for the food items might have been a contributing factor as well. According to Pasquet (2018), the domestication of wild fish modifies their foraging behaviours due to many underlying factors such as absence of substrates, limited movement and restricted choice of prey, and *ad libitum* human-feeding regimes. The spotted grunter has been held captive since 2015 and their first filial generation spawns never exposed to wild experiences, may explain the variation of the foraging pattern to

that of the wild as it was suggested that domesticated wild strains are capable of "*learning cognition*" in new environments (Pasquet, 2018; Saraiva *et al*., 2018). However, a large body of evidence on these modifications of wild fish in domestic conditions and *vice versa* mostly exist on juvenile salmonids *sp.* (Saraiva *et al*., 2018). The grunters swam in an organized coordinated fashion, in school, but this may have been influenced by the restricted space, lack of predators, polyculture species as in the wild and the circular broodstock holding tank. However, they are sensitive to interferences s such as human presence as this broke their swimming pattern unless during feeding. Kaiser *et al*. (2018) collected pigfish from the wild and observed these grunters forming compact school in captivity.

Fish experiences different types of stressors in the wild environments and in captivity. They may be environmental, pathological, social, and physical stressors (Bernier, 2006) and leads to reduced food intake levels and or disruption of feeding behaviours, suppressed growth rate and reproduction and impairment of other physiological function such as immune response (Bernier, 2006; Martin *et al*., 2010; Schreck, 2010). Marais (1984) reported that the spotted grunter vomited after a period of 12 h of stress induced by gillnetting. Even in the present study, the grunter vomited a limited portion of their diet after handling. The acute stress caused by the catching, and handling of the spotted grunters after 2-phenoxyethanol exposure, resulted in profound effects on the daily food and cumulative intake levels: the fish completely lost appetite for the first three to five days post handling (and spawning induction) and partial feeding response to complete recovery were observed onlyin the following second week. This observation was also reported by Kaiser *et al*. (2018) who reported that the grunter, *O*. *chrysoptera,* actively fed a week later after capture.

Juveniles of European sea bass, *Dicentrarchus labrax,* and gilthead sea bream (*Sparus aurata)* resumed feeding and normal behaviour within day one after each individual species was anesthetized with optimum doses of 30 to 55 mg/ℓ of clove oil and phenoxyethanol (300 to 450 mg/ℓ) for maximum of 15 minutes (Mylonas *et al*., 2005). Therefore, the prolonged fasting tolerance of the grunter observed in the present study may only concern its physiological stress response related to reproduction activities.

Deacon *et al*. (1997b) found no significant difference on growth rate, condition, feed conversion ratio and mortalities between spotted grunter juveniles treated with 2- phenoxyethanol and the untreated control group, suggesting that greater stress was induced during handling and consequent reproduction activities. Radull *et al*. (2002) also reported that handling resulted in elevated stress levels after the oxygen consumption rate was significantly higher following chase and capture of the juvenile spotted grunter.

During starvation periods, it is generally accepted that the stress hormones catecholamine and cortisol are secreted to aid survival. The dietary and energy requirements to sustain metabolic and physiological functions are compensated by reduced metabolic activities and sparing use of nutrients caused by these hormones, while energy is mobilized through the catabolism of stored glycogen in the liver and tissue, plasma proteins, and lipids (Waagbø *et al*., 2017). Endocrine response to mobilize energy during stress conditions differs across freshwater and marine species. For instance, Costas *et al*. (2012) and Polakof *et al*. (2006) reported elevated levels of plasma cortisol in marine *Solea senegalensis* and gilthead seabream respectively, following starvation. Retrospectively, earlier studies found no significant cortisol changes in salmon species during short and long-term starvation (Kakizawa *et al*., 1995; Pottinger *et al*., 2003), suggesting non-endocrine pathway to liberate energy reserves. The physiological effects of stress induced by the transportation and handling of the spotted grunter are still unknown. Mbona (2017) only investigated these biochemical glucocorticoids on spotted grunter exposed to different dietary feeds. While it may be so, the spotted grunter did not reflect compensatory feeding once they resumed feeding in contrast to the hyperphagic activity of sea bass (Rubio *et al*., 2010; Leal *et al*., 2011; Azodi *et al*., 2016). Rubio *et al*. (2010) reported fourfold higher re-feeding food intake on the first day following nine days of food withdrawal compared to the pre-feeding levels. The spotted grunter however, exhibited gradual feed acceptance in the second week post handling. Also, the neuroendocrine response to stress is still to date poorly understood and reported (Schreck, 2010; Milla *et al*., 2021). The susceptibility of the grunter to prolonged stress and the depressed feeding recovery periods may disfavour the manual (e.g., injection) application of priming and immediate resolving hormone dosages for optimizing egg procurement. As mentioned earlier - the scientific evaluation of a proper dietary

requirement for gonad maturation in domesticated sexually mature *P. commersonnii* is still to be investigated.

A stained section of the mature ovary (Figure 6) in spotted grunter was characterized by prominent cortical alveoli in both primary and secondary oocytes, late vitellogenesis, and hydrated oocyte thus indicating an asynchronous ovarian development (DiMaggio *et al*., 2014). An unplanned thermal shock (temp = 16 °C) fortuitiously revealed the surprising result of three episodes of ovulation of small quantities (< 20) eggs spaced by three to four days in a separate tank of spotted grunters after temperature was again increased to 20 °C. These incidents confirmed that temperature is the predominant cue for grunter gonadal conditioning, and that the species is an asynchronous multibatch spawner; the latter was further confirmed by histological observations (albeit on only two female specimens) of multiple batches of oocytes in successive developmental stages, with the matured stage cohorts presumably ovulating upon spawning induction. The asynchronous oocyte development and multiple spawning characteristics of the spotted grunter agrees with reports for other *Haemulidae* species, *Haemulon plumieri* (Palazón- Fernández, 2007), *H. melanurum* (Granado,1989), *H. steindachneri* (Rodriguez, 1985), *H. Sciurus* and *H. album* (García-Cagide, 1986a,b).

Pigfish (DiMaggio *et al*., 2014) and other finfish species such as yellowtail (Stuart *et al*., 2016), not only undergo vitellogenesis under captivity but readily completes the process spontaneously by ovulation and spawning. The spotted grunter, however, failed to spawn spontaneously as no eggs or larvae were observed throughout the conditioning period or any other time in the broodstock tanks under constant or fluctuating temperature regimes – except in the one unplanned event. It can be speculated that levels of luteinizing hormone necessary to induce ovulation and spawning may have not been met, hence the reproductive failure. Both the mature and maturing ovaries lacked atretic oocytes. Atresia forms during pre-spawning because of lack of consistent gonadotropic stimulation to maintain maturing vitellogenic oocytes (Guraya, 1976) and is common during spawning and postovulatory periods as well. Sullivan and Yilmaz (2018) stated that limited atresia is a normal process by which females adjust their fecundity, but massive preovulatory atresia entails reproductive failure. Otherwise, failure of ovulation

of matured vitellogenic oocytes leads to regression and reabsorption of oocytes, hence manifestation of atretic oocytes (García-López *et al*., 2007; Adebiyi *et al*., 2013). Environmental conditions have been reported by Patiño *et al*. (2003) as a contributing factor to the development of atretic oocytes too. The latter authors conducted a study on *Cyprinus carpio* from two sites in Lake Mead, Nevada and found that high incidences of atresia were associated with higher temperature above optimum spawning range of the species resulting in incomplete spawning and poor nutritional status (Patiño *et al*., 2003). In King Lake, Australia, atresia has been linked to imbalance of sex ratio in white sucker study where the removal of males from the Lake resulted in increased incidences of atretic oocytes up to 70 % (Trippel and Harvey, 1990), implying the lack of males to fertilize the ripe oocytes. Early indicators of atresia such as follicle layer vacuolising, or disintegration of the vitelline wall were not present in the ovaries of the dissected grunters, pointing out that the conditioning temperature, feeding ration and sex-ratio stocking density were appropriate.

As mentioned, the oocytes of spotted grunter were not all in the same stage of development since immature oocytes with cortical alveoli, and late vitellogenic oocyte stages both co-existed in the same ovary, which indicated that the maturation of the spotted grunter broodstock is not synchronised. This may have been determined by the non-synchronised onset of sexual maturity not synergistically distributed within the population, resulting in a greater condition factor for conditioned fish with ovaries containing mature oocytes. A proportional population of mature oocytes in ovaries of none-synchronous spawners is indicative that breeders with a relatively low GSI may still spawn. This conforms to the finding that a GSI of only 2% or less still yielded spawning ovaries during the peak spawning season of *P*. *commersonnii* (Al-Nahdi *et al*., 2011), *P. stridens* (Amtyaz *et al*., 2013) and *P*. *maculatum* (Amtyaz *et al*., 2014). However, this observation and speculation should be investigated further in controlled and replicated design featuring a larger sample of dissected spotted grunters.

Advanced ovarian maturation in domesticated breeders is not uncommon. Milla *et al*. (2021) reported advanced reproductive capacity of domesticated sea bream and striped bass compared to wild strains. This may be influenced by the consistent

optimal photothermal regimes, absence of competitors and predators, balanced nutrition and feeding to satiation - which may be somewhat compromised in the wild because of long term seasonal impacts on the marine environments.

In conclusion, male spotted grunter broodstock released running milt when they were stripped while females exhibited some abdominal swelling and the presence of mature oocytes in the ovarian sac when oviduct opened under gentle antero-posterior abdominal pressure. Yet, no spontaneous spawning activity was observed in either of the conditioning thermal regimes. Development of a technique for the oral delivery of spawning induction agent is recommended since spotted grunter broodstock is highly susceptible to capture and handling stress (as necessary for hormone injections). Poor feeding response and prolonged recovery to normal feeding appetite levels were observed after handling the broodstock for conditioning purposes.

CHAPTER 3: INDUCED SPAWNING

3.1 Introduction

Reproductive dysfunction of fish species in unnatural captive settings is addressed via the selective employment of hormonal therapies and manipulation of the rearing environments (Bromage, 2001). However, manipulations of environmental conditions to mimic natural spawning grounds does not necessarily result in control of the reproductive function, and in land-based aquaculture facilities it is not easy to simulate the changing marine environment which is important to promote gonad maturation especially in migratory fish (Fontaine, 1975). Domesticated sea bass, salmonids, and *O. chrysoptera* for example, readily undergo vitellogenesis under captive culture conditions, however final maturation (oocyte maturation and spermiation), ovulation and spawning are restricted or unpredictable (Sower *et al*., 1982; Woods and Sullivan, 1993; DiMaggio *et al*., 2014).

The correction of the reproductive pathway dysfunction in fish through exogenous hormonal administrations has been reviewed (Zohar and Mylonas, 2001; Mañanós *et al*., 2008; Mylonas *et al*., 2010). The authors further outlined factors that may significantly affect egg quality during the application of the hormonal therapies to include: the developmental stages of oocytes at the time of hormone applications, the type of hormonal therapy, induced stress due to manipulation of the environments in preparation of the hormonal therapy, and latency period between hormone administration and stripping of gametes for artificial fertilization (Mañanós *et al*., 2008; Mylonas *et al*., 2010).

Spawning aids (e.g., substrates) are useful tools to also synchronize spawning for coordinated hatchery management control (Zohar and Mylonas, 2001) and have valuable fisheries conservation implications. Endangered fish that are of commercial interest in aquaculture and fisheries can be salvaged under intensive fish breeding programs (Mijkherjee *et al*., 2002; Borsa *et al*., 2019).The success of LH preparations from crude and purified carp pituitary extracts and purified human chorionic hormone (hCG) in

freshwater and marine teleost finfish are delimited by (i) the risk of transmission of diseases from donor to recipient broodstock fish, (ii) the efficacy of the donor hormone as it is dependent on the age and maturity of the donor fish, (iii) unquantified LH and possible amino acid sequence substitutions that may result in high species-specificity when using a heterologous or uncharacterized crude pituitary extract, (iv) bioavailability, and (vi) high cost (Zohar and Mylonas, 2001; Broach, 2015a).

Human chorionic gonadotropin (hCG) mimics LH function andinduced ovulation in Australian sea bass (*Macquaria novemaculeata*), mangrove red snapper (*L. argentimaculatus*), cobia (*Rachycentron canadum*), spotted sea bass (*Lateolabrax maculatus*), and striped bass (*M. saxalitis*) through single but pharmacologically high dose injections (200 to 2000 IU/kg) (Zohar and Mylonas, 2001; Lee and Yang, 2002; Mylonas *et al*., 2010). A haemulid species *O. chrysoptera* attained maximal total egg spawned at 2000 IU/kg (DiMaggio *et al*., 2014). Similar dose results were reported by Lee and Yang (2002) for spotted sea bass (*Lateolabrax maculatus*). Effective spawning induction dose ranges of 2 to 10 mg/kg has been reported for 20 fish species treated with hCG, whilst 12 species were successfully induced with crude pituitary extracts (Zohar and Mylonas, 2001). The high doses needed with hCG in fish and the corresponding high total cost of treatment and possible immune responses introduced in fish broodstock, are major negatives to consider against successful induction of spawing with this preparation. Similarly, the many drawbacks of using ill-available and expensive purified or crude pituitary extracts, are constraints that cannot be overlooked in aquaculture production concerns. This has favoured the exclusive use of commercially available GnRH analogues (GnRHa) in spawning induction of aquaculture fish.

The hormone analogue is synthesised with two changes to the amino acid sequence of the natural GnRH peptide of salmon species: (i) the sixth residue is substituted with a dextrorotatory (D) amino acid, and (ii) the tenth amino acid is substituted with an ethylamide group (Goren *et al*., 1990). The resulting GnRHa can no longer be cleaved by natural endopeptidases between positions 5-6 and 9-10, which leads to an improved half-life of the peptide mimetic and an increased potency over that of endogenous

GnRH,thus stimulating an intense and more prolonged and release of endogenous gonadotropins from the hypothalamus (Habibi *et al*., 1989; Zohar *et al*., 1990; Lovejoy *et al*., 1995; Zohar and Mylonas, 2001; Lethimonier *et al*., 2004). One of the first used species to have demonstrated ovulatory response to GnRH analogues is goldfish (Lam *et al*., 1975, 1976). In these studies, synthetic LHRH- releasing hormone (i.e., GnRH) injected into mature female goldfish resulted in ovulation (>75 %) and the later study (1976) replicated the effectiveness of the synthetic hormone at lower dosages. 0.1-1.0 mg/kg intraperitoneally injected fish achieved 33 – 80 % ovulation, whilst 100 % ovulation was achieved when similar dosages were administered intra-cranially (Lam *et al*., 1976).

Although the hormone-delivery system is not pertinent to the scope of the present study, it requires attention. The method of the delivery-system is influenced by the ovarian development type: the economic consequences of multiple handling (in the case of multiple batch spawners) not only induce stress but promote the susceptibility to disease infections which may result in devastating mortalities of valuable broodstock (Haddy and Pankhurst, 2000; Schreck *et al*., 2001); this led to the invention of slow-release GnRHa implants to advance final oocyte maturation and ovulation in female fish following the once-off handling of the broodstock using (Goren *et al*., 1995). For example, a single GnRHa injection resulted in 20 % of female gilthead seabream undergoing final oocyte maturation and spawning as compared with 80 % of females responding positively when GnRHa implants were used (Zohar, 1988; Zohar *et al*., 1995; Barbaro *et al*., 1997, Zohar and Mylonas 2001). The use of GnRHa implants also saves repetitive expenses such as anaesthetics and labour (Mylonas and Zohar, 2001;2007).

Studies carried out by Sokolowska *et al*. (1984, 1985) on goldfish detected the presence of gonadotropin-releasing inhibition factors (GRIF) identified as dopamine (Peter *et al*., 1986), which blocks the release of gonadotropins from the pituitary. The use of dopamine antagonist (DOPA) to block the pituitary dopamine effect has been applied (Billard *et al*., 1973; Lin *et al*., 1986a; Van Der Kraak, 1986; de Leeuw *et al*.,1985). The availability and absence of dopaminergic inhibitor, such as metoclopramide and

domperidone, have been demonstrated to have no significant effect on reproduction for some species, especially marine species. For example, cyprinids *Tinca tinca* were successfully spawned with and without metoclopramide in combination with GnRHa: LH levels increased gradually with a peak close to ovulation time when cyprinids were offered GnRHa alone, whereas in GnRHa + metoclopramide induced fish, an immediate LH surge peak with gradual decline was observed, indicating a degree of dopaminergic control (Podhorec *et al*., 2012). On the other hand, common carp did not ovulate with the administration of GnRHa alone but when combined with domperidone, and double injection of the conjugate resulted in an even higher spawning rate than a single injection (Dorafshan *et al*., 2003).

Peter *et al*. (1988) reviewed the efficacy of the application of GnRH analogue (LHRHa or sGnRHa) and a dopamine antagonist (e.g., pimozide, reserpine or domperidone) to induce ovulatory response and spawning of eight freshwater finfish by the Chinese. Co-administration of domperidone and gonadotropin analogues demonstrated shorter time to ovulation or spawning in cyprinids. In some species ovulation was only achieved after two-timing separated injections of the treatment and at high concentrations of the analogues when domperidone is combined with LHRHa (GnRHa; Lin *et al*., 1985, 1986a,b, 1988; Peter *et al*., 1998). GnRH analogues are generally more potent than endogenous GnRH either alone or combined with dopamine inhibitor (Peter *et al*., 1985; Lin *et al*., 1988).

A large body of evidence exists for freshwater and marine species that were successfully bred through domperidone inclusion in LHRHa induction therapies (Halder *et al*., 1991; Jansen, 1991; King *et al*., 1994; Leu and Chou, 1996; Powell *et al*., 1998; Pironet *et al*., 1999; Dorafshan *et al*., 2003; Arabaci and Sari, 2004; Park *et al*., 2007; Ibarra-Castro and Alvarez-Lajonchere, 2009; Vazirzadeh *et al*., 2008). Over the past years the hormone preparation, Ovaprim, have yielded enormous success in exogenous hormone therapy experiments. Ovaprim is a mixture of salmon GnRHa (sGnRHa) and domperidone (Leelapatra, 1988). The sGnRHa (Glu-His-Trp-Ser-Tyr-DArg^{6}-Trp-Leu-Pro-NH-CH_2-CH_3) is stabilized by an ethyl amide replacing the tenth amino acid (Glycine) and is a slight

modification of the less stable native GnRH structure (Glu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly- NH_2) (Martínez-Rodríguez *et al*., 2010). D-amino acids at the 6^{th} position of decapeptides have increased resistance to proteolytic cleavage, thus offering an extended half-life in the blood (Martínez-Rodríguez *et al*., 2010).

DiMaggio *et al*. (2014) investigated Ovaprim doses on spawning induction and egg and larval quality of pigfish, *O. chrysoptera* and found that the minimal dose of 0.25 mℓ/kg Ovaprim was efficient to induce spawning, and superior spawning performance was recorded at 0.50 mℓ/kg. Similar inducing concentrations were implicated in several finfish species: the carp species *Cirrhinus mrigala* and *Labeo rohita* (rohu) responded positively to minimal doses of 0.25 to 30 mℓ/kg and 0.30 to 0.40 mℓ/kg respectively, while *Aristichthys nobilis, Ctenopharyngodon idella, Hypophthalmichthys nobilis, Hypophthalmichthys molitrix* were induced from 0.40 - 0.50 mℓ/kg of Ovaprim (Nandeesha *et al*., 1990).

Spawning induction does not entirely facilitate a complete release of developed gametes in female *O*. *chrysoptera* (DiMaggio *et al*., 2014), pinfish (*Lagodon rhomboides*) (DiMaggio *et al*., 2013) and male African catfish (*Clarius gariepinus*) (Viveiros *et al*., 2002). DiMaggio *et al*. (2013) induced the spawning of female pinfish (*L*. *rhomboides*) with 500, 1000, 2000 and 4000 IU/kg hCG and found that eggs obtained by the manual stripping of 4000 IU/kg treated fish was significantly larger in volume when compared with the stripped egg volumes from the other hCG treatments. On the other hand, the volume of stripped eggs was not significant different between Ovaprim treatments (0.25, 0.50, 1.00 and 2.00 mℓ/kg) however, 54 % of all the fish released eggs upon manual stripping. Pigfish were manually stripped after the volitional release of eggs, and on average the stripped eggs from the different Ovaprim treatments (0.25, 0.50, 1.00 and 2.00 mℓ/kg) were not significantly different from each other.

The preceding overview clearly indicates the efficiency of Ovaprim as GnRHa spawning induction agent. The use of this compound at dosages consistent with literature-based quantities has been applied to the spotted grunter in the current investigation.

3.2 Objectives

i. To investigate the efficacy and minimum dosage of the commercially available analogue of salmon gonadotropin-releasing hormone (sGnRHa) (Ovaprim) in inducing the highest yields of viable spawned *P. commersonnii* eggs.

ii. To determine the latency time required between induction and spawning of broodstock exposed to t constant and fluctuating temperature regimes.

3.3 Materials and methods

3.3.1 System design

Four circular tanks (2.2 m diameter: 1.1 m deep and 4 kℓ volume) were used. The filtration system, photoperiod, and temperature regimes (constant and fluctuating) used in the experimental set-up is the same as described in section 2.3.4.

3.3.2 Assessment of spawning readiness and distribution of fish over experimental tanks

After the broodstock was conditioned for four months (see Chapter 2 section 2.3.5), the water in the 12 kℓ broodstock holding tank was lowered to 30 cm and the fish were anaesthetized with 200 ppm of 2-phenoxyethanol treatment prior to removal and further handling. Immediately when the fish lost equilibrium, soft material scoop nets were used to scoop them up, and the gender of the fish was identified by visual examination of the urogenital opening before transferring them respectively as per protocol into four circular experimental tanks. When the fish were gently massaged along the superior abdominal cavity in an anterior-posterior line towards the urogenital vent, males could be identified when producing running milt. Sexually mature females could be identified by their conspicuously distended bellies, and with a slightly swollen gonophore, compared to males having a small papilla shaped urogenital organ. The randomly selected fish were

transferred to the respective experimental tanks in a female to male ratio of 1:3, totalling two females and six males per tank. Females of between 39 and 42 cm caudal body length and body weight of 1.58 ± 2.45 kg (mean ± standard error, *n* = 4) were distributed over two tanks (F2 generation) while the remaining two tanks had females ranging between 39 and 53 cm caudal body length with a body weight of 2.64 ± 4.57 kg (mean ± standard error, *n* = 4) (F1 generation). In all the tanks, male spotted grunters ranged from 29 to 38 cm caudal body length and a mean weight of 1.03 ± 4.90 kg, *n* = 24). The fish were allowed to acclimatise four weeks prior to spawning induction. The fish were allowed to acclimatise four weeks prior to spawning induction; active feeding started five days post handling when the spotted grunter recovered from the stress of being physically handled, with the same dietary ration of 1 % body weight of 1:1 combination of squid and pilchard (over two sessions: morning at 09:00 (0.5 %) and afternoon at 17:00 (0.5 %) for the rest of the acclimatisation period (as described in section 2.3.5). It is worth mentioning that the diet of the broodstock was not consistent throughout the broodstock conditioning program for photothermal programs due to the inconsistent availability of squid. The latter was then replaced by sardine, which is also, an equipotent contributor of unsaturated fatty acids (HUFA and PUFA).

3.3.3 Spawning induction

3.3.3.1 Hormonal preparation and administration

Ovaprim® (Syndel, USA) is a liquid with propylene glycol as carrier of sGnRHa at 20 µg/mℓ and domperidone (10 mg/mℓ). The latter is an antagonist of dopamine, a brain neurotransmitter. The sGnRHa in Ovaprim® elicits the release of stored gonadotropins (FSH and LH) from the anterior pituitary (Mañanós *et al*., 2008), whilst the dopamine antagonist (DOPA) prevents dopamine to block the release of gonadotropins in gonadotrophic cells of the anterior pituitary (Donaldson and Hunter, 1983 Leelapatra, 1988; DiMaggio *et al*., 2014).

Following the conditioning of the broodstock of the spotted grunter at constant temperature regime (24 ± 0.5 °C) for four months, the fish were starved for 24 h prior to handling and hormone injection (subsection 3.3.3.2 below). The recirculating system was shut down, and the seawater in individual tanks were timeously lowered to 30 cm depth and the fish anaesthetised with 200 ppm of 2-phenoxyethanol. Immediately when the fish lost equilibrium, soft material scoop nets were used to transfer them into a prepared 1.2 kℓ water tank. The latter with similar water temperature as in the broodstock tank (for induction purpose), also pre-treated with 2- phenoxyethanol (200 ppm) and continuous aeration (20 mm leaky pipe diffuser loop). Whilst the fish were kept in sedated state in the 1.2 kℓ tank, the water in the experimental tank was quickly drained (bottom drain effluent valve) before the tank was filled with water from the recirculating system.

3.3.3.2 Hormonal induction of fish at constant temperature exposure

Four different doses of Ovaprim® were firstly tested on fish exposed to the constant thermal setting (24 ºC): females in the same tank received the same hormone dose, viz. 0.25, 0.50, 0.75- and 1-mℓ/kg, respectively. All the male spotted grunters were injected with a single dose of 0.25 mℓ/kg. The Ovaprim® was administered intramuscularly (dorsal) once using 1mℓ injection syringe. A direct control set-up in which broodstock did not receive hormonal treatment, was not carried out in parallel due to the lack of sufficient tanks; we did, however, have unequivocal evidence from the four-month broodstock conditioning period (under similar holding conditions as for the hormone-induced spawning experimental set-up) that showed that the spotted grunter did not undergo spontaneous spawning in captivity (Chapter 2). Following the injection, males and females were returned to their experimental tanks and left to spawn naturally. The lights were switched off to simulate the dusk spawning conditions of haemulids, following reports from Maurer *et al*. (2020) and Barden *et al*. (2014). The lights were switched back on when the fish ceased to spawn, and the squid and sardine diet was resumed.

This hormonal induction of fish at constant temperature exposure was repeated four times spaced by three to four weeks. The same anaesthetising protocol described above was

followed in all the repetition of the spawning inductions. The tested effective dose of 0.25 mℓ/kg of Ovaprim®, which equates to 5 µg GnRHa and 2.5 mg domperidone per kilogram of fish was used to induce the spawning of fish exposed to fluctuating temperature during further broodstock conditioning trials.

3.3.3.3 Hormonal induction of fish exposed to fluctuating temperature

In following trials, the spotted grunter broodstock were exposed to a fluctuating temperature program as described in section 2.3.4. Briefly, the fish were subjected to a gradual flux in temperature from 24 ± 0.5 °C to 19 ± 0.5 °C at a daily rate of 1.5 °C per day and then a gradual rise to 24 ± 0.5 °C at a similar daily change rate one day after 19 ± 0.5 °C had been it reached. This fluctuating temperature regime was initiated eight weeks after previous trials conducted for fish exposed to a constant thermal setting (24 ± 0.5 °C). Broodstock was then exposed to the fluctuating temperature program 3 weeks prior to the spawning induction event. The tested effective dose of 0.25 mℓ/kg of Ovaprim® was administered to finally induce broodstock spawning. This hormonal induction of fish exposed to the fluctuating temperature program mentioned in section 2.3.5 was repeated four times and spaced by three to four weeks.

3.3.3.4 Assessment of spawning success

At 24 h post-injection with Ovaprim®, visual checks were carried out on a 2 h basis to monitor spawning and to calculate the latency period towards spawning in both constant and fluctuating temperature regimes. The latency period is the time between the hormone injection to ovulation (Rottmann *et al*., 1991). Floating eggs were retained in a 500 µm terylene curtain type net bag fitted into the afore-mentioned egg collector. The net bag was removed from the egg collector, and carefully inverted and washed into a 25 ℓ bucket with clean water from the recirculating system. Viable eggs were floating while dead eggs were allowed to sink first to the bottom. The viable eggs were decanted into a large scoop net with fine mesh positioned slightly above a 25 ℓ water filled bucket so that excess water could overflow but the floating eggs retained in the scoop net. The scoop net was used

to dip and wash the eggs in a series of five respective prepared laboratory glass bowls (5 ℓ) filled with clean and 80 µm filtered marine water. A final dip was performed to disinfect the eggs in 0.1 % formalin bath for five minutes (Russel, 2013).

The weight of the viable eggs was measured using analytical balance (Radwag AS 220R2 Plus-Series, Lasec, South Africa). The bucket with sunken eggs was emptied through another fine mesh scoop net to collect the dead eggs, which were also weighed, and data recorded. A sample of the viable fertilized eggs (buoyant) from the spawner tanks were examined and photographed using a light microscope (Discovery V8, Zeiss, South Africa) to determine the diameter of the eggs and their developmental stage while the none-viable eggs were only photographed. Eggs evaluated as viable were buoyant and characterized by its transparency and a centred oil droplet, whilst non-viable eggs were without an oil droplet and sank. Three sets of 100 eggs were separated from the sampled floating eggs using a sterilised 200 µℓ pipette under the Discovery V8 Stereo microscope. The sets were respectively transferred into 500 mℓ borosilicate glass beakers filled with sea water. The beakers were placed in a temperature-controlled room set at 21 ºC - the same temperature as maintained in the stretcher tarpaulin tanks (see chapter 4) and they were offered faint aeration supplied through air stones. All the respective fertilized egg groups were transferred into new beakers to determine their hatching rates in the temperature-controlled room. The fertilization and hatching rates were calculated according to Brian (2015):

$$\text{Fertilisation rate} = \frac{\text{Number of viable eggs}}{\text{Total number of eggs}} \text{ X } 100$$

$$\text{Hatching rate} = \frac{\text{Number of hatched eggs}}{\text{Total number of fertilised eggs}} \text{ X } 100$$

3.3.4 Statistical analysis

The means of total fecundity, relative fecundity, floating eggs and sinking eggs between constant and fluctuating temperature regimes were compared using student *t*-test at

$p < 0.05$. All statistics were performed using SPSS version 27 (IBM SPSS Statistic, New York, USA).

3. 4 Results

The spotted grunter did not undergo spontaneous spawning in captivity during the four-month broodstock conditioning period under the constant temperature regime and during the eight weeks phasing in the fluctuating temperature regime. After the administration of Ovaprim® to the broodstock exposed to the constant temperature regime, the social and courtship behaviour of the spotted grunter changed significantly. The fish swam close to the surface area in a slow, coordinated and school fashion about two hours following the Ovaprim® administration. About four hours later, the spotted grunters moved to the bottom, and the swimming speed was improved. Two hours before the actual spawning, the fish were pursuing one another and started settling in groups. Released eggs (spawns) were observed in the egg collector about 30 -32 h post injection and the spawning lasted for three days. The total spawned eggs declined over the spawning period. The spawning temperature was set between 23 and 24 °C. Spawning was not successfully induced in all the tanks; only one tank of the small sized spotted grunters (two females and four males) that had received receiving 0.25 mℓ/kg of Ovaprim®, spawned. Other than that, no broodstock mortalities were observed throughout the spawning inductions. Similar social and courtship behaviour were observed when the fish were spawned repetitively (eleven weeks apart) after exposure to the fluctuating temperature program. Again, spawning only occurred in one tank that housed the same breeders that had previously spawned during the induction of fish exposed to constant temperature program (four episodic repetitions). The post-induction latency period was not affected by the temperature regimes or the broodstock spawning experience and a fertilization rate of 98.66 and hatching rate of 89.53 % were achieved as mean value between spawning events. Larval hatching occurred 18 to 21 h post spawning. The fertilized eggs were spherical and measured 1.52-1.60 mm (mean ± SE = 1.5 ± 0.01 mm, $n = 10$) in diameter and the chorion was transparent surrounded by perivitelline spaces (discussed extensively in the next chapter).

The hormonally induced spawning output data for *P*. *commersonnii* conditioned at constant and fluctuating temperatures, respectively, are presented in Figure 7. When the fish were exposed to constant temperature (24 °C), the total number of spawned eggs (305239) was considerably lower than the 1070808 eggs produced after the broodstock conditioning with fluctuating temperature regime (a gradual flux from 24 ± 0.5 °C to 19 ± 0.5 °C at a daily rate of 1.5 °C per day and then a gradual rise to 24 ± 0.5 °C at a similar daily change rate one day after 19 ± 0.5 °C had been it reached). The ratio of floating to sinking eggs was better in spawns for the fish consistently exposed to the same temperature (43:1) compared to an 18:1 ratio from the same broodstock spawned after exposure to the fluctuating temperature program (Figure 7). The results also demonstrated a significant difference ($p < 0.05$) between the spawn volumes of the same breeders respectively exposed to constant and temperature fluctuation (Figure 7). Total batch spawn fecundity was significantly higher in spawners exposed to the fluctuating temperature program ($t = 2.05$, $p < 0.05$), with spotted grunter females producing egg numbers that are almost three-fold higher than their constant temperature exposed broodstock counterparts. The same tendency was observed for the collective total of floating eggs between treatments ($t = 2.15$, $p < 0.05$).

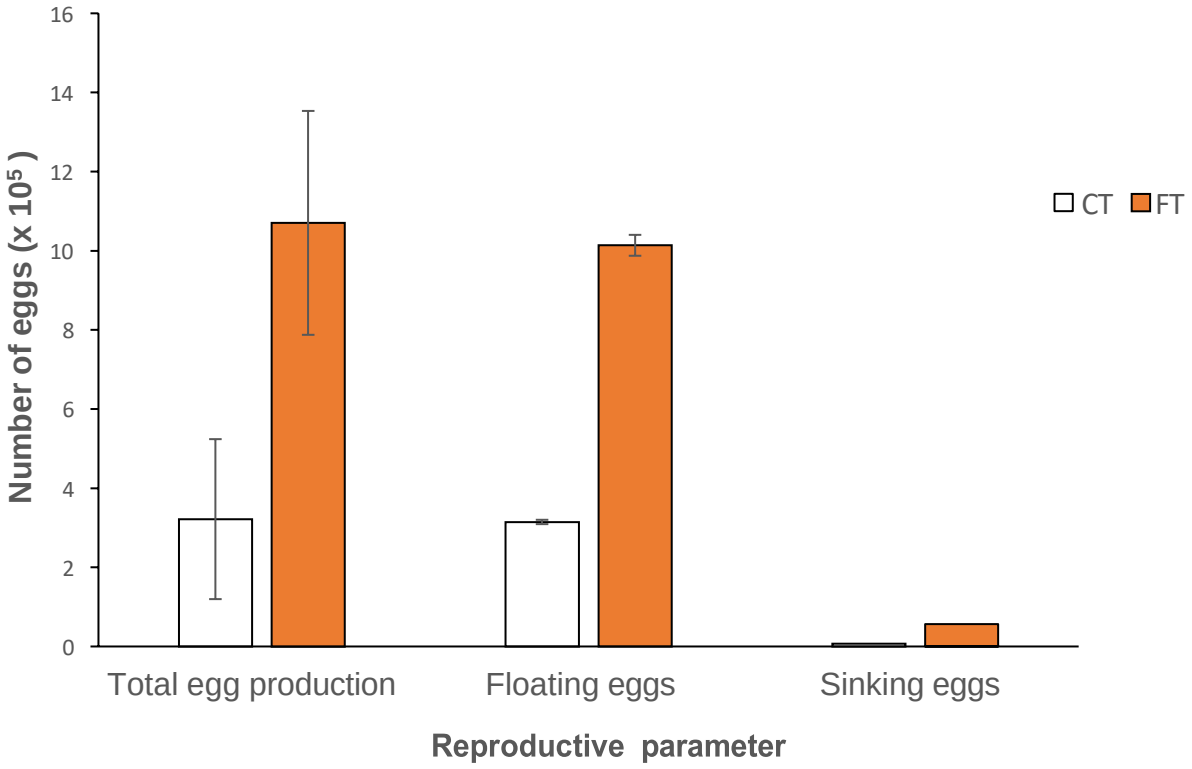

Figure 7: The reproductive parameters of the *P*. *commersonnii* broodstock spawned with induction agent Ovaprim® (0.25 mℓ/kg), that represents 5 µg salmon GnRH analogue and 2.5 mg domperidone per kilogram of fish. CT represent spawning induction after exposure to constant temperature while FT is fish spawned after fluctuating temperature regime. Data are shown as Mean ± S.E., where the spawned broodstock consisted of the same set of six individuals (two females and four males) that were induced to spawn multiple times following conditioning under constant environmental temperature (CT), and under a fluctuating temperature (FT) set-up.

3.5 Discussion

The application of hormone therapies is a necessary tool to induce the spawning of fishes that exhibit reproductive dysfunction in captivity such as the spotted grunter. For the first time, conditioned spotted grunter breeders were successfully induced to spawn using a commercial GnRHa agent (Ovaprim®). The lack of luminescent biomarkers to visibly discriminate between the genders and the dark shading hindered the observation of female to male pairing and the courtship ritual. The induction initialization time (11:00 - day 1) was selected to accommodate a 32-h latency time and to co-incide with next day dusk conditions - considering that some other haemulid species also spawn during dusk. Spawning events at dusk were also reported in the studies of Oriental Sweetlips, *Plectorhinchus vittatus* (Leu *et al*., 2012); French grunter, *Haemulon flavolineatum* (Maurer *et al*., 2020) in recirculating systems, thus spawning ascent could not be observed due to the dark photoperiod (Barden *et al*., 2014). These species were reported to have spawned volitionally while the grunters failed to spawn naturally under the constant temperature regime, and even when the environment was manipulated (fluctuating temperature).

In the current investigation female breeders only responded to induction in one out of four tanks for all four consecutively used trial groups respectively treated with an increasing hormone dosage. The spawning females present 20 % of the available spawning cohort. It was conspicuous that the abdomen of the larger female size group of 2.64 ± 4.57 kg was not distended, which is the primary indicator that their ovaries were not fully packed with vitellogenic oocytes at maturation stage. The bigger females could have been the latest spawners, having recovery-ovaries with premature clusters of oocytes that will become the next generation of mature eggs, though not synchronised with other females of the population, since the spotted grunter is a group asynchronized spawner. Therefore, such as in the wild, final gonad maturation and spermiation is also asynchronized in artificially controlled broodstock keeping environments. This is confirmed given that the larger size of *Pomadasys sp*. generally spawn earlier than smaller breeders within the same season (Darcy, 1983; Ohs *et al*., 2011). Bacela (1998) and Childs (2005) reported that wild spotted grunter attains sexually maturity at 280 - 420 mm total length, same as the size

class of the spawning group used in the current investigation. This is indicative that small females are conditioning more rapidly than the bigger fish (Dr Fouche, personal communication).

Maternal age is reproductive parameter affecting the frequency of spawning output (Targońska *et al*., 2012). The egg quality of *Leuciscus idus* broodstock older than nine years were significantly poor, and only 67 % of the females broodstock ovulated (Targońska *et al*., 2012). In captive striped Bass *Morone saxatilis,* higher sperm production and increased sperm longevity was superior in three years old fish compared to one- and twelve-years age groups (And and Zohar, 1999). It is evident that the four to five years old female spotted grunters in the current investigation grunters may have reduced reproduction capacity or may be completely dysfunctional. The ovarian histology a larger size-class female spotted grunter, was unfortunately not performed since female numbers were limited for experimental use.

DiMaggio *et al*. (2013; 2014), Broach *et al*. (2015), Broach and Ohs (2020), confirmed no more than 50 % of *Orthopristis chrysoptera* or pinfish females injected with hormonal spawning aids will ovulate and spawn even under the optimal recommended Ovaprim dosage of 0.5 mℓ/kg. However, Broach and Ohs (2020) observed an improved number of maturing pinfish and pigfish females when chicken gonadotropin release analogue (cGnRH IIa) was administered too, suggesting that they were in the process of conditioning, but still not ready to spawn. It's assumed that cGnRH IIa leads to higher expression of cGnRH IIa receptors in reproductive tissues (Lin *et al*., 2010), leading to multiple spawning frequencies compared to single spawning events for Ovaprim-treated fish. The results prompt further investigation into alternative inducing agents such as cGnRH IIa or testing different delivery systems such as slow-release hormone therapies. In terms of the latter application for example Mylonas *et al*. (2010) reported that 20 % of gilthead seabream spawners ovulated daily after receiving a single dose of gonadotropin analogue, whereas the use of a slow release GnRHa-delivery system resulted in 70 % spawners. Matsumaya *et al*. (1995) reported that immature *Pagrus major* underwent

oocyte maturation and ovulation for 12 to 14 days in response to LHRHa implants while the control group that received no treatments did not spawn.

Failure of gametogenesis, final maturation of oocytes and spermiation in captive fish is a feature of both sexes (Mylonas *et al*., 2010). Ovarian biopsy as a pre- selection method to assess the readiness of the broodstock was neglected as it may interfere with the reproductive performance of the spotted grunter. Handling grunter leads to slow feeding response recovery, which negatively affect feeding (long delayed response) and consequently oogenesis and final oocyte maturation. In some other investigated species, handling stress inhibits oocyte development, and final maturation, therefore negatively affecting the timing of reproduction (Morgan *et al*., 1999; Cleary *et al*., 2000; Schreck, 2010).

The efficacy of the 0.25 mℓ/kg Ovaprim® is a demonstration of the potency of the hormone to inducing spawning in spotted grunter, considering that the manufacturer recommends 0.5 mℓ/kg Ovaprim®. The lower required dosage could also be a result of the near absence of inhibitory dopaminergic activity as asserted by Mañanós *et al*. (2008), who suggested that active dopaminergic systems may be weaker or lacking in marine fish. Ovaprim potency is also higher in African catfish (Dr Fouche, personal communication), and the pituitary receptor of goldfish was reported to have higher affinity for Ovaprim than mammalian analogous (Habibi *et al*., 1989).

The efficacy of the minimum dose is identical to the findings for *Orthopristis chrysoptera* (DiMaggio *et al*., 2014), *Cirrhina mrigala* (Nandeesha *et al*., 1990), and black sea bream, *Acanthpagrus berda* (Abbas *et al*., 2019). Meagre, *Argyrosomus regius* on the other hand was spawned with a lower concentration of 0.01 - 0.05 mℓ/kg Ovaprim (Pastor *et al*., 2013). Mata *et al*. (2004) achieved the spawning of the grunter *Orthopristis ruber* using 50 and 25 ng/kg of GnRHa (LHRHa) and quality eggs were only produced from females injected with 50 ng/kg (DiMaggio *et al*., 2014). Grunters such as *P*. *vittatus*, *Anisotremus scapularis, and H*. *flavolineatum*, were reported to spawn spontaneously in captivity (Leu *et al*., 2012; Barden *et al*., 2014; Hauville *et al*., 2016; Montes *et al*., 2019; Maurer *et al*., 2020),

whilst the spotted grunter failed to spawn naturally for all photothermal broodstock conditioning programs tested. The spawning of the spotted grunter occurred 30 - 32 h post-injection, while *Orthopristis ruber* was reported to have spawned after 28 h (Mata *et al*., 2004). Latency time may be unique to species since *O*. *chrysoptera* spawned only within 48 h from induction (Broach and Ohs, 2020). In other species, the latency period of *Channa punctatus,* injected with 0.3- and 0.5 mℓ/kg of Ovaprim, was similar to the spotted grunter (Kather Haniffa and Sridhar, 2002). These studies demonstrated that latency period is species dependent, and dependant on the type of the spawning agent (Okere *et al*., 2015).

The spawning frequency and the oocytes at different stages of development confirmed the spotted grunter as a multiple-batch asynchronous species. This asynchronous nature and spawning frequencies are consistent with wild *P*. *commersonnii* in the waters of Arabian Sea Off Oman where Al-Nahdi *et al*. (2011) suggested that the spotted grunter may spawn more than once within a year and is consistent with the spawning frequency of *Pomadasys stridens* (Vahabnezhad *et al*., 2018). The spawning frequency is similar to that of the French grunter, which was reported to spawn spontaneously after two weeks of captive acclimatization to 24 °C and photoperiod of 12D: 12L (Barden *et al*., 2014), as well as for *Orthopristis chrysoptera* exposed to the same conditions (Kaiser *et al*., 2018). The spawning of asynchronous fish is unpredictable due to the cohorts of eggs that are in different stages of development, and some retained eggs when stripping was not applied (DiMaggio *et al*., 2014).

It is unclear if the spotted grunter is a capital or income-breeder in terms of energy sourcing during oocyte maturation. Egg production of income breeding type relies on the rapid mobilization of energy reserves stored during oocyte maturation while capital breeder come from ovarian, or liver energy stores acquired over long periods (Higuchi *et al*., 2018). Other non-related fish species such as Japanese anchovy (Yoneda *et al*., 2014) and medaka, *Oryzias latipes* (Hirshfield, 1980) spawned for several weeks despite being starved but, their batch fecundity and spawning frequency declined as well. The spawning frequencies of the grunter may suggest predominantly capital

energy resource breeding nature, since multiple spawning will exhaust energy reserves that is only available at oocyte level. Yellowtail *Seriola quinqueradiata* was demonstrated to exercise both breeding energy resource categories (Higuchi *et al*., 2018).

In spotted grunter, the total number of spawned eggs gradually increased per spawning cycle. However, the relative fecundity of the individual females could not be assumed to be equal, hence the failure to establish the statistical significance of the batch fecundity between spawning inductions per photothermal program treatment. However, the total yield of spawned eggs of the first two spawning inductions of breeders exposed to the constant temperature conditioning program, was distinctively low (n=30239 and 55680) whilst the follow up inductions respectively yielded 215040 and 920000 eggs. Poor yield of first egg batches was also reported by Trippel (1998) in Atlantic cod study where first-time spawners produced low batch fecundity, low fertilization rates and hatchery rates, exhibited smaller egg size, and spawned for short time, relatively to repeat spawners. The biochemical composition (total lipids, triacylglycerol, sterol, essential amino acids) of Atlantic halibut, *Hippoglossus hippoglossus*, was significantly lower in eggs procured from first time spawner than repeat spawners, leading to poor fertilization in first time spawners (51%) compared to the 81% achieved from repeat spawners (Evans *et al*., 1996).

The fluctuating temperature program yielded a first spawn collective total of 873600 eggs post induction, which is a dramatic increase when compared to the indicated yield of first batch spawns in breeders exposed to the constant temperature program. Also, follow up inductions of breeders that were exposed to the fluctuating temperature program, were consistent in mediating the highest fecundity in spawning yields. The spawned batches collective totals were up to three-fold more ($p < 0.05$) than batches collected from the constant temperature conditioning program for the same females. The significantly low total number of sinking eggs in the spawns of both temperature control programs is indicative that broodstock conditioning was complete and that the respective collective egg outputs for both programs was a function of the program itself and not factors like feed quality and quantity. The reproductive dysfunction of the larger fish size was obviously attributed to factors other than the conditioning program. Properly conditioned

breeders that delivered a relatively low output of sinking egg spawns were reported in other marine and freshwater finfish treated with Ovaprim (Nwokoye *et al*., 2007; DiMaggio *et al*., 2015; Broach and Ohs, 2020).

It is worth mentioning that the diet of the broodstock was not consistent throughout the broodstock conditioning program for photothermal programs due to the inconsistent availability of squid. However, sardine proved to be a good alternative since relative high spawn yields were still maintained for the same fish exposed to the photothermal programs. The spawning inductions were spaced by almost three to four weeks; therefore, the diet composition variation and timing of spawning induction may have influenced to some extent the inconsistency in fecundity yields as marked by the relatively high standard error from the average between the collective spawned egg outputs of the respective spawning induction trials (Figure 7). However, the fluctuation in diet type availability did not affect the superior spawning performance of broodstock exposed to the fluctuating temperature program.

In the wild, the spawning of the spotted grunter occurs in the summer period which coincides with high temperatures (Day *et al*., 1981; Webb, 2002; Al-Nahdi *et al*., 2011), suggesting that spawning is temperature dependent. Webb *et al*. (1999) found that when white sturgeon, *Acipenser transmontanus* was exposed to oscillating temperature of between 10 - 15 °C and two constant temperatures (15 and 18 °C) and spawned through priming and resolving hormone dose, ovulation only took place in the fluctuating thermal regime while oocytes development was inhibited in both the constant temperatures. Senegal sole, *S*. *senegalensis*, was spawned in captivity under a naturally fluctuating temperature regime (Anguis and Cañavate, 2005). The authors found that when the temperature was increased up to 2.5 °C two to three days before the spawning induction, the total fecundity increased to 65-73 % while constant or declining temperature led to decreased daily fecundity. Similar improved fecundity was reported by Argüello- Guevara *et al*. (2013), who induced the spawning of oyster, *Striostrea prismatica* through thermal shock by fluctuating water temperature up and down by 10 °C for an hour and temperature change between 30 and 20°C for 30 minutes.

Therefore, it is probable that the temperature fluctuation in the present study may have led to a higher frequency pulse stimulation of the hypothalamic-pituitary axis, which regulates the production of gonadotropin-releasing hormone, maturation agents and luteinising hormones which are in turn, associated with maturation and ovulation (Mylonas *et al*., 2010).

The high hatching success from this study is consistent with the finding of Broach and Ohs (2020) in their pigfish experiment. The hatching rate were significantly high, 70.67 ± 2.15 % for Ovaprim treated females (0.5 mℓ/kg) than either 25, 50, 100 and 200 µg cGnRH IIa- treatments (Broach and Ohs, 2020). Unfortunately, the effect of hatchery temperatures above 22 ºC consistently caused the outbreak of a bio-contaminant that compromised the eggs and larvae so much that 100 % mortalities were experienced, hence the single representation of corresponding fertilization and hatching rates. This problem of bio-contamination was peculiar to the Sea Point Marine Research Aquarium and will be further discussed in the next chapter.

Okere *et al*. (2015) reported adverse health concerns using Ovaprim following the mortalities of all post-spawn catfish broodstock injected with 0.5 mℓ/kg Ovaprim while the control fish administered pituitary extract completely recovered. Earlier studies reported no mortalities of catfish species (Kather Haniffa and Sridhar, 2002; Olubiyi *et al*., 2005) using similar treatment as Okere *et al*. (2015). In the present study, the broodstock completely recovered post spawn in all the spawning induction with no visible adverse conditions, which demonstrate that neither the lowest concentration and the highest dose of 1 mℓ/kg were toxic and lethal. A surveillance studies involving 39982 fish from 25 species injected with Ovaprim reported a general low post spawn mortality of about 1.3 % and were either caused by poor water quality, egg binding condition, and classified as ‘‘unknown” (Hill *et al*., 2009). The hormone Ovaprim is a “Legally Marketed Unapproved New Animal Drugs” status in the United States of America by the Food, Drug and Administration since 2009 for ornamental fish breeding purposes while in countries like Chile and India is indexed for food fish consumption (Hill *et al*., 2009).

In one incident of the current investigation, following spawning induction procedure, there was a failure of the system clean water pump serving the inline heat pump before the water is introduced into the holding broodstock tank. This caused a steep temperature decline from 24 to 16 °C within 13 h (overnight), resulting in 93 % mortalities of breeders during the last trial conducted. There was otherwise no deviation in the physico-chemical properties of the water. As mentioned in the previous chapter, the spotted grunter is highly susceptible to stress. It was therefore deducted that the temperature- induced stress, combined with the stimulus of spawning and spawning related stress, may have contributed to the mentioned mortalities. Their wild counterparts were reported to avoid below 16 °C areas during the “grunter run” (Childs, 2005), thus indicating sensitivity to low temperatures.

Lowered temperature in the current investigation creates physiological shock in spotted grunter is a stressor which can stimulate low level spawning fecundity (≤ 30 eggs) induction when the temperature is gradually increased over two days. This phenomenon was observed when spotted grunter broodstock recovered from cold shock (17 ºC) when the temperature was increased by 1 °C per day. However, egg output volumes were obviously very low and non-efficient as a tool for hatchery programs. This observation is asserted by Ohs *et al*. (2011) who suggested that a cold front and decline of water temperature may trigger spawning. Further investigations are needed to establish whether a positive correlation exist between the batch fecundity and the biochemical composition of the spotted grunters eggs for both photothermal programs used. However, the high yield output of quality fertilized eggs may be indicative that the biochemical composition of ovulated eggs is consistent. It is therefore recommended that the fluctuating thermal program of the current investigation be applied to maximize fecundity outputs in future hatchery spotted grunter breeders.

In conclusion, this study developed an efficient programmable broodstock conditioning method which will facilitate hatcheries to produce seed stocks at high quantities when required. The commercial GnRHa analogue developed for salmon (Ovaprim®) has been

tested and proven to be a potent inducer of spawning in pre-conditioned spotted grunter. Broodstock exposed to fluctuating temperature regime yielded significantly high numbers of viable eggs and low volumes of sinking eggs. Contrastingly, broodstock conditioned at a constant temperature spawned lower numbers of viable eggs and high volume of sinking eggs. It was demonstrated that the spotted grunters are multiple batch asynchronous spawners, which allow the species to have multiple spawning sessions within one season (wild), or continuously – when exposed to photothermal programs in captivity.

CHAPTER 4: LARVAL REARING TECHNIQUES

4.1 Introduction

The larvae are stocked at very low densities in extensive larval rearing practices (0.1- 2/ℓ), and therefore in relatively large water bodies with conditions less manipulated (more natural) (Dhert *et al*., 1998; Divanach and Kentouri, 2000). Extensive larviculture is also mostly practiced by first pre-fertilizing the water body and culturing of phytoplankton, prior to the synchronised stocking of zooplankton and eventually fish eggs/larvae (Dhert *et al*., 1998; Divanach and Kentouri, 2000). Since conditions are kept mostly natural, larvae have more living space and are not subjected to stressors such as high densities in stringent rearing conditions (Dhert *et al*., 1998). Because of less control this technique has a very low productivity output (temperature and predator pressures) which can be a challenge in commercial aquaculture (Dhert *et al*., 1998). With this technique, fish larvae are strictly dependent on the endogenous bloom of zooplankton which develops on endogenous primary food chain productions (autotrophic and heterotrophic) (Divanach and Kentouri, 2000).

Semi-intensive larval rearing techniques are intermediate between extensive and intensive techniques, hence the name (Divanach and Kentouri, 2000). These types of hatchery techniques can either be indoors or semi indoors. They have advantages from both the extensive and intensive techniques without their inconveniences (Divanach and Kentouri, 2000). In these rearing techniques, rearing densities of larvae are a bit higher than in extensive techniques but still relatively low (2 - 8/ℓ) in relatively big (30 - 100 m^3), deep (1.5 - 2.5 m) well shaped tanks (Divanach and Kentouri, 2000). A high number of fry can be produced per tank in semi-intensive rearing systems.

Environmental conditions in the semi-intensive technique are both natural and artificial providing a variety of possible culture scenarios, with limited climatic, seasonal, or geographic influences, allowing also for optimization of operational costs, with relatively low production risk (Divanach and Kentouri, 2000; Mozes *et al*., 2011). Factors such as

photoperiod and salinity of the water are artificially controlled and well managed (Mozes *et al*., 2011; Divanach and Kentouri, 2000). Larvae depend on both exogenous and endogenous feeding in different stages; this ensures a good matching of larval energy requirements (Divanach and Kentouri, 2000). The semi-intensive production technique can accommodate methods like natural algae bloom, pseudo-green water, green water, and clear water methods (Divanach and Kentouri, 2000).

Intensive larval rearing techniques are sophisticated and the most expensive rearing techniques with production entirely depending on intervention from man and technology (Dhert *et al*., 1998; Divanach and Kentouri, 2000). Unlike extensive and semi-intensive rearing, intensive larval rearing has high larval rearing densities in small, well-shaped tanks, under very strict specific hydraulic, thermal, light, and feed conditions (Dhert *et al*., 1998; Divanach and Kentouri, 2000). Larvae depend on exogenous feeding in these techniques, and they are mostly restricted to a diet of *Branchionus* and *Artemia*. The latter species are enriched with proteins, highly unsaturated fatty acids and vitamins (Divanach and Kentouri, 2000). Besides being expensive, the downside of these techniques is that when the technical supply does not totally match the biological demand, reared larvae have no other option than to adapt or die (Divanach and Kentouri, 2000). The metabolic requirements of the larvae are better maintained when nourishing first feed organisms like rotifers/copepod nauplii with a continuous availability of unicellular algae in the exponential phase of proliferation. This method is applied in the pseudo-green water technique initially developed in sparid hatcheries (Rubio da Costa, 2012).

4.2 Objectives

i. To record microscopic embryonic and post-embryonic ontogenetic stages until the exogenous feeding stage in larvae, to identify and monitor morphological changes required for larval survival.

ii. To produce plankton species with predictability and as pre-requisite for larval rearing.

iii. To determine the survival rate and growth of *P*. *commersonnii* larvae reared in pseudo-green water conditions as the preferred method for increased larval survival to the weaning stage.

4.3 Materials and methods

4.3.1 Hatchery design

A total of eight 1.3 kℓ rectangular fiberglass tanks (1.3 m x 1.3 m x1 m) with shallow conical floor were used to support the four legs ends of a rectangular stretcher tarpaulin tank (STT) (Figure 8A,B). The STT were used to rear the larvae of the spotted grunters. The fibreglass tanks and STT's were disinfected using Virkon® disinfectant (South Africa). An overhead illumination of 750 lux was provided for hatchery tank as there is no reference for the rearing of the spotted grunter's larvae from eggs to juvenile stages (Figure 8D). Each of the tarpaulin slings consisted of an exit 250 µm screen connected with an L-shaped pipe (Figure 8C) below it to control and adjust the flow of water out of the slings into the tanks. The RAS filtration systems were in principle similar to the system described in Section 2.3.4 - system design, except that the heat pump (Model SF040P, Cape Town, South Africa) was set to 21 ± 0.5 °C. Treated water was finally introduced into the tanks via controlled valve (Figure 8E) and slings. The tanks were filled with water up to 70 % of their capacity to generate a water bath, as means to ensure constant temperature of 21 ± 0.5 °C in the slings. Aeration was supplied into the individual slings using an airline connected glass pipette to produce weak to mild bubbles (Figure 8F), and through valve-controlled air supply from the facility's main side channel blowers.

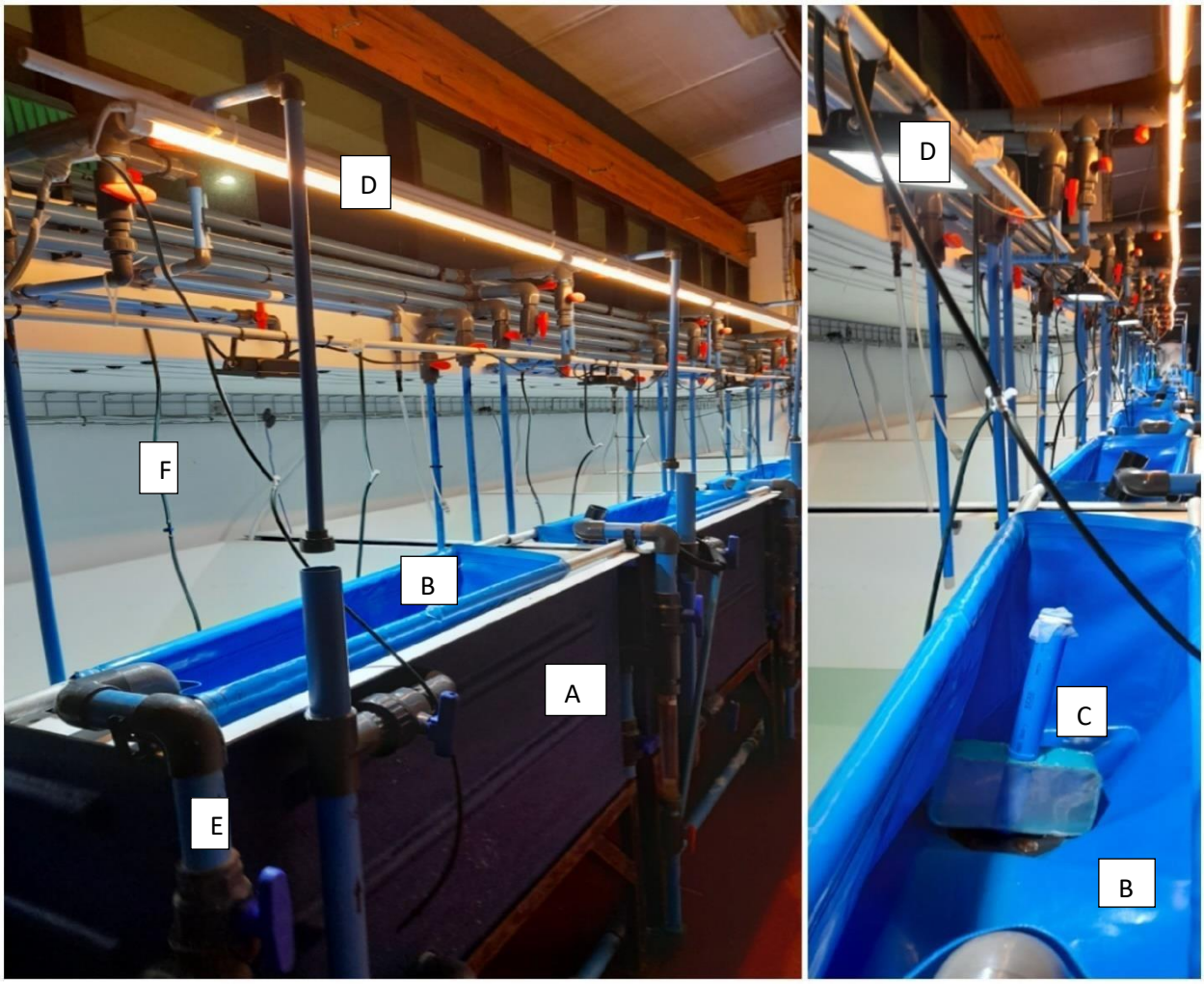

Figure 8: The hatchery system used for the rearing of *P. commersonnii* larvae. The 1.3kℓ tank (A); Stretcher tarpaulin tank (B), 250 µm screen (C), overhead 750 Lux LED illumination maintained on 12D: 12L photoperiod (D), pump supply pipe and valve (E), aeration line valve controlled (F).

4.3 2 Algae and Rotifer production

4.3.2.1 Algae production

Stock cultures of *Nannochloropsis oculata* were obtained from the in-house algal laboratory. Three aliquots of 200 µℓ were enumerated using a haemocytometer and placed under compound microscope (AEC 158, Zeiss, South Africa) to determine the

density of the stock culture (8.9 X 10^6 cells/mℓ). The stock cultures were kept in four round bottom flasks (volume 4 ℓ each) sterilized (autoclave) and fertilized with 1 mℓ of Guillard's F/2 growth medium (Guillard and Ryther, 1962) prepared from the commercially available Cell-Hi F2P powder (Varicon aqua Solutions, Malvem, UK) at a dosage of 1 g/ℓ of distilled water. The bottles received pre-filtered (10 µm) aeration supplied through glass pipettes, and the top of the flasks were covered with tinfoil. The flasks were then positioned next to horizontally installed fluorescent tubes (30 W cool white) on a culture rack. Light intensity was 455 lux, measured with a luxmeter (MS-6610 Mastech, United States). The stock culture was left to mature for two weeks at 0D: 24L and was consistently kept a 21 °C laboratory temperature (Figure 9A).

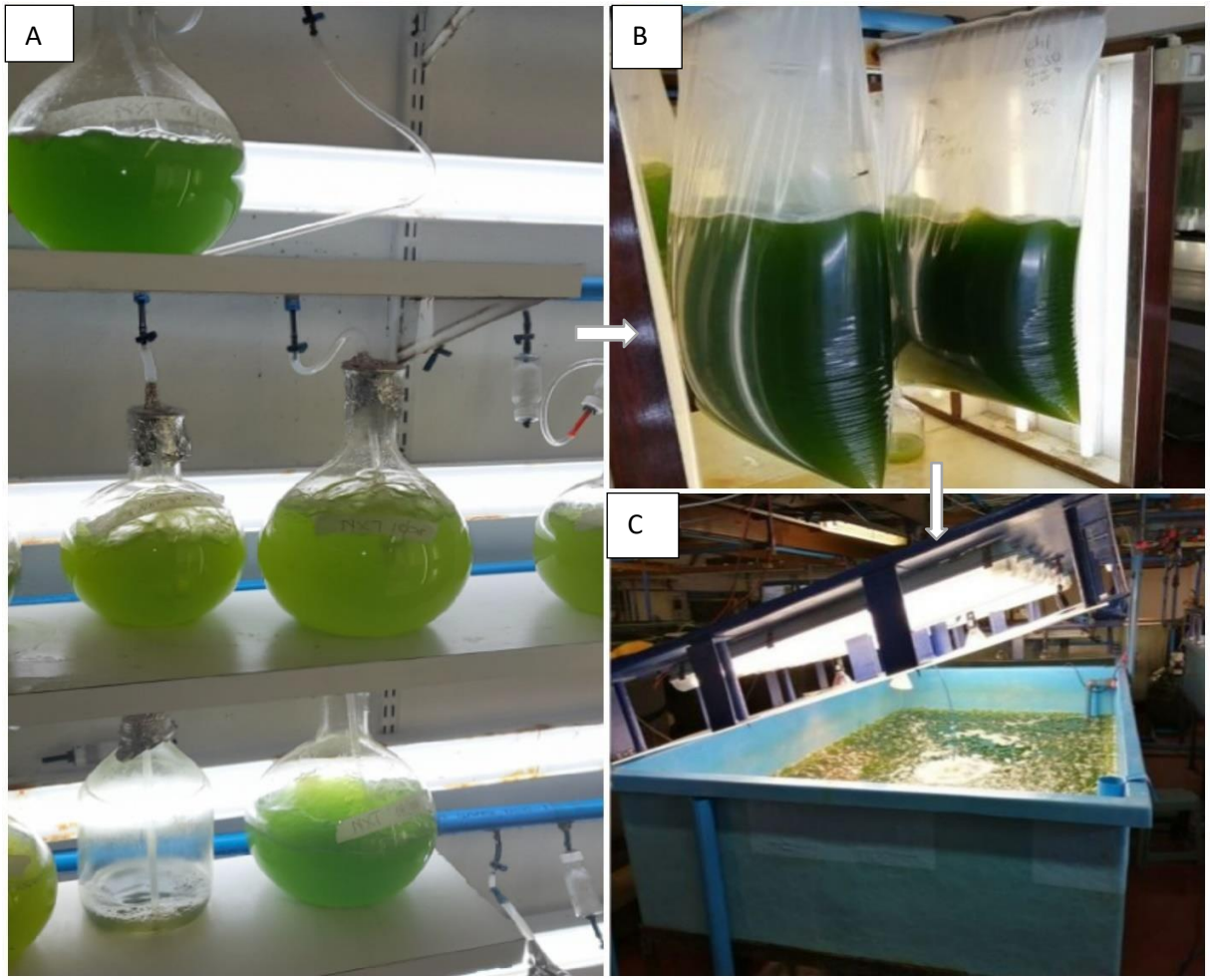

Figure 9: Biomass production of *Nannochloropsis oculata*. Stock cultures of the algae (A), the mature algae transferred into maturing bags (B), upscaling of the algae in 2.2 kℓ fibreglass tank (C).

Two transparent plastic bags were filled with 60 ℓ of 0.2 µm filtered sea water and were individually chlorinated with 100 mℓ of 12 % sodium hypochlorite solution (Figure 9B). The bags were then supplied with aeration through 1 000 µℓ pipette tips for 2 h and chlorine neutralized with 84 g sodium thiosulphate ($Na_2S_2O_3.5H_2O$). A pair of the culture matured flasks were later transferred into each of the plastic bags, fertilized with 60 mℓ of the prepared Guillard's F/2 growth medium and the bags were left to mature for two weeks (24-h photoperiod at 455 lux, and 21 °C).

A 2.2 kℓ fiberglass tank covered with an overhead fiberglass lid was used for mass culture of algae. The lid was equipped with 18 x 1.2m LED T8 lighting tubes (22 W and 2400 kW each), mounted with lighting side facing the tank water. The lid also accommodated 3 x infrared globe heaters (Figure 9C). The lighting components were protected by a perspex sheet also attached to the lid with extension leg connections. The tank was also equipped with a temperature probe that was connected to a control panel (PC SIMATIC HMI panel, Siemens, South Africa). The latter controlled all lighting, heating, and pump transfer of tank contents. The tank was filled with 1000 ℓ of one micrometre filtered sea water, chlorinated with 1667 mℓ of 12 % sodium hypochlorite solution and received mild to strong aeration for 2 h and later neutralized with 1400 g sodium thiosulphate ($Na_2S_2O_3.5H_2O$). The sea water was fertilized with a formulated industrial fertilizer (Dr Fouche, personal communication [Table 1A]). The mature algae (from the plastic bags) were then introduced into the tanks with algae culture diluted in a ratio of about 1:5. The culture matured within two to threeweeks at 22 ± 1°C and continuous 24 h lighting (600 lux). The algae culture was then maintained by implementing a weekly 30 % total replacement exchange with pre-fertilized dechlorinated water, following the maturation period. After each replacement, the tank was re-fertilized (Table 1B). The culture tanks also received a floor mounted ring aeration supply through a 32 mm pvc pipe with a 1 mm aperture spaced 100 mm apart. The ring mounted in the corner line of the rectangular tanks (L = 268 cm; W = 170 cm; D = 40 cm). The aeration “curtain” created a bubble wall that lifted bottom water to the top and back in a vertical convection current created, so that all algae will be surface positioned for light exposure over time.

Table 1: The composition of the industrial fertiliser used for mass production of the algae, *Nannochloropsis oculata*.

Ingredients of the fertilizer	**A (1000 ℓ)**	**B (100 ℓ)**
Phosphoric acid (85%) in mℓ	4	0.4
Nitric acid (55%) in mℓ	160	16
Vitamin B12 (mg)	1	0.1
Sodium bicarbonate (g)	1000	10
Fe-EDDHA (Ferric ethylenediamine-N, N′-bis -(2-hydroxyphenylacetic acid) (g)	0.5	0.05
Thiamin (mg)	20	2
Nutrifeed®* (g)	50	5

* A Starke Ayers product

4.3.2.2 Mass production of rotifers

Two cylindrical fiberglass tanks (1 m^3) were prepared for mass rotifer culture. The tanks were first rinsed with warm to hot tap water and filled with 800 ℓ of 0.1 µm filtered sea water chlorinated with 1333 mℓ of 12 % sodium hypochlorite solution. After 2 h of chlorine treatment, the tanks were aerated with open pipe (no diffuser) bubblers for 2 h. Any remaining residual chlorine (HACH test strip reading) was neutralized with sodium thiosulphate ($Na_2S_2O_3.5H_2O$) in a ratio of 5:1. The tanks were then illuminated throughout the course of the rotifer production with a 120 W metal halide lamp and water temperature maintained at 22 °C, using a suspended 2 kW hotrod heater (Metalquip, SA) controlled by a digital temperature sensor/controller (Model STC1000).

Tank cultured rotifers were fed 20 ℓ of bag cultured *N. oculata* (Figure 9C) every other day and a 30 % water exchange were implemented twice a week per rotifer tank. Top up sea water was kept in an adjacent tank and pre-treated with 0.1 µm filtration and only dechlorinated prior to use. Rotifer culture aliquots of 200 µℓ were enumerated by haemocytometer subjected to the compound microscope to quantify the stocking density of the rotifers. The indicated compound microscope was used to monitor the development of the rotifers.

4.3.3 Experimental design

The spotted grunters' eggs were procured from the spawning induction protocols reported in Chapter 3 (section 3.3.3). About 5 g of disinfected eggs (approx. 8000) were weighed using an analytical balance, and then respectively distributed into the eight STT's (Figure 8) with a water volume of 90 ℓ. The temperature was set at 23 °C and photoperiod was maintained at 12D: 12L (750 lux) to promote swim bladder inflation (Foscarini, 1988), while salinity was similar to that of the broodstock conditioning. One STT was placed into the 2.2 kℓ tank (exposed to the same conditions as the tarpaulins held in the tank banks), which also received 5 g of the disinfected eggs, therefore totalling to nine STT's. When the spotted grunters started spawning, eggs were collected from the external egg collector and the diameter of the fertilsed eggs and length at hatch were measured to the nearest millimeter using a light microscope (Axio Lab A1, Zeiss, Cape Town, South Africa) fitted with a camera (Axiocam ERc 5s, Zeiss, Cape Town, South Africa).

Three of the STT's served as the control treatment (clear sea water treatment), the next triplicate was subjected to *Nannochloropsis oculata* treatment at a final concentration of 500 x 10^3 - 1 x 10^6 cells/mℓ, whilst the remaining three slings received 0.125 g/ ℓ of bentonite clay (Natures Choice, Randvaal, South Africa) introduced twice per day (Stuart *et al*., 2015). Feeding in all the treatments commenced from three DPH (days post hatch) with the initial rotifer density set at 0.5 individual/mℓ, increased to 1 individual/mℓ from four to five DPH. Then maintained on 2 rotifers/mℓ until seven DPH. The fish larvae were fed in the morning and in the afternoon to always maintain the densities. No water exchange was performed for the first two DPH in all the slings. Daily water exchange

commenced from three DPH (18H00 to 06H00 am) at a rate of 10 ℓ/ h (trickle). A pipette (1000 µℓ) was used to withdraw three aliquots from three different positions in all the algae slings in the morning and enumerated on a haemocytometer staged on the mentioned transmission microscope and the number of algae cells were counted to replenish and maintain the above-mentioned algae stocking densities. Similar routine was followed to replenish and maintain the stocking density of rotifers in all the STT's.

In an additional trial, about 1 g of reserve eggs from the spawning induction were cultured in a 5 ℓ beaker with full strength *N. oculata* culture equating 35 - 40 million cells/mℓ (3.5 ℓ of algae, at 24 °C room temperature and 350 lux illumination), further supplied with strong aeration through glass pipette to produce bubbles and rigorous agitation. Rotifers were added with similar protocol as for the trails done in the STT incubators previously discussed. The beaker was gradually topped up with algae daily to maintain culture density. One of the 2.2 kℓ fibreglass tank - set with the same photothermal regime used for mass algae production, was filled with 100 ℓ of *N*. *oculata* at a density of 6 - 10 million cells/ mℓ, were inoculated with uncounted spare eggs (and rotifers). These scenarios were not statistically analysed but were kept to determine if the algae stocking density affected larval survival or not

4.3.4 Statistical analysis

Repeated analysis of variance (ANOVA) was used to determine the significant difference ($p < 0.05$) between the means of the number of larvae and the growth rate between the treatments (clear water, algae and bentonite clay), followed by Tukey post hoc multiple comparison ($p < 0.05$) when significant differences arose. Non-parametric test Kruskal Wallis was used to compare the means between the treatments at $p < 0.5$ if the data did not meet the assumptions of ANOVA (Levene's test for homogeneity of variance). All statistics were performed using SPSS version 27 (IBM SPSS Statistic, New York, USA).

4.3.5 Water parameter testing and monitoring

The dissolved oxygen (DO) was measured weekly using a DO meter (YSI 85 DO/SCT

meter, Ohio, USA). Total ammonia nitrogen (TAN; the total amount of nitrogen in the forms of ammonia, nitrite, and nitrate in water) was calorimetrically determined twice a week using SERA® test kits (Heinsberg, Germany). The frequency of monitoring was suitable since cultures consisted of live algae, prey, and fish larvae. The algae utilized metabolic waste and the cultures were stable (more than three days) in terms of water quality parameters tested.

4.4 Results

The number and the size of *P*. *commersonnii* larvae are respectively presented in Table 2 and 3 and are expressed as mean ± standard error (*n* =3 per treatment). The number of larvae declined gradually between day one to three post hatch, and then steeply from day four to six, with small quantity (<20) of spotted grunter left at the 7th day. Larval size measurements for the indicated period (µm) are summarized in Table 3. The number of larvae amongst treatment trials and control (clear water) at six days post hatch (DPH) were not significantly different ($p > 0.05$) within the individual treatments (Table 2). Approximately 5.1 % larval survival was measured at six DPH for both the control and *Nannochloropsis* treatment groups, whilst survival of the bentonite treated group was slightly better at 8.5 %.

The initial number of larvae of one to two DPH larvae were not significantly different ($p > 0.05$) amongst the treatments (Table 2). However, on three DPH, the control treatment demonstrated significantly higher number of larvae of 5198 ± 458.31 compared to 2843 ± 564.33 of *N*. *oculata* and 3092 ± 407.41 bentonite clay enriched water. On the following day to four DPH, clear water treatment mortalities were two-fold higher than in *N*. *oculata* while the mortalities in bentonite clay treatment were slightly higher than *N*. *oculata* enriched water treatment. Therefore, the number of larvae were no longer significantly different amongst the treatment on day four and this trend continued until day six post hatch. By the end of the larval survival period on day 6 post hatch, bentonite clay treatment demonstrated moderately higher number of larvae of 8.5 %, while clear water and *N. oculata* were 5 % and 5.6 %, respectively, but were not significantly different from each other (Figure 10).

The diameter of the fertilized eggs was 1.52 -1.60 mm (mean ± SE = 1.54 ± 0.01 mm, *n* = 10) and the length of the larvae 5 h after hatch was 2.44 - 2.51 mm (*n* =10). Larval size (weight gain) were not significantly different between treatments (Table 3). Larvae exposed to clear water treatment obtained a final total length of 3285 ± 41.32 µm ($F_{18,2}$ = 0.49, p = 0.62) while bentonite clay and *N*. *oculata* achieved 3106 ± 25.35 µm ($F_{18,2}$ = 0.60, p = 0.94) and 2907 ± 9.20 µm, ($F_{18,2}$ = 0.47, p = 0.64), respectively.

The size of the *P*. *commersonnii* larvae of the respective treatments were significantly different ($p < 0.05$) amongst each other from day three to seven DPH (Table 3). The size of the larvae on three DPH for both bentonite clay and *N. oculata* treatments were not significantly different, but with growth performance significantly less than those fish larvae exposed to the control treatment ($p < 0.05$). The control treatment larvae continued to grow fastest until five DPH, whilst bentonite clay treated larvae grew the slowest (Figure 11 and 12). By the end of the experiment (five to seven DPH), the growth performance of the *P*. *commersonnii* larvae were still significantly higher in the control treatment while *N*. *oculata* treated larvae had grown significantly less compared to larval cohorts in bentonite clay. The development of eye pigmentation and the onset of exogenous feeding were observed on three (Figure 12) and four DPH, respectively. Swim bladder inflation started on five DPH.

Monitoring of the water quality in the hatchery slings revealed the pH in all the slings was between 6.7 to 7.6 while the dissolved oxygen ranged between 4 to 5 mg/ℓ. The total ammonia nitrogen ranged between 0 and 20 ppm.

Concerning the rearing of the fish larvae in the 5 ℓ beaker and the algae production tank; 250 hand counted larvae were harvested (50 larvae per litre) after 12 DPH while 300 larvae were harvested 15 DPH from the algae production tank and they survived for more than 21 DPH.

Table 2: The number of *P*. *commersonnii* larvae within the individual treatments over the rearing period of six days. The data are presented as mean ± standard error, *n* = 3 per treatment.

Treatment	Day 1	Day 2	Day 3	Day4	Day 5	Day 6	$F_{(15,2)}$	p-value
Clear Water	7854 ± 27.17	6185 ± 64.70	5198 ± 458.31[a]	3484 ± 501.10	1336 ± 80.16	403 ± 30.82	0.06	0.94
N. oculata	7882 ± 1.20	5595 ± 163.76	2843 ± 564.33[b]	2033 ± 74.20	1244 ± 169.11	443 ± 218.80	0.01	0.99
Bentonite	7883 ± 1.20	5605 ± 281.20	3092 ± 407.41[b]	1991 ± 713.21	1537 ± 354.36	675 ± 209.84	0.15	0.86

Repeated measure ANOVA to compare means between treatments at p =0.05. Notes: Means with different superscripts within columns of the treatments indicates significant difference ($p < 0.05$).

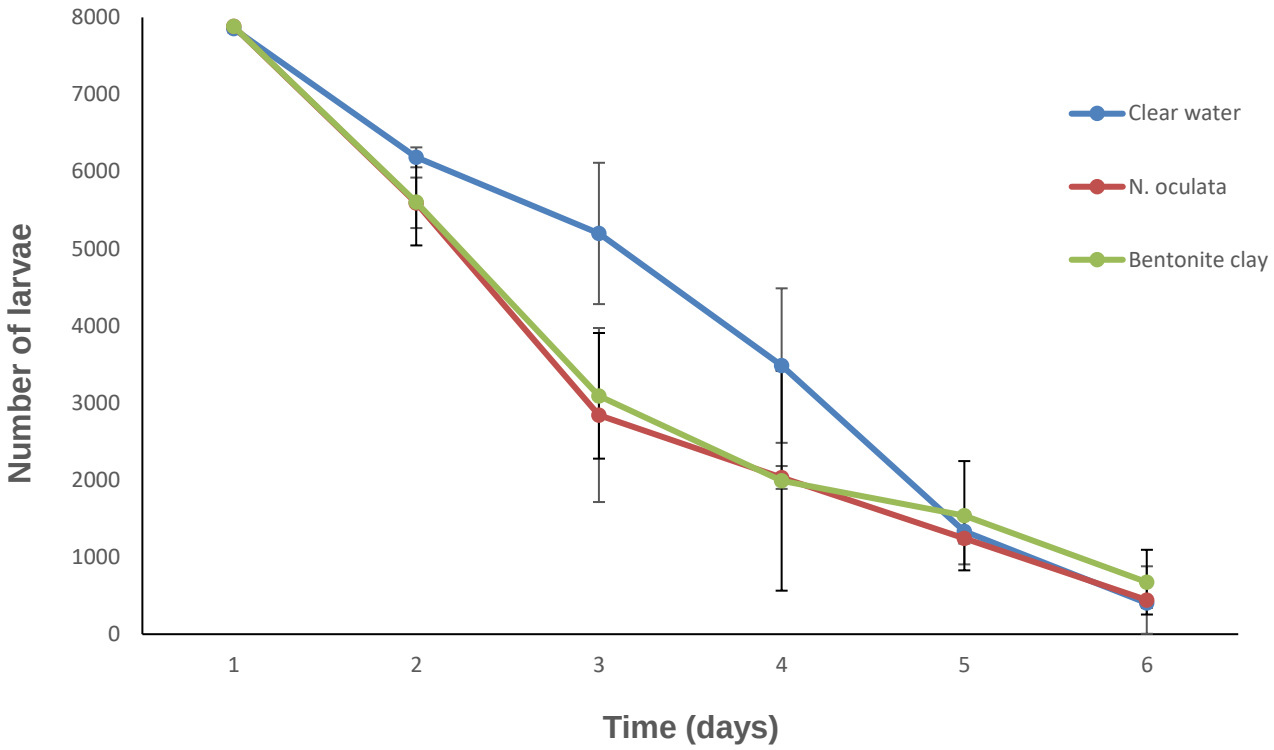

Figure 10: The number of larvae of the *P. commersonnii* larvae amongst the treatments over the six days of rearing.

Table 3: The size of the *P. commersonnii* larvae within the individual treatments, the data are presented as mean ± standard error (µm).

Treatment	Day1	Day 3	Day 5	Day 7	$F_{18,2}$	p-value
Clear water	2481 ± 21.34	2783 ± 17.42[a]	3036 ± 18,64[a]	3285 ± 41,32[a]	0.49	0.62
N. oculata	2473 ± 23.11	2683 ± 17.74[b]	2867 ± 18.07[b]	2908 ± 9.20[c]	0.47	0.64
Bentonite	2484 ± 22.60	2631 ± 24.11[b]	2772 ± 40.46[c]	3106 ± 25.35[b]	0.60	0.94

Repeated ANOVA, followed by Tukey-D test to compare the variances of the means between the treatments at p = 0.05. Footnote: Means with different superscripts within columns of the treatments indicates significant difference ($p < 0.05$).

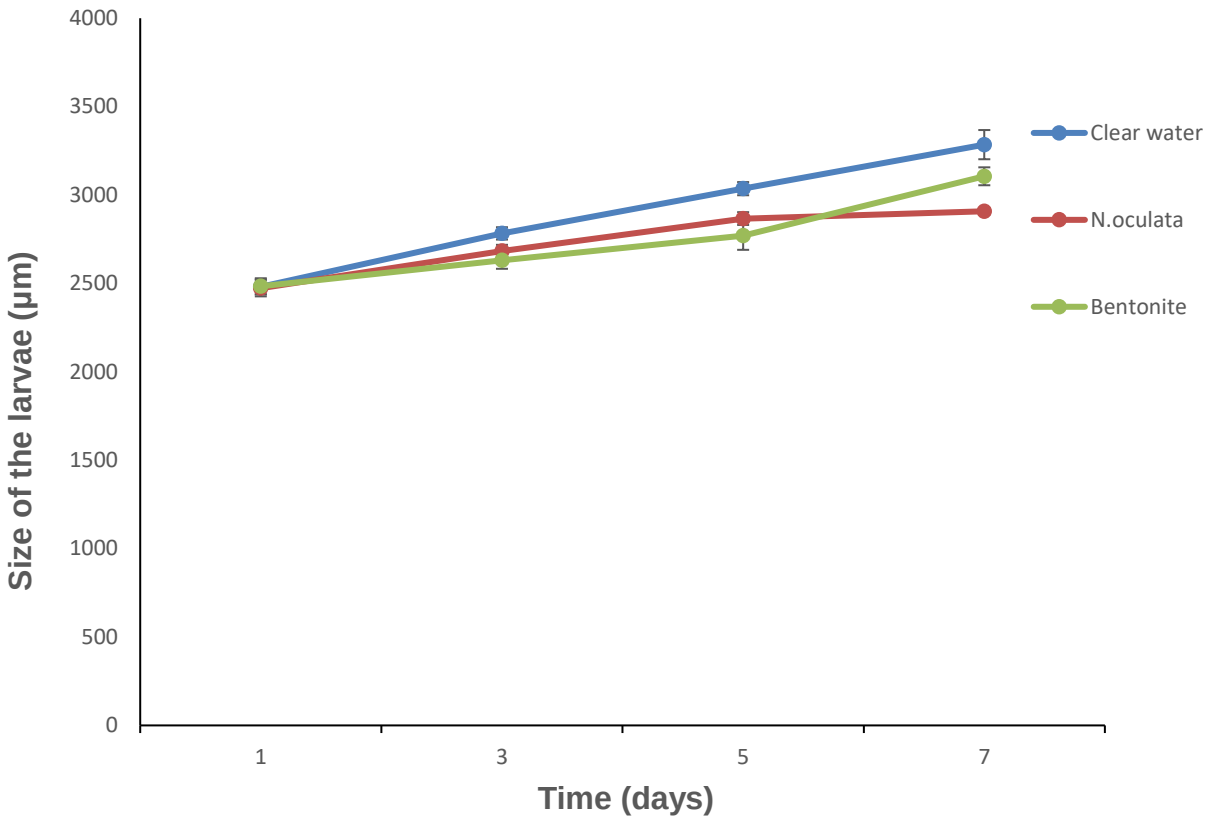

Figure 11: The size of *P. commersonnii* larvae over the seven days culture period.

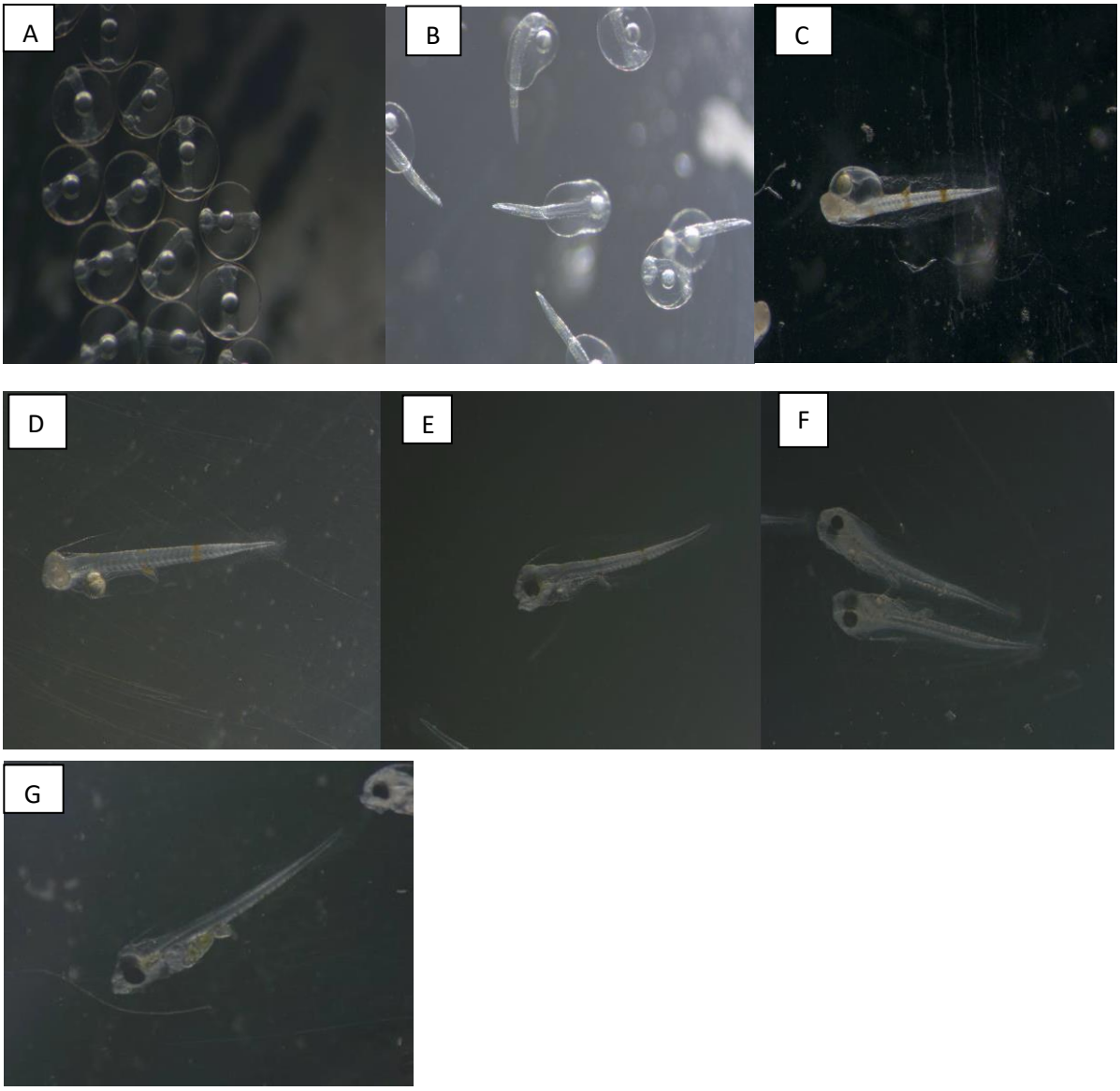

Figure 12: The early life stages of *P. commersonnii* larvae. (A) Fertilised eggs. (B) Hatching of eggs to larvae. (C) A one-day-old larva, characterised by oil globule enveloping the yellowish yolk sac. (D) A larva at two days post-hatch (DPH). (E) The development of eye pigmentation (three DPH). (F) Four DPH larvae. (G) Larvae that ingested enriched rotifers- six DPH.

4.5 Discussion

The fertilized eggs of the spotted grunter were spherical and measured 1.52 - 1.60 mm (mean ± SE = 1.54 ± 0.01 mm, *n* = 10) in diameter and the chorion was transparent surrounded by perivitelline spaces. This size is larger than that of laboratory reared *H. flavolineatum* 0.96 ± 0.03 mm (Hauville *et al*., 2017); white grunter, *Haemulon plumieri* 0.90 - 0.97 mm (Saksena and Richards *et al*., 1975); Oriental Sweetlips, *Plectorhinchus vittatus* 0.76 - 0.83 mm (Leu *et al*., 2012), all having pelagic eggs. The size of haemulid fertilized eggs is dictated by the perivitelline space and not associated with the nutritional composition of the egg nor predict the growth and survival patterns of hatched larvae. For example, the newly hatched larvae of *H. flavolineatum*, 2.69 ± 0.05 mm notochord length (Hauville *et al*., 2017) is larger than that of *P. commersonnii,* yet its fertilized egg is smaller. A similar observation was recorded for hatched larvae of *H. plumieri* - that measured 2.7 - 2.8 mm (Saksena and Richards, 1975). DiMaggio *et al*. (2014) noticed that the average vitellogenic oocyte diameter differed from cohort to cohort in asynchronous spawners and suggested that final maturation may be reached but without eggs ovulating, therefore retaining larger vitellogenic oocytes from lagging cohorts which would then eventually be released by spontaneous spawning. The study also confirmed release of relatively large diameter eggs during spontaneous spawning in pigfish (*O. chrysoptera*). It is plausible to speculate that smaller diameter eggs may predominate in ovaries of the currently investigated spotted grunter due to artificially induced spawning by an administered bio-active peptides (GnRHa). Should forced spawning induction have not taken place, then the larger grown oocytes may have been eventually absorbed via atresia, but this remains to be confirmed in future studies.

The morphology and feeding behaviour of the hatched spotted grunter larvae were observed microscopically as the first feeding period and the inflation of the swim bladder are critical periods for commercial operations with marine larvae. The appearance of eye pigmentation in spotted grunter larvae initiated three DPH and the onset of exogenous feeding was observed four DPH; this necessitated the low stocking density of the rotifers at 0.5/ℓ on day three post hatch to prevent proliferative propagation of rotifers with low

nutritional value. Preliminary observations by Fouche *et al*. (2018) indicated larval spotted grunter feeding to commence at three DPH. This discrepancy in the same fish species may be explained by the rearing temperature differences: the larvae of Fouche *et al*. (2018) were raised at 24 °C, whereas the present study maintained the fish larvae at cooler conditions (23 °C). *Pomadasys sp*., Corocoro grunter (*Orthopristis ruber*) and *O*. *chrysoptera* were reported to start feeding on day 2 post hatch (DiMaggio *et al*., 2013; Mata *et al*., 2014). Other grunter such as French grunter, *H. flavolineatum* began feeding on day three DPH (Hauville *et al*., 2017). Similar first feeding was reported for yellowtail species *S. lalandi* (Stuart and Drawbridge, 2012).

The daily mortality rates of *P. commersonnii* larvae were relatively high from the first day of exogenous feeding regardless of the treatment tested (clear water, greenwater, or bentonite clay) with mortalities somewhat reduced from five DPH, but with declining trend towards seven DPH. In addition, Fouche and Goodman (2017) maintained spotted grunter larvae on a rotifer diet in clear water and reported a high mortality rate trend towards 10 DPH. These findings conform also to other hatchery studies of haemulids, with similar trends in mortalities recorded for *P. vittatus* (Oriental sweetlips) larvae, reared in green water (*Nannochloropsis sp*.), also fed with algae enriched rotifers (Leu *et al*., 2012).

The survival of the spotted grunter, commencing at three DPH was not significantly different between algae and bentonite clay treatment trials ($p = 0.05$). Retrospectively, the survival rate was significantly higher in control treatments. Unidentified contaminants within the algae cultures or bentonite clay of the current study were evidently impacting larval viability. The presence of the contaminant was conspicuous and marked by a rapid change from a translucent water medium state into an opaque milky appearance. The identification of the contaminant is still ongoing with investigations by an inhouse specialist microbiologist. From the 4th DPH, which included first feeding, survival rates were no longer significant between the clear water and other treatments, suggesting that live feed feeding of control treatments introduced survival impacting contaminants. A similar finding for cod larvae (*P*. *vittatus*) was experienced by van der Meeren *et al*. (2007), who used *I*. *galbana* as algal resource, with rotifers and *Oithona* sp. nauplii as

preferred prey.

The inflation of the swim bladder of spotted grunter larvae commenced at six DPH, compared to five DPH for French grunter (*Haemulon flavolineatum*) and nine DPH for pigfish at similar temperatures (Hauville *et al*., 2017; DiMaggio *et al*., 2013). Swim bladder inflation failure is usually marked by larval lordosis and buoyancy imbalance, resulting in sinking of larvae to the bottom - eventually leading to mortality (Woolley and Qin, 2010). Mortalities experienced in the current study were not related to dysfunctional swim bladder, since the mortalities were already high from hatch to three DPH in all the treatments. In fact, the high fertilization and hatching rates achieved in this present study is an indication of high-quality eggs (Bromage *et al*., 1994). Therefore, the high mortalities of the larvae of the spotted grunter were probably associated to the culture conditions. The identification of the mentioned contaminant is still under investigation.

At the end of the experiment (seven DPH), average survival was discernibly higher in bentonite treated larvae though not significantly different compared to the other treatments, but clear water exposed larvae (control) survived best with significance. Rubio da Costa (2012) reported that when *S. auratas* larvae were exposed to the daily addition of *Chlorella minutissima*, the growth performance was significant while the survival was not significantly different to clear water. On the other hand, when Navarro and Sarasquete (1998) and Papandroulakis *et al*. (2002) offered *N*. *oculata* and *C. minutissima* to *S. auratus*, respectively, survival was twofold higher in algae treatments compared to clear water. Again, growth was significantly improved in the pseudo-green water treatments than in the control. Similar larval performance was identified with *Hippoglossus hippoglossus* larvae exposed to natural phytoplankton (Naas and Harboe, 1992). The better growth performance of bentonite treated larvae over the algae treatment was recorded in larval rearing trials for yellowtail kingfish (*S. lalandi*) conducted by Stuart and Drawbridge (2012), mean growth performance was reduced (TL= 5.81 ± 0.55 mm) using live algae, when compared to the growth of bentonite exposed larvae (TL = 6.23 ± 0.56 mm), though statiscally insignificant ($p > 0.05$). The highest length gain was, however, not a function of high survival rate, with similar tendency experienced for spotted grunter in the current investigation. In the present

experiment, the prolonged suspension of bentonite clay in the water column was not achieved despite vertical column aeration.

The density of the algae and bentonite clay induced turbidity with reduced light scattering, also further inhibited by the blue colour of the tarpaulin sling incubators. Insufficient light exposure can negatively influence algae culture welfare impacting on photosynthesis therefore favouring the culture and proliferation of pathogenic microbes (e.g., *Vibrio sp*.) (Ina-Salwany *et al*., 2019). Retrospectively, the selected 750 lux light condition of the hatchery in the current study is of relatively low intensity when compared with 1500 - 3000 lux radiation intensity exposure to *S. lalandi* larvae in bentonite trials (Stuart *et al*., 2015). A study using *Seriola rivoliana* larvae required 1000 - 3500 lux light intensity exposure to maintain a quality pseudo-green water culture by using *Nannochloropsis sp*. (Roo *et al*., 2014). Turbidity and illumination intensity are critical in prey localization or even foraging behaviour of haemulids/ finfish larvae since they are visual feeders (Palmer *et al*., 2007).

The survival and growth of milkfish *(Chanos chanos)* larvae reared in *Chlorella salina* was significantly higher in tanks with yellow background colour than in blue or white backgrounds (Bera *et al*., 2019). This was synchronised with significant standard growth rate, lower prey abundance of rotifers and Artemia in the rearing tanks and higher gut content of the prey (Bera *et al*., 2019). In clear water demonstrations, Ferosekhan *et al*. (2020) found growth performance of the *Clarias magur* larvae reared on white background to be significantly higher while *Pangasius pangasius* larvae maintained in black tanks had better growth outcomes than blue tanks or others (red, green). Higher survival rate was recorded in black tank for C. *magur* while *P. pangasius* was favoured in green tanks (Ferosekhan *et al*., 2020). Larval performance of marine and freshwater finfish is dependent upon background colour (McLean, 2021). The blue-coloured hatchery stretcher tarpaulin tanks used in the present investigations may not have been the most-suited for optimal rearing of spotted grunter larvae, and this pilot study has revealed several areas for future improvement.

The mouth gape of any fish larvae is a limiting factor in early feeding days, hence the offering of graded rotifers and copepod nauplii (or sub-adults) as food items. The use of calanoid copepods is increasingly becoming the preferred live feed option in commercial marine finfish hatcheries since it increases larval survival multi-fold when compared to rotifer culture (Dr Fouche, personal communication). French grunter reared on *Tisochrysis lutea* was reared on three feeding regimes, single diets of *B*. *plicatilis*, *Oithona colcarva* and their combination (Hauville *et al*., 2017). On 15 days post hatch, standard length was not significantly different between the treatments while survival was significantly higher in rotifer and copepod combination compared to non-significant single diets. The inclusion of the calanoid copepod *Pseudodiaptomus pelagicus* in the diet of *O*. *chrysoptera* increased survival while growth was not significant (DiMaggio *et al*., 2013). Although Fouche and Goodman (2017) successfully weaned spotted grunter larvae fed exclusively with *B*. *plicatilis*, the substantial benefits (e.g., larval survival increase) of feeding with dietary calanoid copepods still must be tested. The sustainable mass production of calanoid copepods is also still requiring further research.

At this point, it is difficult to exactly determine what could possibly have led to the poor survival of the grunter's larvae as it is dependent upon several cofounding biotic and abiotic factors such type of diet, optimum light intensity, background colour and the physical properties all of which are yet to be investigated for this species. The quality of larvae on the other hand is linked to the nutritional composition of the egg, and nutritionally balanced fed broodstock (Readings *et al*., 2018; Izquierdo and Hernández-Palacios, 1997). The high fertilization and hatching rates achieved in the present study is an indication of sufficient broodstock nutrition. Nonetheless, *Vibrio sp*. are pathogenic opportunists and common in larvae hatcheries (Attramadal *et al*., 2012; Novriadi, 2016; Stuart *et al*., 2016). In most of the spotted grunter trial, the treatments were polluted with an unidentified bio-contaminant that developed an acute cloudy surface layer thus resulting in severe mortalities especially in pseudo-green treatments within two DPH. Though the present trial survived seven DPH, weaker cloudiness was still observed. In two instances during the larval rearing of the spotted grunter, the introduction of rotifers at three DPH resulted in total mortalities across all the treatments by the following day regardless of the 100 % trickling water exchange overnight, suggesting that the rotifers too

could have been vector carrier of the bio-contaminant. Interestingly, Hauville *et al*. (2017) reported the appearance of increased turbidity from three DPH when calanoid copepods were offered to French grunter larvae throughout the entire experiment, though it was not observed when rotifers were fed alone. The authors implicated the filter feeding abilities of rotifers in controlling the cloudiness (Hauville *et al*., 2017). Effective methods of complete disinfection of rotifers without compromising their nutritional status or killing them remains a challenge (Munro *et al*., 2020; Suga *et al*., 2011). The observation from the two scenarios suggested that strong turbulence may be necessary to improve the survival rate of the spotted grunter. Furthermore, the high stocking density of algae controlled the proliferation of the unidentified bio-contaminant as no cloudiness was observed in both scenarios.

Strong aeration turbulence currents were positively impacting larval growth and survival, a phenomenon also being demonstrated in the early developmental studies for striped bass (Chapman *et al*., 1988). Survival was increased by agitated turbulence in hatchery water while its absence led to failure of swim bladder inflation (Chapman *et al*., 1988). In gilthead seabream studies, Tander *et al*. (1995) found that when salinity was reduced from 40 to 25 ppt, the rate of swim bladder inflation was significantly increased from 65 to 92.5 % and to an extent, led to the improvement of survival of 18.6 from 5.3 %. These studies alluded the successes to the reduction of the water-surface tension, therefore enabling the larvae to inflate their swim bladder with the atmospheric air. Therefore, the use of calanoid copepods (nauplii and sub-adults) co-fed with graded rotifers should be investigated in the case of the spotted grunter's larvae rearing in aeration agitated turbulent environments. The turbulence may not only reduce the water- surface interference tension but may sustain the suspension of the bentonite clay as well, if usage should be preferred above pseudo green water culture.

CHAPTER 5: CONCLUDING DISCUSSION

This study is a first to experimentally develop procedures for controlled reproduction of captive reared spotted grunter, and with tested repetitiveness. Highlights firstly include the successful rearing and photothermal programmed conditioning of captive raised spotted grunter broodstock (F1 and F2). Secondly, the endocrine facilitated induction of spontaneous spawning and production of high percentage (>95 %) fertilized eggs were achieved. The latter outcome is an indication that broodstock conditioning was properly manipulated and that high quality gametes were produced.

The current study further confirmed histologically that spotted grunter is a multiple batch asynchronous spawner as ovaries were in different stages of development and did not show atretic oocytes (Figure 6) and organismally it was observed that spotter grunter could also release egg batches multiple times considering that consecutive experimental induced spawning sessions were closely spaced. Pre-conditioned breeder males were selected when the gonopore expeled milt when slight pressure is simultaneously exerted on the mid-lateral sides of the abdomen. The dominant presence of such males was indicative that females were also ready for spawning but only if hormonally induced under captive conditions since natural cues for spawning induction were not present under captivity conditions. When males do not have ripe running milt then the females were also not prepared for spawning as no spawning activity occurred after GnRHa hormonal induction (empirical data on the same breeders). Ovarian biopsies will also confirm this tendency (Hurvitz *et al*., 2007; Wylie *et al*., 2013; Fakriadis *et al*., 2020).

The current investigation also developed an efficient broodstock conditioning program using firstly a consistent thermal program (24 ± 0.05 °C), followed by a three-episode fluctuating thermal program (24 ± 5 °C) during the third week (Figure 7), and at a consistent photoperiod simulating equinox (12D: 12L). Temperature is known to stimulate the hypothalamic-pituitary axis to release gonadotropins, influence final oocyte maturation and ovulation, timing of reproduction, quality of the gametes (Mañanós *et al*., 2008; Mylonas *et al*., 2010; Reading *et al*., 2018), therefore possibly explaining the spawning output differences observed between the spawns of the

constant and fluctuating temperature regimes.

Broodstock could also be conditioned to pre-spawning status by feeding them squid and/or sardine at 1 – 2 % body mass/day. This observation compares with those of other haemulids kept under captive conditions - such as the pigfish, French grunter, and oriental sweetlips (Leu *et al*., 2012; Barden *et al*., 2014; Broach and Ohs, 2020). Although the optimum dosage of salmon gonadotropin releasing hormone (sGnRH) (Ovaprim®) in inducing high yield of viable eggs was not determined, the spotted grunter was successfully spawned using the minimum dose of 0.25 mℓ/kg, as opposed to the more expensive prescribed dosage of 0.5 mℓ/kg by the manufacturer.

Although the gonadal histological examination of the larger/older broodstock females ranging between 39 and 53 cm caudal body length with a body weight of 2.64 ± 4.57 kg (mean ± standard error, n = 4) were not conducted, they failed to spawn regardless of the Ovaprim® dosage. The frequency of the spawning females of between 39 and 42 cm caudal body length and body weight of 1.58 ± 2.45 kg (mean ± standard error, n = 4), in the two- to three-year-old age range, were performing reliably with predictable spawning output success. Young spawners in this age group spawned eggs with increased quantities when uninterruptedly exposed to the conditioning programs applied. The results clearly indicated that the reproductive capacity of spotted grunter broodstock is size and age related. One unique observation from this study is the susceptibility of the spotted grunter to handling stress. The feeding levels of the spotted grunter returned to satiation levels a week after handling. Therefore, the prolonged stress of spotted grunter after handling must be considered in broodstock management strategies.

The spotted grunter larvae died following the addition of the rotifers, which suggested that the rotifers may have been carriers of pathogenic microbes. Though advanced hatchery methods were applied to rear and grow spotted grunter larvae, as for those exercised in most marine finfish research and industrial hatcheries, it was clear that microbial species such as the ubiquitous *Vibrio*, adversely impacted larval survival during the first seven DPH, and during consecutive trials.

Vectors for such microbial invaders are mainly the added zooplankton (rotifers) as available first feed for larvae, followed by the proliferation thereof in the culture tank as a result of the unavoidable increase in organic waste (uneaten food and larval metabolic waste). Bio-contamination in marine finfish hatcheries is a general problem and it is currently a common challenge to overcome. Strategies to control or eliminate plankton associated microbial pathogens are still in process and much focus is given to test the efficacy of probiotics or rotifer disinfectants. However, the current investigation demonstrated an option to suppress microbial proliferation by using an undiluted density of cultured phytoplankton (*Nannochloropsis oculata* > 10^6 x 10 cells/mℓ). The algae at such densities obviously suppressed microbial proliferation as demonstrated by many other workers (Çağatay *et al*., 2021; Sharifah and Eguchi, 2011; Jusidin *et al*., 2022). Survival of spotted grunter larvae to three weeks old, weaned juveniles was multi-fold increased from the usual 1 juvenile/ℓ to 15 juveniles/ℓ (Dr Fouche, Personal communication). The downside of using high density algae culture is that larviculture on a commercial scale will require huge volumes of high-density algae cultures, which may be both too costly and unpractical to maintain.

Enriched rotifers (PUFA and HUFA) are still considered to be inferior live feed for marine larvae compared to marine copepods (Chen *et al*., 2019). Although the mass production of copepods remains a challenge in commercial hatcheries (Chen *et al*., 2019), small scale culture of copepods nauplii and sub adults is necessary to co-feed the larvae of the spotted grunter with rotifers. This is necessary during the early feeding days of marine larvae due to the restricted feeding incidences caused by the mouth gape and morphological development of the digestive tract (Das *et al*., 2012). The marine research aquarium is in the process to establish a calanoid copepod integrated multi-trophic aquaculture (IMTA) system for future mass production of calanoid copepods, which have a significantly better nutritional and larval acceptance profile when compared to rotifers.

The SST (Figure 8) as a primary hatching microenvironment offers several advantages: better control of feed; observation of the larvae at its sensitive stage of gut and morphological developments at ease; siphoning of the dead larvae, remnants of algae, precipitated bentonite; low volume of algae - depending on its maturation status.

Part of the rearing protocol was to transfer the larvae at 12 DPH into the 1000 ℓ grow-out tanks (Figure 8). The success of the passive transfer of larval was demonstrated by Stuart *et al*. (2016) where *Vibrio sp*. colonies were significantly reduced in transfer tanks and the larval survival performance was almost two-fold compared to the control tank (Stuart *et al*., 2016). This application of this method is important considering the unidentified bio-contaminant in all the treatments. In addition, the blue colour of the stretcher tarpaulin tanks, and the poor illumination (750 lux) are hypothesized to have greatly influenced the feeding behaviour of the larvae. Poor illumination negatively affects the scattering of light throughout the water column, prey identification and localization (McLean, 2021). Therefore, it is proposed that further spotted grunter larval rearing experiments be conducted in white background tanks and increased illumination.

Since proven methods of disinfecting rotifers are still lacking, it is advised that the design of the rotifer culture system must include recirculating water passed through a biological filter and treated with ultra-violet radiation. A further addition of probiotics may prove suppression of pathogenic microbes. Since the survival rate of the clear water treatment trial was superior to algae and bentonite treatments during the early feeding days (Table 2), it is recommended that algae and bentonite clay must only be added on the day coinciding with first larval feeding incidence. This reduces the early bacterial and organic debris load in the culture environment during the larval hatching period.

For further studies a proposed two step solution to increase larval survival, without using high density algae cultures, will be to feed algae-enriched calanoid nauplii and to disinfect the nauplii with a non-lethal agent against *Vibrio sp*. in future studies. Non-lethal (dose dependent) disinfectant agents such as bronopol and sanucare are promising and were tested to disinfect rotifers and *Artemia* (Mzimba and Steinarsson, 2013). These disinfectants should also be tested on calanoid nauplii. The use of effective probiotics or selected cilliate bacteriovores may also reduce or exclude the presence of pathogenic microbes.

Concerning the sustainable mass production of algae, the industrial fertilizer (Table 1) should be further exploited in other species. This is advantageous as its constituents largely available and accessible in laboratories. Prior to that, small scale experiments should be conducted to compare the growth rates of the algae fertilized with F/2 growth medium (Guillard and Ryther, 1962) Vs the industrial fertilizer. There is a need to stress the use of conical shaped tanks to grow the algae and to flush settled waste at ease as it was difficult to do so in this present study (Figure 9C).

In conclusion, the broodstock of the spotted grunter was conditioned successfully on a diet of squid and sardine, and programmed photothermal cues. The latter included a double step approach that commenced with a 14-day photoperiod exposure of 12 Dark: 12 Light (250 lux) and constant temperature of 24 ± 0.05 ºC, followed by three thermal fluctuation cycles (1.5 ºC change per day from 19 to 24 ºC) of about 2 days each in the third week prior to spawning induction. The minimum dosage of 0.25 mℓ/kg of salmon gonadotropin-releasing hormone analogue (sGnRHa) (Ovaprim®) was sufficient to induce spawning of *P. commersonnii* in both constant and fluctuating temperature regimes. In the latter, the collective fecundity yield of ovulated eggs was significantly higher compared to constant temperature exposed broodstock. This efficient thermal fluctuation effect should be further exploited for its cost-effective benefits such as reduced conditioning time, and reduction of costly dietary rations for broodstock conditioning. The results and observations also demonstrated that the pseudo-greenwater technique (undiluted algae culture) is employable to raise larvae and should be exploited further in hatcheries. Also, the broodstock conditioning regimes, and spawning induction protocols were effective and are transferable for industrial use.

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