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Evaluation of the Biological Feasibility of white stumpnose,
Rhabdosargus globiceps, as a Potential Aquaculture
Candidate in South Africa.

THESIS

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Plagiarism declaration

I know the meaning of plagiarism and declare that all of the work in the dissertation (or thesis), save for that which is properly acknowledged, is my own.

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Abstract

Sustainable diversification and the biological viability of selected aquaculture species are now viewed as being crucial factors that must be assessed in order to introduce the candidate species into commercial production. The biological feasibility of white stumpnose, *Rhabdosargus globiceps*, as a potential aquaculture candidate, was assessed by examining the ability of the broodstock to be manipulated to spawn in captivity and the viability of the larvae from 0 days post hatch (DPH) to 30 DPH under varied conditions. These factors are recognised as the most significant bottlenecks of development for a potential aquaculture candidate.

A compressed photothermal cycle successfully brought about the reproductive maturation of both male and female broodstock. The natural, seasonal reproduction cycle, of the population of *Rhabdosargus globiceps* in Langebaan Lagoon, and manipulated cycles were comparable with regard to oocyte maturation. Under the compressed cycle an increase in the plasma estradiol 17 β concentration correlated with the increase in oocyte diameter and the presence of late vitellogenic and hydrated oocytes. Reproductively mature broodstock were injected twice with LH-RHa on a 24 hour interval, which successfully induced spawning over four consecutive 24 hour periods. This method of manipulation and induction of spawning was successful twice and the resultant eggs and larvae were used to examine the ontogeny and the optimal rearing temperature of *Rhabdosargus globiceps*. In the first larval rearing experiment the stages of development from egg to 30 DPH larvae were recorded at 20°C and showed similar developmental stages as other sea bream aquaculture species. The developmental stages were noted as being highly influenced by the available food sources with elevated mortalities recorded during the transitional phase between endogenous food reserves and exogenous feeding. The growth pattern showed the same reduced growth present for other successfully cultured sea bream larvae, suggesting that first feeding and swimbladder inflation was relatively successful. At 30 DPH the larvae reached a mean total length of 11.14 ± 0.44 mm. To determine the optimal rearing temperature and the effect of temperature on embryonic development eggs were placed at three different incubation temperatures (25°C, 20°C and 15°C) and the timing to the developmental stages was recorded from 0 DPH to 30 DPH. Measurements indicated that the larvae reached and

exceeded their thermal tolerance at 25°C, with larvae only surviving to 6 DPH at this temperature, while at 20°C the larvae had better growth, and significantly earlier indications and higher percentages of feeding, swimbladder inflation and notochord flexion than at 15°C. The results suggest that the highest quality larvae are achieved at a rearing temperature of 20°C.

This thesis describes the induction of spawning and larval rearing of *Rhabdosargus globiceps*. The ontogeny of the larvae as well as the critical stages of first feeding, swimbladder inflation and notochord flexion are discussed and described in detail.

Overall, the life cycle from adult to juvenile was successfully closed and the experiments showed very positive results regarding *Rhabdosargus globiceps*' biological capacity and feasibility to become a potential aquaculture candidate in South Africa.

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CHAPTER 1

Introduction

Aquaculture has grown rapidly in the last 50 years, and production has increased substantially, even though it is still regarded as a new food production sector. Aquaculture has the specific aim of maximizing the yield of useful organisms, such as algae, crustaceans, molluscs and fish, from the aquatic environment (Beveridge, 1987). The farming of aquatic organisms is achieved via manipulation of the organism's life cycle and control of the environmental factors that influence it (Beveridge, 1987). In 1950 one million tonnes of annual production was recorded through aquaculture. In 2010 total aquaculture production reached approximately 78.94 million tonnes (FAO, 2010b). The growth rate of production over this period is three times the rate of world meat production, including poultry and livestock (FAO, 2010a).

The rapid growth of the aquaculture industry contrasts greatly with the world capture fishery, which has seen little to no growth since the mid-1980's (FAO, 2010a). The increased production of recognised aquaculture species and the constant introduction of new species for diversification have significantly increased worldwide aquaculture production over the last 30 years (Asche *et al.* 2009). The Food and Agricultural Organisation of the United Nations recorded that in 2010, 46% of the fish consumed by the human population was produced by methods of aquaculture (Asche *et al.* 2009; FAO, 2010a). The overexploitation and stagnation of international fisheries as well as the increase in per capita consumption has resulted in a shortfall of fish products. This has pushed the aquaculture industry to produce at a greater scale and introduce more species to meet the high demand. This has led to worldwide food fish production in aquaculture increasing at an average annual rate of 7 to 8.3 % from 1970-2008 (Duarte & Holmer 2007; FAO, 2009), indicating great economic potential. It is hoped that aquaculture will address this shortfall and continually supplement the declining capture fisheries.

1.1. Current status of sea bream aquaculture

A family of fish that is considered to be of significant importance to the aquaculture industry is the family Sparidae, due to their wide geographic distribution, favourable growth rates and high market demand (Hernandez-Cruz *et al.* 1999; Oliva-Teles 2000; Mylonas *et al.* 2004). Species in the Sparidae family belong to the order Perciformes and are more commonly known as sea breams or porgies (Basurco *et al.* 2011).

The farming of sea bream species has produced an important commercial industry in many parts of the world. Japan is known as the foremost producer of red sea bream, *Pagrus major* (Temminck & Schlegel, 1843) and has been farming this species since 1958, when the first tonne was produced (Foscarini, 1988; Basurco *et al.* 2011). *Pagrus major* is recognised in Japan as the most important cultured species of the Sparidae family and in 2006 a total of 71,141 tonnes was produced (Foscarini, 1988: FAO-FishStatPlus, 2008). This makes Japan the biggest producer of farmed sea bream. However, there has been major growth in the production of sea bream species in Europe over the last decade, due both to technical advances, high demand, and to investment in increased production capacity. Aquaculture in Europe has been based on the farming of the gilthead sea bream, *Sparus aurata* Linnaeus, 1758, which was first produced in Italy in 1970 (Shields 2001; Liao *et al.* 2001; Quemener *et al.* 2002; Basurco *et al.* 2011). Together with the sea bass, *Dicentrarchus labrax* (Linnaeus, 1758) it now forms the largest commercial aquaculture industry in the Mediterranean Sea, where other commercially important sea bream species such as the red porgy, *Pagrus pagrus* (Linnaeus, 1758) common dentex, *Dentex dentex* (Linnaeus, 1758) common pandora, *Pangellus erythrinus* (Linnaeus, 1758) and blackspot sea bream, *Pagellus bogaraveo* (Brünnich, 1768) are also being cultured (Shields 2001; Zaki *et al.* 2007; Chavanne *et al.* 2008; FAO, 2009; Basurco *et al.* 2011).

In 2006, *Sparus aurata* production in the Mediterranean Sea was as wide spread as Tunisia, Algeria and Albania, and it reached 106,987 tonnes, with the main contributor being Greece, with 43,916 tonnes (FAO-FishStatPlus, 2008).

The total quantity of Sparidae species produced by aquaculture globally in 2006 was 244,153 metric tonnes. When this total was compared to global Percoidei production and value, it made up 6.8% and 14.5%, respectively (FAO-FishStatPlus 2008). In 2009 total

production increased to 261,000 tonnes (FAO, 2010a). The average annual growth rate of sea bream aquaculture between 1958 and 2006 has been 10% (FAO-FishStatPlus 2008; Basurco *et al.* 2011). From these production results it is clear that some sea bream species, especially *Sparus aurata* and *Pagrus major*, make good aquaculture candidates and are commercially viable.

The 2006 FAO data shows that 75% of all production was made up of *Sparus aurata* and *Pagrus major*. Porgies made up 23% and the remaining species made up 2% of total aquaculture production (Basurco *et al.* 2011). In the past the supply of fish was solely based on catching wild juveniles from the sea and increasing their growth until marketed. Now, the majority of sea bream farms use controlled reproduction techniques under intensive conditions to generate a reliable supply of juvenile fish via methods of larval rearing (Shields 2001; Basurco *et al.* 2011). The commercial application of enhanced larval rearing techniques has led to the growth of the industry and has allowed the overall improvement of culture techniques (Foscarini, 1988).

Many sea bream species are regarded as being commercially important to the aquaculture industry and as a result, many commercial hatcheries and farms have experimented with their broodstock management, larval rearing and grow-out to assess their biological viability as aquaculture candidate species. Two of these species that have shown great potential are *Pagrus pagrus*, and the Australasian snapper, *Pagrus auratus* (Forster, 1801) (Battaglione & Talbot 1992; Kentouri *et al.* 1995; Battaglione 1997; Kolios & Kiritsis 1997; Mylonas *et al.* 2004). One of the reasons why these two species have aquaculture potential is their close resemblance to *Pagrus major*. Experimentation with sea bream species that show similar characteristics to successfully cultured sea bream species is essential for the future of aquaculture, sustainable resource management and production.

The FAO has a list of 17 sea bream species that are currently being farmed. However, some are still in the experimental phase and have subsequently been removed from the list (FAO, 2009; Basurco *et al.* 2011).

To introduce a new species to the aquaculture industry there are many factors that must be taken into account in order for it to be recognised as a potential candidate. Many of the

long-used cultured species were selected after consideration of a limited number of factors. Farms only took into account a high selling price, demand for the product, and a sufficient supply of juveniles and adult fish from the wild (Quemener *et al.* 2002; Suquet *et al.* 2002; Lee 2003). Today, low production costs and high growth rates are still the most important factors for targeting the market. However, biological viability and sustainable diversification of the selected species are now viewed as being a crucial factor that must be assessed. A good example of this is the Norwegian salmon farming industry, where salmon was chosen because of its market price. If biological considerations were made prior to this, it would have been found that Arctic charr, *Salvelinus alpinus* (Linnaeus, 1758) which has a better disease resistance and tolerance of fluctuations in temperature, would have made a better aquaculture candidate (Lee 2003).

1.2. New candidate criteria

Before a species can be introduced to the aquaculture industry there should first be an experimental phase that evaluates its potential for domestication, management and sustainable production (Liao & Huang 2000). Other important considerations are the market potential, technical viability, production economics, production potential and compatibility with current aquaculture methods and industries.

The sustainable development of aquaculture is dependent on the creation and advancement of new technologies and methods for the diversification and rearing of new species (Papandroulakis *et al.* 2004a; Papandroulakis *et al.* 2005). Diversification of aquaculture species is one of the main reasons why new species are introduced, but it is also one of the challenges. At the moment most regions are limited by the number of species in production (Hernandez-Cruz *et al.* 1999). This is problematic, because with the increase in supply of highly valued species there will be a drop in prices. Introducing new species into the aquaculture industry allows for greater profit (Kentouri *et al.* 1995). It is essential that diversification takes place in order to expand consumer markets by bringing in new products.

There is great interest in fish species from the family Sparidae to be used for diversification of aquaculture, as many of these species are already produced around the world (New

1991; Stephanou *et al.* 1995; Hernandez-Cruz *et al.* 1999). The reasons for diversification of aquaculture species are stated below.

Firstly, certain species are better adapted to specific conditions and environments. To be able to apply these conditions the distribution of the candidate must be known. This is significant, as only indigenous fish species should be selected for their local areas (Bell 1999; Liao & Huang 2000; Quemener *et al.* 2002). This will reduce the introduction of new pathogens and competition with indigenous species.

As poikilothermic animals, a fish's body temperature is highly sensitive to that of the surrounding water, making development and growth highly dependent on the water temperature (Patino 1997; Quemener *et al.* 2002; Suquet *et al.* 2002; Devlin & Nagahama 2002). Aquaculturists want a species of fish that is the best suited for their local environment. Warm water tolerant species are grown extensively in parts of the Mediterranean, but these will fair badly in the colder northern waters. Having a larger selection of species will narrow the choices and make it easier to choose a species better adapted to the prevalent environmental conditions.

Secondly, the selected species should be tolerant of a wide range of rearing methods, techniques and constraints (Quemener *et al.* 2002; Suquet *et al.* 2002; Lee 2003). With recirculation systems being introduced and used in the rearing process, it has provided a reliable method that can produce fish efficiently and can be more cost effective than using a flow-through system (Blancheton 2000). However, the use of this system in a smaller area means that the density of fish is increased. The density can be almost double in some cases (Blancheton 2000). The new candidate species must be tolerant of a higher stocking density in these systems and other systems, such as off-shore cages. Extensive methods that have been applied in fjords and lagoons must also be tolerated by the new species.

Thirdly, the new candidate should have good aquaculture potential and show high growth performance when compared to already farmed species (Quemener *et al.* 2002; Suquet *et al.* 2002). The introduction of a faster growing species has the capacity to develop a more profitable aquaculture industry (Suquet *et al.* 2002; Asche *et al.* 2009; Basurco *et al.* 2011). However, this candidate should be able to show a high performance and growth rate

without the use of controversial techniques that can be detrimental to human health and the environment (Suquet *et al.* 2002; Read 2003). The most sustainable production techniques must be used. For the new candidate to have a strong potential for culturing, the larval stage must be described in detail, so as to be able to assess the performance, growth and development. If the larvae show slow growth and low survival, the candidate's potential is drastically reduced (Moretti *et al.* 1999; The Research Council of Norway 2009). However, with the improvement of broodstock conditioning, larval rearing techniques and feeds there is still a possibility to improve their growth and survival thereby improving their potential as a candidate species.

Fourthly, the introduction of a new species can reduce the outbreak of diseases. Having a larger number of cultured species can lower the effect of a disease outbreak, as shown by the outbreak of the nodavirus in 1994 in the Mediterranean (Quemener *et al.* 2002; Lee 2003). Furthermore, using alternating crops in cages reduces the infection pressure of potential species-specific pathogens that may have accumulated in the environment during the previous production cycle. This is a manner of fallowing a grow-on area without losing its production potential.

Fifthly, production of the new candidate must be able to adapt to the trends in the consumer market. There has been great success in the white fish market, where fish flesh is cheap and can be prepared easily (Asche *et al.* 2009). Production should be able to be diversified (frozen, fillet, whole, smoked etc.), thus allowing the market to change without a detrimental effect on the sale of the products (Suquet *et al.* 2002). Asche *et al.* (2009) state that if there are few species in a limited market and there is an increase in production, there will be a sharp decline in prices as the increased supply from all the producers will decrease the demand for the product. Alternatively, in a market that is not limited with a larger number of cultured species, a producer can supply a smaller amount while still having a profitable market share, because there should only be a weak or even no price effect on the product (Asche *et al.* 2009). Diversification can be regarded as a recovery strategy if the market prices for some species decline due to market saturation, as this initiates the avoidance of overproduction (Lee 2003).

Even if the limiting factors related to the biology of the species have been dealt with successfully, it is ultimately the market that provides the possibility for diversification and selection of potential aquaculture candidates for use in commercial production.

Species in the Sparidae family were chosen as potential new candidates for diversification because many have high economic value and there has been great success and development in the production of some members of the Sparidae family, namely *Pagrus major* and *Sparus aurata* (Basurco *et al.* 2011). The introduction of new species for aquaculture could create new opportunities to further the industry's development and growth. By using other sea bream species that have close biological and phylogenetic characteristics there is a greater possibility of success of production (Hernandez-Cruz *et al.* 1999).

1.3. Broodstock management

The success of culturing starts with broodstock management. Aquaculturists put in place appropriate measures to make it possible for the captive fish to become reproductively mature and to spawn. The use of exogenous hormones and manipulations of environmental conditions are commonly used in broodstock management today (Zohar & Mylonas 2001). However, the protocols for the use of hormones, the type of hormones and the methods used to assess reproductive maturity vary between the cultured species, because of their different reproductive strategies. If the broodstock do not fare well under captive conditions and do not mature, the potential for this species to become an aquaculture candidate is decreased. Appropriate management of the broodstock is made more possible with a comprehensive understanding of the selected species' cycle of gametogenesis, final maturation and spawning potential (Mylonas *et al.* 2010).

1.3.1. Domestication

In general, domestication in aquaculture refers to the acclimatisation of the species to captive conditions, which provides the platform for the potential of rapid growth and control of the reproductive cycle (Liao & Huang 2000; Mylonas *et al.* 2010).

Control of growth and reproduction are the main benefits of domestication in aquaculture. These factors are seen as prerequisites for a species to become domesticated (Bilio 2008; Mylonas *et al.* 2010). Success in domestication has allowed aquaculture production to increase to a point where the maximum sustainable yields from the capture fishery of some species have been surpassed (Bilio 2008). However, for such success the correct species must be chosen first. As with diversification, there are certain criteria that the selected species must meet. These criteria are made up of biotic and abiotic factors.

The biological considerations include various factors, which include the organisms' health, fecundity and viability for successful production. The growth rate, fecundity, food conversion rate (FCR), resistance to disease and minimum size are the most common factors that are assessed during the start of domestication (Liao & Huang 2000; Bilio 2008). Other biological considerations are high economic value, resistance to stress, a known reproduction and life cycle, acceptance of artificial feed, physical characteristics that are appealing to the customer and the ability to maintain genetic variability (Liao & Huang 2000; Bilio 2008).

The non-biological considerations include the environmental aspects that can affect the positive traits of the captive population (Liao & Huang 2000). Favourable conditions will improve the expression of positive traits of the population, such as growth, development and resistance to disease. These conditions can be controlled by the culture types and the facilities used in the production process. Outdoor culture, indoor culture, monoculture, polyculture, intensive or extensive culture will create conditions that suit the needs of different culture species. Within this range of culture types and facilities, domestication should be possible for most sea bream species (Liao & Huang 2000).

Domestication can however cause problems. The cages, raceways or tanks that are used as housing create a small environment, which reduces the size of the parent population. This can lead to problems with genetic variation and inbreeding (Falconer 1989; Agnese *et al.* 1995). A disease outbreak under these conditions is also more likely to affect the entire population.

1.3.2. Conditioning and Reproduction

The most essential aspect of broodstock management in modern aquaculture involves the control of reproduction (Bilio 2008; Mylonas *et al.* 2010). This can be achieved via conditioning techniques, which involve the manipulation of the photothermal cycle and the use of hormones. This allows for quality seed production and the sustainable production of new fish in season and out-of-season, which could later become new broodstock or be sold to the consumer (Bromage, 1995). Using appropriate conditioning techniques to control the timing of reproductive maturation can ensure a better quality of egg. A reduced supply and low quality of eggs have been recognised as two of the main factors that impede the establishment of a potential industry (Battaglione & Talbot 1992; Cleary *et al.* 2000; Zohar & Mylonas 2001).

In order to effectively condition and culture sea bream species, knowledge of their biology, reproductive style, factors governing reproduction, breeding behaviour and the optimal conditions required for the species to become reproductively mature and able to produce seed of a good quality, is needed (Kokokiris *et al.* 2006; Mouine *et al.* 2007). With this knowledge the appropriate control measures can be applied (Kokokiris *et al.* 2001). Different reproductive strategies are present in the sea bream family, and these need different control methods (Buxton & Garratt 1990; Kokokiris *et al.* 2001). Hermaphroditism is commonly found in the 127 species that make up the Sparidae family, but of these 127 species some are gonochoristic, some are rudimentary hermaphrodites (late gonochoristic), whilst others are either protogynous or protandrous hermaphrodites (Buxton & Garratt 1990; Kokokiris *et al.* 2006; Attwood *et al.* 2010). This factor incurs different methods of management as this knowledge is vital in being able to spawn broodstock of a particular species.

Sparus aurata is a protandrous hermaphrodite species. Under farm conditions mature males, at approximately 20-30 cm in length, undergo sexual inversion between their second and third years (Moretti *et al.* 1999; Chavanne *et al.* 2008; Basurco *et al.* 2011). However, the sexual inversion of *Sparus aurata* is socially determined. The presence of young, potential males during the spawning period increases the number of older fish that become females. The presence of older females will inhibit sexual inversion in younger fish, which

will remain functional males (Moretti *et al.* 1999). This behaviour results in the female broodstock being older, which helps produce a greater quantity of eggs, but the quality of these is reduced, making them less viable (Moretti *et al.* 1999). To rectify this problem the farmer annually replaces the oldest females with younger ones either from the wild or other farms. The broodstock maturation and spawning of *Sparus aurata* in captivity is noted by Basurco *et al.* (2011) to be technically feasible.

With a protogynous species, such as *Pagrus pagrus* the opposite methods are used. After three to four years of age the females change sex to males (Kokokiris *et al.* 2006). However, conditions in captivity can prevent sexual inversion from occurring. This marine species is commercially important and there is great interest in establishing captive broodstock and farming this fish, but in order to do so, the environment in which it is kept must be suitable for the reproductive strategy of this protogynous species (Kolios & Kiritsis 1997; Kokokiris *et al.* 2006).

Gonochoristic species such as the *Pagrus major* and *Dentex dentex* have hermaphroditic juveniles (Matsuyama 1988; Morales-Nin 1997; Loir *et al.* 2001). However, as the fish get older, the sexes become determined, making the broodstock management and conditioning tasks easier.

Another aspect of the reproductive biology of the female fish that must be taken into account is whether the fish is a synchronous or asynchronous spawner. These different reproductive strategies have implications for broodstock management. Cultured sea bream species are either asynchronous or group synchronous (Basurco *et al.* 2011). *Pagrus major*, *Pagrus pagrus*, *Dentex dentex* and *Sparus aurata* are all batch spawners, which means their spawning is asynchronous and they can spawn for 3-4 months during their spawning season, as their ovaries produce oocytes at different stages of maturity (Loir *et al.* 2001; Mylonas *et al.* 2004; Basurco *et al.* 2011) (Figure 1.1). The oocytes that mature first and become hydrated are then released during spawning. This has aided the use of these species in aquaculture, as a constant supply of eggs can be collected over their spawning period. This type of spawning is frequent and regular, and can be a daily occurrence for the spawning period of 3-4 months (Mylonas *et al.* 2004).

Using asynchronous species in aquaculture can be advantageous, as the spawning period occurs over an extended period unlike that of synchronous species that have a confined reproductive season. Stocks of asynchronous fish can be photoperiodically manipulated to produce eggs in 9-12 months cycles (Morehead *et al.* 2000; Zohar & Mylonas 2001; Pankhurst & Porter 2003). To achieve similar production using a synchronous species such as *Dicentrarchus labrax*, six to eight stocks must be maintained throughout their spawning period using different photothermal manipulations, which incurs a higher cost to the facility (Mylonas *et al.* 2010).

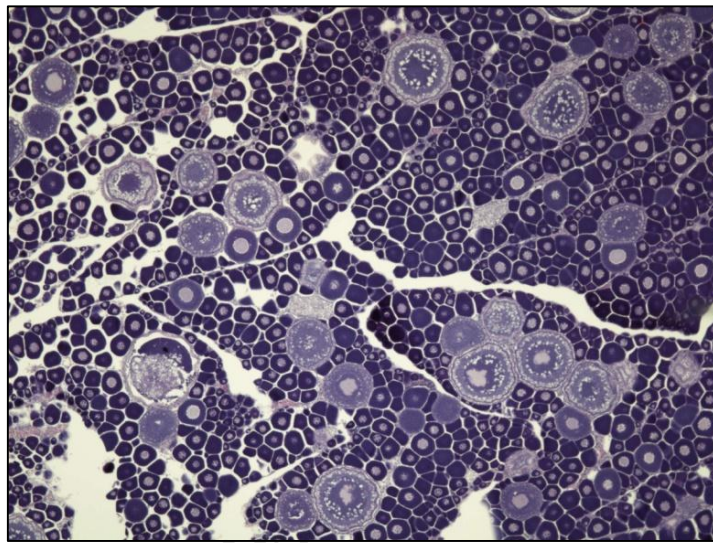


Figure 1.1: Asynchronous ovary of *Rhabdosargus globiceps*. The ovary contains oocytes at the previtellogenic, cortical granule and endogenous early vitellogenic stage. Photograph taken during the assessment of broodstock maturation.

Although cultured sea bream species can have varying life-cycles, the reproductive cycle of gametogenesis and final maturation are the same. This cycle is separated into two distinct phases. The first phase is a growth phase that sees the differentiation of the gametes (spermatogenesis and vitellogenesis), and the second phase is a maturation phase that consists of the gametes of spermatozoa and oocytes, preparing for release and fertilisation (spermiation and oocyte maturation) (Mylonas & Zohar 2007; Mylonas *et al.* 2010). If the cultured fish are kept under optimal conditions, spermatogenesis and vitellogenesis occur without there being any significant problem (Rottmann *et al.* 1991; Buchet *et al.* 2008).

However, there can be problems with the second phase, and dysfunction has been shown to occur (Zohar & Mylonas 2001). Reduced sperm volume and quality is a common reproductive dysfunction in males, and females can have a total failure of oocyte maturation or a very unpredictable occurrence of mature oocytes (Zohar & Mylonas 2001; Mylonas & Zohar 2007). This results in the failure to spawn, even if the fish have gone through vitellogenesis and ovulation (Zohar 1989), thus limiting effective production. The reasons for these dysfunctions have been suggested to be a combination of lack of a natural environment in which to spawn and stress brought about by the conditions of captivity (Pankhurst & Van Der Kraak 2000; Schreck *et al.* 2001; Biswas *et al.* 2010).

Capturing and handling of fish increases stress levels, which causes an increase or decrease in certain hormones, which in turn reduces the quality of the eggs (Cleary *et al.* 2000; Schreck *et al.* 2001). This effect of stress has been noted in cultured *Sparus aurata* and *Pagrus auratus* individuals. The results of this showed that circulating levels of gonadal steroids, particularly androgens (testosterone) and oestrogens (17 β -estradiol) have significantly reduced levels after stress from handling or capture (Carragher & Pankhurst, 1991). This would greatly impair reproduction, as these hormones control the development and maturation of the gametes (Cleary *et al.* 2000; Zohar & Mylonas 2001). Affected hormone levels can lead to ovarian atresia and result in the reabsorption of unspawned vitellogenic oocytes (Schreck *et al.* 2001). Loss of oocytes will not only decrease the number of eggs released, but will also reduce the quality of the eggs that will be released. Abnormal larvae can also be produced when hormone concentrations are affected (Clearly & Pankhurst 2000).

The use of appropriate conditioning techniques and hormonal therapies have been shown to prevent the reproductive dysfunctions in both male and female cultured fish (Mylonas *et al.* 2010).

Sea bream species are conditioned in captivity to become healthy and reproductively active (Leu & Chou 1996; Bilio 2008) by changing the feeding regime, manipulating the environmental conditions and using hormones to promote reproductive maturation (Bromage *et al.* 2001; Mylonas *et al.* 2010). These techniques make it possible for the broodstock to either spawn spontaneously or be induced to spawn when their gametes are

at their final stage of maturation (Mihelakakis *et al.* 2001; Zohar & Mylonas 2001; Sheaves 2006). However, not all the effects of the interactions with the environment on all the cultured species are known (Mylonas *et al.* 2010). Some of these other interactions could be water currents, food availability or even spawning migrations. A viable species for culture would not be one that has limit to its reproductive capacity.

Evidence has shown that sea bream reproduction cycles are heavily influenced by photoperiod and temperature, and with the manipulation of these parameters and the use of appropriate hormones the broodstock can be successfully spawned (Morehead *et al.* 2000; Mihelakakis *et al.* 2001; Zohar & Mylonas 2001; Sheaves 2006; Bilio 2008; Mylonas *et al.* 2010). These techniques will allow for continuous controlled reproduction, ensuring the sustainability of a viable sea bream aquaculture industry (Bilio 2008).

1.3.3. Induced Maturation and Spawning

Techniques that are used to bring about spawning in captivity, both in season and out of season, involve the manipulation of exogenous cues (environmental conditions of temperature and photoperiod) and endogenous cues (various inducing hormones that affect the endocrine system), which trigger a response from the brain-pituitary-gonad axis (Leu & Chou 1996; Moretti *et al.* 1999; Mihelakakis *et al.* 2001; Zohar & Mylonas 2001; Zaki *et al.* 2007; Hodson & Sullivan 1993). With these techniques in mind, the mechanism is to induce maturation and spawning in captive fish species that do not become reproductively active, and to control the time to spawning of species that can become reproductively active in captivity (Donaldson & Hunter 1983). Even though these techniques of manipulation have been improved, natural spawning occurs annually and aquaculturists still rely on these events, but many of the techniques for controlled or semi-controlled breeding of sea bream species have been perfected and adapted for large-scale production of seed. Even though sea bream reproduction cycles have been manipulated in captivity with the use of temperature, photoperiod, substrate and tank volume/depth, the presence of the artificial environment created by humans, and their presence, have been noted as inhibiting factors on reproduction (Bilio 2008; Mylonas & Zohar 2007).

1.3.3.1. Environmental manipulation

The external parameters of the environment control and trigger the internal reproductive mechanisms in fish species. Manipulating ambient temperature and photoperiod to induce gametogenesis in fish can be said to be natural, as fish do spawn spontaneously due the optimal temperature and photoperiod being used over a certain period. However, these conditions can be created in aquaculture systems and by emulating the conditions of the spawning season with the optimal temperature and photoperiod the fish can be induced to spawn in captivity (Mihelakakis *et al.* 2001; Bromage *et al.* 2001).

All fish have very similar internal mechanisms, but the external factors that drive these can differ considerably between species. Some of the factors that play a role in the reproduction cycle of fish are; photoperiod, water temperature, water quality, water current, food availability, tides and lunar cycles, spawning substrate, parasites and disease (Rottmann *et al.* 1991; Mylonas *et al.* 2010; Biswas *et al.* 2010).

Photoperiod and temperature have been noted as having the most significant effect on reproduction in sea bream species (Pankhurst & Porter 2003; Bromage *et al.* 2001), and experiments have been conducted that have allowed the reproduction cycle to be reduced (Morehead *et al.* 2000). Fish display both seasonal (day length-induced) and daily (light/dark cycle-influenced) endogenous rhythms (Pankhurst & Porter 2003). Appropriate use of temperature and photoperiod modifies the cycle of reproductive rhythms, brought about by the change in seasons, and helps to induce the fish to start producing the hormones used to produce gametes (Rottmann *et al.* 1991; Bromage *et al.* 2001; El-Sayed & Kawanna 2007). Temperature and photoperiod can be manipulated to promote and induce gonad development, timing of sexual maturation and hormone production in both males and females (Rottmann *et al.* 1991; El-Sayed & Kawanna 2007). This effect is more prominent in fish from higher latitudes where seasonality is more marked, making temperate sea bream species more susceptible to environmental manipulation.

For temperate sea bream species, such as *Sparus aurata*, *Pagrus pagrus* and *Rhabdosargus globiceps* (Valenciennes, 1830) spawning is an annual event (Mihelakakis *et al.* 2001; Attwood *et al.* 2010; Basurco *et al.* 2011). Photoperiod has been recognised as the main driving factor of sexual maturity. However, water temperature is highly influential and

through interaction with photoperiod it helps to synchronise final maturation of the gametes (Van der Kraak & Pankhurst 1996; Mihelakakis *et al.* 2001).

The use of a controlled photoperiod regime by Kadmon *et al.* (1985) was shown to delay the spawning of gilthead sea bream and also affect their sexual differentiation. This method of postponement of gonadal development has great potential and economic benefits, as it saves energy reserves that would have been used in this process, which enables overall weight gain, thereby increasing the market value of the fish (Kissil *et al.* 2001).

This method of induced spawning is regarded as a “natural method”, that does not require any veterinary medicines, and for certain species it is a very effective method. Temperature and photoperiod conditions are crucial and are needed even when hormones are used as a stimulus for final maturation and spawning (Henderson-Arzapalo & Colura 1987; Leu & Chou 1996; Sheaves 2006; El-Sayed & Kawanna 2007).

1.3.3.2. Use of Hormones

Hormonal therapies have been extensively used, not only to control reproduction, maturation and spawning, but in the case of exogenous hormones, to manage broodstock maturation and improve the efficiency of egg production. This has improved production and reliability of hatchery operations (Mylonas & Zohar 2007), and reduced reproductive dysfunction of the broodstock (Zohar & Mylonas 2001).

In the initial trials of induction of maturation and spawning in fish, exogenous hormonal treatments of pituitary homogenates were used (Fontenele 1955). It is now known that dopamine inhibits the gonadotropic hormone release (Abraham 1987). This has led to dopamine antagonists being added with the pituitary homogenates to act as a suppressant of GnRH-inhibitor and a stimulant of Gnt-release (Abraham 1987; Hodson & Sullivan 1993). Naturally occurring hormones such as human chorionic gonadotropin (hCG), gonadotropin-releasing hormone (GnRH) and pituitary extracts are commonly used to induce sexual maturation and spawning of broodstock. Induction of spawning can only be achieved when the fish are sexually mature and at the advanced stage of reproductive development (Rottman *et al.* 1991; Zaki *et al.* 2007; Hodson & Sullivan 1993). Follicle Stimulating Hormone (FSH) and Luteinising Hormone (LH) bring about gametogenesis and maturation in

both male and female fish (Mylonas *et al.* 2010). Zohar *et al.* (1995) concluded from an experiment of *Sparus aurata* that LH is secreted from the pituitary to trigger oocyte maturation and ovulation during vitellogenesis, to coincide with the peak spawning season. Stimulating the release or direct addition of these hormones initiated these sequences.

A diminished release of LH from the pituitary at the end of vitellogenesis has been noted as the reason why some cultured sea bream species fail to undergo oocyte maturation and spermiation, which has led to the use of exogenous LH solutions. These have a direct effect on the gonad and initiate maturation (Mylonas & Zohar 2007; Mylonas *et al.* 2010). GnRH was used to initiate the release of endogenous LH from the pituitary, which in turn acted on the gonad. Today, there are many forms of potent antagonists of GnRH (GnRHa) that are used to influence the endogenous endocrine process (Lee & Yang 2002; Zohar & Mylonas 2001). The solutions of Luteinising hormone include two main components. Firstly, carp or salmonid purified pituitary extracts with a high concentration of LH, that come from reproductively mature fish, and secondly, a highly LH active form of purified human Chorionic Gonadotropin (hCG) (Donaldson & Hunter 1983; Donaldson 1996; Mylonas *et al.* 2010). The addition of exogenous LH serves as the artificial surge that would occur in the wild population, which accompanies oocyte maturation and ovulation in females and spermiation in males (Mylonas & Zohar 2001).

There are different methods of administering hormones. The most common method is via injection, but there are cases where the hormone has been added to the water, or the feed and even by implanting pellets into the fish (Leu & Chou 1996; Mylonas & Zohar 2001; Hodson & Sullivan 1993). Two types of pellets are implanted, one with fast releasing GnRH, and the other with slow releasing GnRH (Hodson & Sullivan 1993). An injection of hCG or LH during the final stages of oocyte and sperm maturation induced the fish to spawn (Hodson & Sullivan, 1993). The injection can be either intraperitoneal (within the body cavity) or intramuscular (within the muscle) (Hodson & Sullivan 1993). Intraperitoneal injections are given through the ventral part of the fish behind either the pectoral or pelvic fin and intramuscular injections are commonly done on the dorsal part of the fish above the lateral line and below the anterior part of the dorsal fin (Hodson & Sullivan 1993).

The method of implanting pellets that provide a GnRHa-delivery system over time has seen the best results and this method has been used to induce ovulation and spawning in many fish species including the Atlantic Bluefin tuna, *Thunnus thynnus* (Linnaeus, 1758) and Southern Bluefin tuna, *Thunnus maccoyii* (Castelnau, 1872) (Mylonas & Zohar 2007). This method of long-term administration has shown better results than a single injection, because it provides a prolonged hormonal treatment (Fontenele 1955). Multiple injections at maturation allow for similar results, but the stress from repetitive handling can have negative effects on the reproductive performance of the fish (Mylonas & Zohar 2007).

Overall, the objective of hormonal therapy in broodstock is to increase sperm production in males and bring about ovarian maturation in females (Mylonas & Scott 1997; Mylonas *et al.* 2010), but because fish have different reproductive strategies the application of the hormones must be different. Synchronous fish species can effectively be made to spawn with one or two injections of GnRHa and dopamine antagonists. However, with asynchronous species, a more effective way of achieving maximum fecundity is through a prolonged GnRHa release system (Zohar & Mylonas 2001).

South-East Asia produces yellowfin porgy, *Acanthopagrus latus* (Houttuyn, 1782) and this is one of the aquaculture species that has been induced to spawn (Leu & Chou, 1996). In this case, as with many of the cultured species, seed supply is a problem, so induced spawning is a good alternative to harvesting seed from the wild. A combination of salmon gonadotropin hormone analogue and domperidone (suppressant of dopamine production) were used and 0.5 ml per kg of body weight was injected into each fish (Leu & Chou 1996). Eggs were successfully released and fertilisation took place and the fertilised eggs were transferred to a hatchery (Leu & Chou 1996).

The main purpose of induced spawning is to create a reliable supply of new fish that can increase and sustain the supply of fish products for the consumer market and to reduce the amount of wild fish caught for ranching and grow-out. The successful implementation of projects that do this could greatly help to improve the numbers of wild stock in the oceans, as there could be a shift to decrease the quantity caught.

1.4. Larval rearing - The foundation of sustainable aquaculture

Most fish species go through a vulnerable larval period. At this time they are not protected inside the egg shell and are very sensitive to external factors. Sea bream species produce a large number of small eggs that hatch early in development and are dependent on nutritional and environmental factors for normal development and survival (Conides & Glamuzina 2001; Shields 2001; Zaki *et al.* 2007; The Research Council of Norway 2009). To evaluate the potential of a possible aquaculture candidate the larval rearing phase provides a good indication of whether the production of the selected species is feasible.

The specialisation of hatcheries and rearing systems at aquaculture facilities allow for controlled larval production through the optimisation of conditions for larval development and survival. This has made it possible for the commercialisation of marine aquaculture, especially for many sea bream species (Shields 2001; The Research Council of Norway 2009). The success of rearing and culturing these fish species on such a scale can be attributed to three important factors: (1) successful broodstock management, including broodstock collection and conditioning, spawning, and egg collection and incubation, (2) complete larval rearing using intensive, semi-intensive and extensive methods and systems, and (3) improvement of techniques of live food preparation for larval feeding (The Research Council of Norway 2009). These improvements have allowed the industry to provide healthier animals in a reliable and constant supply.

1.4.1. Techniques and methods used in sea bream larviculture

The larval rearing phase and the supply of juveniles have been recognised as the major bottleneck in the production process and it accounts for the highest mortality (Dhert *et al.* 1998; Planas & Cunha 1999; Conides & Glamuzina 2001). During the vulnerable rearing phase, the environmental conditions and food availability within the tanks are regarded as the most important factors that the larvae depend on for growth and survival (Papandroulakis *et al.* 2002). The improvement of controlled techniques, live feed preparation, and a greater knowledge of the optimal environmental parameters for larval rearing has allowed production to become reliable, which has enabled the aquaculture industry to generate a consistent, high-quality product. Control and optimisation of the

environmental parameters includes temperature, photoperiod, salinity and oxygen content (Moretti *et al.* 1999; Shields 2001; Fielder *et al.* 2002; Fielder *et al.* 2005).

The rearing method is species dependent and there are larviculture classification schemes that separate the species accordingly. These classification schemes have been based on the prey source (Van der Meeren and Naas 1997; Divanach *et al.* 1998), tank volume, larval stocking density and nature of water supply (clear water, green water, pseudo-green water) (Divanach & Kentouri 2000; Lee 2003). The larval rearing systems for sea bream species range from uncomplicated systems utilizing endogenous plankton blooms to large-scale, intensive hatchery systems that can produce thousands of fry a year (Shields 2001). There are extensive, semi-intensive (mesocosm) and intensive larviculture techniques (Shields 2001; Divanach & Kentouri 2000; Lee 2003), which range in order of magnitude according to larval density. Extensive methods use 0.1-1 larvae l⁻¹, while intensive methods use 150-200 larvae l⁻¹ (Divanach & Kentouri 2000; Shields 2001).

Within each intensity category, there can be variation in water source, extent of trophic autonomy, prey type and presence/absence of phytoplankton. There has been an increase in the production of juveniles over the last decade. However, this is not due to a total control of the intensive technologies applied. This has been the result of repeated applications of rearing methods and progress in the control of reproduction (Divanach *et al.* 1998).

1.4.1.1. Intensive rearing methods

Under intensive rearing conditions all parameters and environmental conditions are kept on strict specific settings in indoor systems, with production dependent on human intervention (Divanach & Kentouri 2000; Mihelakakis 2001; Papandroulakis *et al.* 2002; ICES 2004). This method of absolute control promotes similar behaviour, growth and development of the larvae among the tanks (Divanach & Kentouri 2000). The larvae are kept at high densities that are categorised as semi-intensive (30-50 larvae l⁻¹), intensive (80-100 larvae l⁻¹) and hyper-intensive (150-200 larvae l⁻¹) (Dhert *et al.* 1998). The feed production is synchronised with the larvae's growth and is added to the tanks at the correct concentration. The feed regime begins with the addition of rotifers, followed by *Artemia* (Leach, 1819) at different

instars, then finally the larvae are weaned onto an artificially formulated feed (Shields 2001).

With all parameters under control the problems of food availability and fluctuations in environmental conditions can be minimised, allowing for optimal growth and survival. This method has had the best success on species that are well known and their biology has been studied extensively. Commercial production of only two sea bream species in the Mediterranean, *Sparus auratus* and white seabream, *Diplodus sargus* (Linnaeus, 1830) has been achieved with intensive methods (Shields 2001).

1.4.1.2. Extensive rearing methods

Extensive methods are used for the rearing of sea bream species, where optimal conditions are created naturally (Pillay & Kutty 2005). An ecological approach based on oligospecific pelagic marine ecosystems is used. Instead of adding phytoplankton and zooplankton to the rearing medium when the larvae are ready to feed, as in intensive rearing, phytoplankton and zooplankton are added before the eggs, so that a natural food chain can be created (Divanach & Kentouri 2000).

This natural environment is run on an endogenous food supply that can either be from a natural bloom or from an artificial/domesticated bloom (Shields 2001). This environment allows the larvae to be at the top of the food chain, where they benefit the most (Divanach & Kentouri 2000).

This method is used to produce larvae on a short growth period and a long growth period. The short growth period (1-2 months) is used to provide ready-to-wean post larvae for intensive rearing systems, while the long growth period (2-6 months) supplies extensive systems with fully metamorphosed juveniles (Divanach & Kentouri 2000). At densities of only 0.1-1 l⁻¹, in outdoor tanks that can be hundreds to thousands of cubic metres, there are stable conditions that reduce the effects of environmental change and the larvae are able to adapt to the environment provided.

Under these conditions there can still be sufficient production of good quality larvae, showing few deformities and good growth and development (ICES 2004; Pillay & Kutty

2005), but intensive methods are preferred for commercial production, as there is a much higher survival rate of the larvae and the results are more reliable (Divanach & Kentouri 2000).

Papandroulakis *et al.* (2002) stated that the extensive method is overlooked as its productivity per unit volume is too low to meet with the demands on sustainable commercial production. However, there are suggestions that the extensive methods could meet commercial production challenges if the productivity of the rearing medium is increased, or if the techniques are mastered in very large volumes of water (Divanach & Kentouri, 2000).

1.4.1.3. Semi-intensive (mesocosm) rearing methods

This method integrates both extensive and intensive principles and can solve many problems associated with production. Extensive methods are used during the early developmental stages, when the larvae are still sensitive to the environment and difficult to feed. Only when the larvae are older are more intensive methods used.

There are two variants of the mesocosm method used in tanks of 30-100 m³ with a larval density of 2-10 l⁻¹ (Divanach *et al.* 1998; Shields 2001). A more extensive approach sees the food chain being endogenous and is supplemented with exogenous feed. The more intensive mesocosm method involves the addition of exogenous feed that allows the rotifers and other small prey to reproduce. This is possible due to the low density of the larvae and the existence of phytoplankton in the rearing environment (Divanach & Kentouri 2000; Papandroulakis *et al.* 2004b). This combination of exogenous and endogenous food sources allows for the improved fulfilment of the larval energy requirements (Papandroulakis *et al.* 2004b).

Research from known sea bream species reared in semi-intensive conditions has shown that performance and survival is better than that achieved with intensive or extensive conditions, with total deformities reduced and swimbladder inflation and similar behaviour increased (Divanach & Kentouri 2000). For a new species that has not been reared

previously, the mesocosm method provides a more natural environment with the benefit of added nutrients and food.

1.4.1.4. Addition of microalgae

Sparus aurata and *Pagrus pagrus* larvae in the Mediterranean are reared under extensive conditions using the 'green water' technique. This is characterised by the inclusion of phytoplankton in the rearing tanks, which creates an endogenous bloom and either *Chlorella* or *Nannochloropsis*, and rotifers, *Brachionus plicatilis* (Müller, 1786), during the first month of the larval rearing process (Hernandez-Cruz *et al.* 1999; Stephanou *et al.* 1995; Papandroulakis & Divanach 2002). There is a method called the 'pseudo-green' water technique, used in intensive and semi-intensive (mesocosm) methods, in which phytoplankton and rotifers are added to the rearing tank when the larvae begin feeding on exogenous food sources. The concentration of phytoplankton and rotifers is kept constant by daily additions (Papandroulakis & Divanach 2002).

The main difference between the 'green water' technique and the 'pseudo-green water' technique is that instead of the phytoplankton being produced in the rearing tank it is produced in a separate tank and added to the rearing tank to keep an appropriate concentration (Papandroulakis & Divanach 2002). By using the 'pseudo-green water' technique the characteristics of the 'clean water' and 'green water' methods are integrated. Divanach *et al.* (1998) state that phytoplankton plays a vital role in the development during the early stages and that the addition of phytoplankton can affect several aspects of larval development, including: microfauna in the gut, nutrition, feeding and behaviour (Nicholas *et al.* 1989; Hernandez-Cruz *et al.* 1994; Oie & Olsen 1997; Planas & Cunha 1999). Other research has also concluded that the presence of phytoplankton stabilises the rearing environment, protects the larvae against pathogenic bacteria and has been shown to have a nutritional effect either through direct or indirect means (Papandroulakis *et al.* 2002; Planas & Cunha 1999; Lee 2003).

The presence of phytoplankton in an experiment by Papandroulakis *et al.* (2002), using *Sparus aurata*, resulted in $44 \pm 17\%$ survival and individuals weighing up to 2.0 ± 0.2 mg over a 20-day period of rearing, while using the clear water method resulted in both survival and growth decreasing to $16 \pm 6\%$ and 1.1 ± 0.2 mg, respectively. Together with the increased

survival and growth of the larvae there was a decrease in pathogenic bacteria, which indicates that the inclusion of phytoplankton in the rearing tanks acts as a protection agent (Papandroulakis *et al.* 2002). Using the microalgae in sea bream rearing has impacted the survival and overall health of the larvae, making its use indispensable.

1.5. South African aquaculture

The aquaculture industry in South Africa is dominated by abalone farming, *Haliotis midae*, Linnaeus, 1758, with mussels, *Mytilus galloprovincialis*, Lamarck, 1819, and *Choromytilus meridionalis*, Krauss, 1848, and oysters, *Crassostrea gigas*, Thunberg, 1793, making up a significant part of production (DAFF 2011). The abalone industry made up 93.9% of the total value of the marine aquaculture sector in 2010. In the same year total aquaculture production was 3,133 tonnes (FAO-FishStatPlus 2010).

Marine finfish aquaculture is still in its infancy in South Africa, with only dusky and silver kob *Argyrosomus japonicus* (Temminck & Schlegel, 1843) and *Argyrosomus inodorus*, Griffiths & Heemstra, 1995, showing any promise. Production has been erratic over the past five years with only 2008 and 2009 producing any viable stock (DAFF, 2011). In 2010 no finfish were produced on a commercial scale, however, *Argyrosomus japonicus*, *Argyrosomus inodorus* and yellowtail, *Seriola lalandi*, Valenciennes, 1833, were being produced, though only on a pilot scale (DAFF 2011). Other fish have been experimented with (Harris & Cook 1995; Davis 1996). However none have been introduced as a candidate for potential commercial use.

The Department of Agriculture, Forestry and Fisheries (DAFF) have created policies, one of which states that there is a need to expand the resource base of marine aquaculture from the few species currently being farmed to a more diverse selection of suitable species and farming technologies (DAFF 2009). By carrying out this research *Rhabdosargus globiceps* could potentially be introduced successfully into marine aquaculture, thereby increasing the diversity of species used.

1.6. *Rhabdosargus globiceps* – Biology and life history

There is no information about the early life stages of this species. A detailed account of this could help to identify any problems that may inhibit the mass culture of the species and

hopefully help to introduce it as a new candidate for aquaculture and diversify the aquaculture products of South Africa.

This endemic, temperate species of sea bream is a Perciformes fish belonging to the family Sparidae and to the genus *Rhabdosargus* Fowler, 1933 (Figure 1.2).

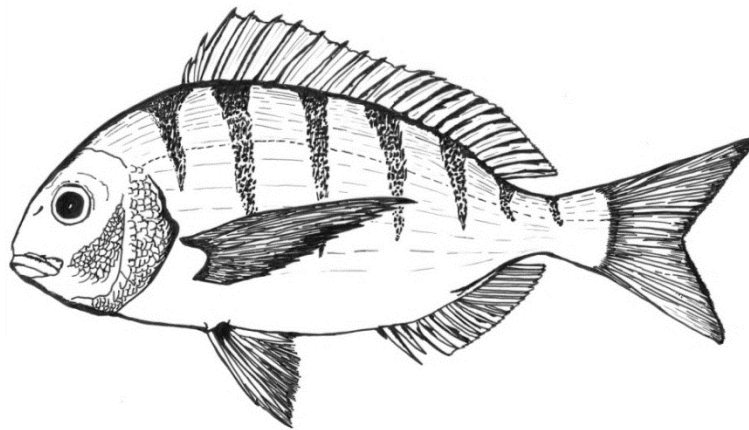


Figure 1.2: White stumpnose, *Rhabdosargus globiceps*. Original drawing by author.

1.6.1. Family description

Representatives of the family Sparidae have oblong to oval bodies that are compressed with large heads and steep foreheads (Smith & Heemstra 1995; Basurco *et al.* 2011). Their mouth is small in relation to their body size. This family is characterised by a groove in the outer end of the premaxilla, into which the maxilla fits. The lateral line is always visible and the caudal fin is moderately forked (Smith & Heemstra 1995). The dorsal fin has 10-13 spines and 8-15 soft-rays. The anal fin has 3 spines. The scales are weakly ctenoid, with the cheeks and opercles also being scaly. Teeth are conical or incisiform. Molars are present in some species (Smith & Heemstra 1995).

Members of this family are found in temperate and tropical waters in all oceans (Basurco *et al.* 2011). They congregate along the shore in relatively shallow water. Some species have been known to live in estuaries and lagoons. Only a few species live in deeper water.

Southern Africa appears to be a main area of distribution, where at least 41 species are present, with 25 of these being endemic to the region (Smith & Heemstra 1995). Sea bream species make up a very important part of the line-fishery, be it subsistence, recreational or commercial (Basurco *et al.* 2011). This family is made up of species that are either protogynous (female to male), protandrous (male to female), or simultaneous or rudimentary hermaphrodites (Basurco *et al.* 2011).

1.6.2. Morphology

Rhabdosargus globiceps is mainly silver with 6-7 black vertical crossbars (Branch *et al.* 2002) (Figure 1.2). Some specimens may appear to be considerably darker in colour. This variation is due to the environment and/or stress. This species can attain 50 cm in total length and 3 kg (Van der Elst 1988; Branch *et al.* 2002). The jaws have 4-8 enlarged incisors and several rows of molars (Branch *et al.* 2002). There are approximately 60 series of scales arranged along the lateral line (Van der Elst 1988). The bridge between the eyes has no scales covering it. All the fins are well developed, with the single dorsal fin having 11 spines and 11-12 soft rays (Van der Elst 1988). The anal fin has 3 spines with 10 or 11 soft rays (Van der Elst 1988). The lower first gill arch is comprised of 7-9 rakers (Branch *et al.* 2002; Van der Elst 1988).

1.6.3. Geographic distribution

Rhabdosargus globiceps is extensively found along the Southern African coast, from southern Angola to the Kei River mouth on the east coast of South Africa (Griffiths *et al.* 2002). Analysis of linefish catches revealed that there are 4 areas of abundance of this species. Two of these populations are found on the West Coast, Saldanha Bay and Langebaan lagoon (Western Cape), and False Bay (South-Western Cape) (Griffiths *et al.* 2002). The other two populations are found off the East Coast, at the central Agulhas Bank (Southern Cape) and the other in Algoa Bay (South-Eastern Cape) (Griffiths *et al.* 2002). It is found over sandy sea beds and rocky areas up to 80 m deep (Branch *et al.* 2002). The populations in the easterly regions have been caught at depths of 130 m. Juveniles are found in estuaries and along sheltered areas of False Bay (Van der Elst 1988). The

population present in the Langebaan lagoon migrate to warmer waters during winter (Attwood *et al.* 2007).

1.6.4. Reproduction

Rhabdosargus globiceps is an asynchronous rudimentary hermaphrodite, whose spawning season starts in September and can last until March (Attwood *et al.* 2010). The population in Langebaan Lagoon has peaks of spawning in October and February (Attwood *et al.* 2010). From April to July the gonads are resting. Griffiths *et al.* (2002) has shown that the populations on the south and east coasts of South Africa have a spawning season from August to April, with one broad spawning peak in October. Juveniles are known to recruit in estuarine nursery areas and move farther offshore as they grow (Branch *et al.* 2002).

Rhabdosargus globiceps is sexually dichromatic. This means that the male and female have different colour markings (Figure 1.3A & B). This suggests that females make a mate selection and paired spawning occurs. The change in silver colouration is apparent on the male. Its head is darker, which makes its white jaw more prominent. It also has a black patch immediately behind its lower jaw (Griffiths *et al.* 2002) (Figure 1.3B).

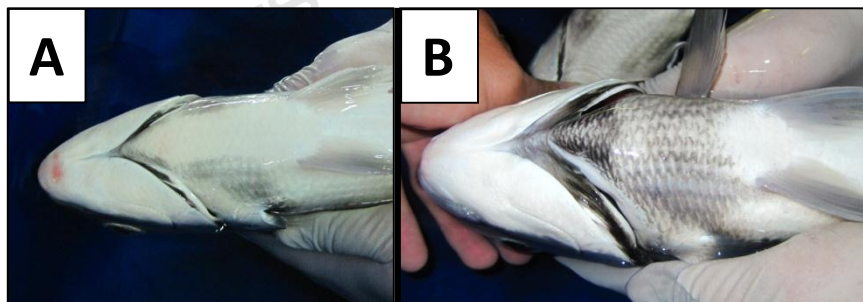


Figure 1.3: Photographs of female (A) and male (B) *Rhabdosargus globiceps* taken during broodstock conditioning experiment.

1.6.5. Fishery

This species is popular with recreational and commercial fishermen and is caught with line and seine-net (Branch *et al.* 2002). This species can also be caught as bycatch by inshore trawlers (Griffiths *et al.* 2002). The estimated annual landing is approximately 40 tonnes by shore-anglers, 147 tonnes by commercial line-fishers, 12.5 tonnes for beach-seine operators

and 14 tonnes for inshore trawlers (Griffith *et al.* 2002). *Rhabdosargus globiceps* populations have been exploited and over time their numbers have been reduced. Decline of the catch rate by as much as 68% off the Western Cape, 52% off the South - Western Cape and 99.8% off the Southern Cape has been recorded by commercial linefishers over the last 70 years (Griffiths *et al.* 2002). This species is currently on the orange list on the SASSI guide (SASSI 2011).

1.7. Project aims and objectives

Intensive and successful work has been done on sea bream aquaculture in the Mediterranean, Japan and Australia, with species such as the *Pagrus pagrus*, *Sparus aurata*, *Pagrus major* and *Pagrus aurata* being cultured successfully (Battaglione 1997; Mihelakakis *et al.* 2001; Shields 2001). The reason for this is that survival, growth rate and condition of the fingerlings have improved considerably due to the improvement of zootechnics, nutritional quality of the prey and hygienic conditions of the hatchery and rearing systems (Planas & Cunha 1999). Similar methods will be used to rear *Rhabdosargus globiceps* larvae, as these species are similar in life histories, reproductive strategies and they react very similarly to the conditions of captivity. This suggests that there may be some potential in the use of *Rhabdosargus globiceps* as a novel candidate species in the aquaculture industry in South Africa.

Information of growth, development and survival during the larval stage of development is crucial for the proper rearing of the fish and provides valuable information allowing for appropriate financial and logistical planning. The main aims of the research were to optimise the conditioning, spawning, and rearing techniques for *Rhabdosargus globiceps* and ultimately close the life-cycle.

Research objectives and hypotheses:

The first objective of the research was to assess the ability of the broodstock to be conditioned to spawn in captivity. This would provide information about whether it is possible for the broodstock to be brought to reproductive maturation under a compressed,

manipulated photothermal cycle and what the effect these variables would have on the spawning frequency, gametes and larval condition. The hypothesis for this section of the research was that the manipulated photothermal cycle would successfully condition the broodstock to become reproductively mature. The null hypothesis was that the manipulated photothermal would not bring about reproductive maturity in the broodstock.

The second objective was to induce the broodstock to spawn successfully and then assess the spawning frequency, fertilisation success, and fertilised egg quality. Best methods of transfer, quantifying and weighing of eggs were also assessed. The success of this objective relied on the successful conditioning of the broodstock. The hypothesis was that if the broodstock were reproductively mature, the appropriate administration of an inducing hormone would result in successful spawning. The null hypothesis was that if the broodstock were not reproductively mature, spawning could not be successfully induced.

The third objective was to perform successful larval rearing. From this the general ontogeny, growth rate, development and the effects of temperature on growth, development and survival were assessed. Larvae were reared through the endogenous feeding stage to the weaning stage at 30 days post hatching. The hypothesis regarding the ontogeny of the larvae was that the growth and development of the larvae would be very similar to that of other sea bream species. The null hypothesis was that the ontogeny of the larvae would not be similar to other sea bream species. The effect of temperature was hypothesised to increase the rate of growth and development as it increased, for both the embryo and larval stage. The null hypothesis was that there would not be an increase in growth and development with an increase in temperature.

This thesis reports on the manipulation of gonadal development in *Rhabdosargus globiceps* broodstock using artificial cycles of photoperiod and temperature, first descriptions of the early life stages of *Rhabdosargus globiceps*, and investigation of the embryonic and larval development under different environmental conditions. This research examines the biological capacity of the *Rhabdosargus globiceps* adults and larvae to meet certain criteria that mirror that of successful sea bream aquaculture candidate species. Through application of the results gained from this research we will gain better knowledge on whether *Rhabdosargus globiceps* fits the candidate criteria for aquaculture in South Africa.

CHAPTER 2

Natural and manipulated reproductive cycles of white stumpnose, *Rhabdosargus globiceps* (Teleostei: Sparidae).

ABSTRACT

The annual reproductive development of female *Rhabdosargus globiceps* from Langebaan Lagoon was investigated, and then compared to a compressed spawning cycle brought about by a manipulated photothermal cycle. This compressed cycle successfully brought about the reproductive maturation of the female broodstock. The natural and manipulated cycles were comparable with regard to oocyte maturation. Under natural conditions the spawning season duration based on histological evidence extends from August to March, with peaks in October and February. The natural conditions found between winter and summer in Langebaan Lagoon were used to create a compressed photothermal cycle to use on three tanks of captive broodstock (15 females, 4 males). The tank number had no significant effect ($P>0.05$) on the estradiol 17β concentration or the oocyte diameter, however over the sampling period there was a significant increase in estradiol 17β concentration ($P<0.05$; $F=105.26$). The increase in the plasma estradiol 17β concentration correlated with the increase in oocyte diameter ($R^2=0.55$, $P<0.05$) and the presence of late vitellogenic and hydrated oocytes. By comparing these data to the data collected from the wild caught fish from Langebaan Lagoon the timing to successful maturation could be recorded. The most mature female broodstock (oocytes $>200\ \mu\text{m}$) were placed into the same tank as four males, at a ratio of 1:1. These broodstock were injected twice with LH-RHa on a 24 hour interval. Broodstock spawned successfully 48 hours after initial induction for a period of four consecutive 24 hour periods. The highest quantity and best quality eggs came after the third 24 hour period.

2.1. INTRODUCTION

The physiological and behavioural changes that occur during the reproductive cycle of fish usually follow a fixed periodicity. These are typically annual, lunar or diel cycles (Billard and Breton 1978; Sheaves 2006). The cycle involves the development of the gametes, changes in the concentration of certain hormones in the blood and can include alterations in behaviour. The period and timing of the cycles are adapted to local circumstances. For example, many reef fish species spawn during high tide, which helps the eggs and larvae to be transported away from the reef and its density of filter feeders and planktivorous fishes (Shibuno *et al.* 1993). However, there are many possible ecological, physiological and phylogenetic reasons for the timing of the spawning season (Billard and Breton 1978; Sheaves 2006). The physiological aspect is connected with a particular time of the year that may provide the best conditions for the sexual maturation of the adults, so that reproductive output can be optimised (Bye 1984). Another likely reason is that the spawning may coincide with suitable environmental conditions for the gametes, eggs and larvae to increase survival and growth (Billard and Breton, 1978; Bye 1984; Bromage *et al.* 2001; Sheaves 2006).

Reproduction in temperate fish species is strongly related to environmental production cycles (Prat *et al.* 1999; Aristizabal 2007). These seasonal cycles have a causal relationship with the concentrations of the circulating hormones and proteins such as estrogen and vitellogenin (Kokokiris *et al.* 2001), which drive reproductive development or gametogenesis (Mylonas *et al.* 2010).

Most teleosts found in temperate regions appear to time their reproduction to the seasonal changes in photoperiod and water temperature, which act to control and initiate endogenous reproductive rhythms (Clark *et al.* 2005; Pillay & Kutty 2005; El-Sayed and Kawanna 2007; Mihelakakis 2001). The external factors of temperature and photoperiod are recognised by the gonadotropin-releasing hormones (GnRHs) in the hypothalamus, which regulate reproduction by controlling the secretion of the pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinising hormone (LH) (Mylonas *et al.* 2010). This action allows for the control of reproduction along the hypothalamic–hypophysial–gonadal axis (Prat *et al.* 1999; Mylonas *et al.* 2010). The release of FSH and LH into the bloodstream acts directly on the gonad, producing the sex steroid hormones (e.g. estrogens), which act as the

main stimulants of gametogenesis (Rottman *et al.* 1991; El-Sayed and Kawanna, 2007; Mylonas *et al.* 2010). By assessing the concentration of estradiol, the main component of estrogen, or vitellogenin, a yolk pre-cursor glycolipoprotein, the effect of the external factors of temperature and photoperiod can be examined and the sexual maturity of a female fish can be assessed (Kokokiris *et al.* 2001; Mosconi *et al.* 2002). Other factors that can be used to check the reproductive maturity of the fish is oocyte size and the specific stage of the oocytes that dominate the ovaries (Mylonas *et al.* 2010).

With knowledge of how specific environmental conditions affect gametogenesis in the natural environment the broodstock can be manipulated to advance or delay their spawning cycle by artificially prolonging or shortening natural cycles (Morehead *et al.* 2000). Hormones such as GnRH and LH-RHa have been used to induce maturation and spawning (Mylonas & Zohar 2007). However, a hormone such as LH-RHa will induce ovarian maturation and hydration of the oocytes only if they are at the late vitellogenic stage (Moretti *et al.* 1999). An artificially created regime of temperature and photoperiod can be used to condition the broodstock to the point of induction. The ability to artificially advance and delay the spawning period for a selected species is a valuable tool for broodstock management and egg production (Zanuy *et al.* 1986). Eggs can be produced out-of-season using a method of artificial stimulation, allowing for continuous production throughout the year (Moretti *et al.* 1999).

Rhabdosargus globiceps is a gonochoristic species (rudimentary hermaphrodite) and spawns between August and February with peaks from September to October (Griffiths *et al.* 2002; Attwood *et al.* 2010). During the colder months, May to July, the females are immature or their gonads are resting. Female *Rhabdosargus globiceps* mature at three years old and males mature at 2 years old (Attwood *et al.* 2010). The size-at-maturity varies greatly between the sexes. The females mature at 210 to 310 mm (FL), while males mature at 180 to 270 mm (Attwood *et al.* 2010). *Rhabdosargus globiceps* is an asynchronous species, which implies the ovaries contain oocytes at different stages of maturity (Murua & Saborido-Rey 2003). The hydrated oocytes are released in batches, while the less developed oocytes remain in the ovary to reach full maturation and become hydrated (Murua & Saborido-Rey 2003).

Asynchronous sea bream species have a regular periodicity in gonadal growth, as they ovulate many times during spawning season (Tyler and Sumpter, 1996; Murua and Saborido-Rey 2003; Aristizabal 2007). However, the number of batches spawned is influenced by the availability of food, temperature and other environmental factors (Bye, 1984; Wootton 1990; Murua and Saborido-Rey 2003; Sheaves 2006).

This present study aimed to use a compressed photothermal cycle to advance the timing of spawning in *Rhabdosargus globiceps*. The development of the oocytes under this cycle was compared to the oocyte development during the course of the annual cycle. A second aim was to examine the correlation between temperature and photoperiod and the reproductive development of female *Rhabdosargus globiceps*, in terms of estradiol 17 β concentration, oocyte diameter and oocyte maturation. The hypothesis for this section of the research was that the manipulated photothermal cycle would successfully condition the broodstock to become reproductively mature and that as the estradiol 17 β concentration increased over time the oocytes would mature and increase in diameter. The null hypothesis was that the manipulated photothermal cycle would not bring about reproductive maturity in the broodstock and that the estradiol 17 β concentration would not increase, thus resulting in the oocytes not maturing.

This research will enhance our knowledge of the reproduction of the *Rhabdosargus globiceps* and determine whether it is possible for the captive broodstock to produce viable eggs out-of-season, through shifting of the spawning period. Comparative analysis of the development and growth of the oocytes would provide comparable results from the natural cycle and the compressed cycle. Blood samples were taken from the captive broodstock and the concentrations of estradiol 17 β were calculated. This was then used to assess whether the external photothermal cycle is having the desired effects. By assessing the quality of the eggs the conditioning protocol can be evaluated.

This work will have application in aquaculture and fisheries management. The influence of temperature and photoperiod during the development of the ovaries and eggs, and the temperature and photoperiod that induces spawning is useful information to those wishing to culture *Rhabdosargus globiceps* specimens kept in aquaculture facilities.

2.2. METHODS

Assessment of reproduction under natural environmental conditions

Study Site

Sampling took place in Langebaan Lagoon, in Saldanha Bay on the cool-temperate west coast of South Africa. The southern part of the Langebaan Lagoon is designated as a Marine Protected Area, while the northern area, around Schaapen Island and further north, is a popular recreational, commercial and subsistence fishing ground. The lagoon is divided into three utilisation zones: a sanctuary, a restricted zone and a controlled zone (Figure 2.1).

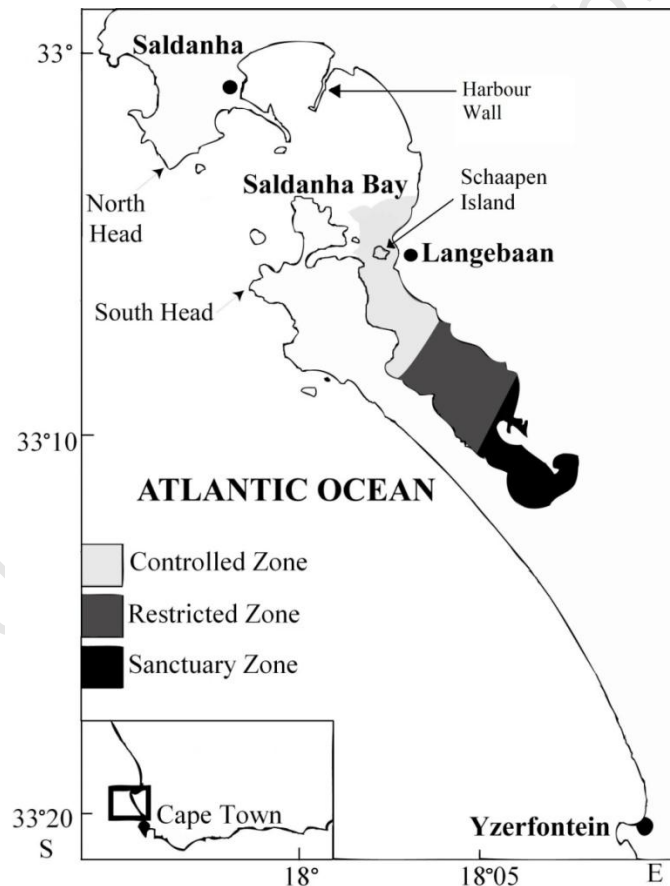


Figure 2.1: The South Western Cape of South Africa, showing the position of Langebaan Lagoon and Saldanha Bay.

Sampling

A total of 66 female *Rhabdosargus globiceps* were sampled from the 28th of April 2010 to the 22nd of September 2010. *Rhabdosargus globiceps* were caught by rod and line baited with the mud prawn, *Upogebia africana* (Ortmann, 1894). The majority of the specimens were caught around Schaapen Island in the warmer months, while in the winter months the majority was caught near the Harbour wall in Saldanha. This movement reflects their migration when the seasons change (Kerwath *et al.* 2009). The sexual markings of the specimens helped to identify whether the fish were male or female, although in the early months it was difficult to distinguish their sex. The females have no sexual markings, while the males have black markings along the pectoral girdle (Griffiths *et al.* 2002).

Temperature

A temperature logger was used to record temperature. A logger was attached to the bottom of a channel marker in the lagoon (33° 06' 29.35" S and 18° 01' 53.41" E). The logger recorded three temperature readings per day. The first set was at 8:00 am, the second set at 16:00 pm and the third set at midnight. The average temperature was calculated for each day. The maximum and minimum daily temperature was also recorded in order to display the variability in water temperature over the sample period. The recorded data described the temperature fluctuations from April to March within the lagoon. The temperature logger was first placed in the lagoon on the 28th of April 2010. It was removed twice over the recording period and another temperature logger was attached to the same channel marker. The last temperature logger was removed on the 29th of March 2011.

Photoperiod

The photoperiod for each day of each month was recorded using the program SUN (Sunrise Sunset calculator, 2012). This program calculated the amount of sunlight available each day. Langebaan (33° 05' 25" S and 18° 02' 07" E).

Morphology and gonad examination

Before the fish were dissected their total length (TL) and the fork length (FL) were measured to the nearest mm. The weight was measured to the nearest 1 g. The fish were dissected and the ovaries were removed, weighed and stored in 10% buffered neutral formalin.

Histological analysis

The ovaries were stored in 10% buffered formalin for at least 48 hours prior to sectioning. A 3-5 mm section was cut from the middle of the left lobe of the ovary. These sections were again stored in 10% buffered formalin. The samples were sectioned at 5 μ m and stained with haematoxylin and eosin. Samples were analysed using the 20X, 40X and 100X objectives of the microscope (Olympus BX51). The ovary and oocyte development was categorised using the histological characteristics described by Jackson and Sullivan (1995) and Kokokiris *et al.* (2001) (Table 2.1). The ovary stages were determined according to the presence and relative frequency of the most advanced oocytes (Figure 2.9).

Digital images were recorded using a Nikon Digital Sight Fi 1 Digital camera and analysed using NIS elements basic research software. The stage of maturity of each female was determined by classification of the most advanced oocytes from each ovary slide. The imaging software was also used to measure the area and diameter of the oocyte cells. The mean oocyte diameter for each oocyte stage was determined from the mean diameter of 20 randomly selected oocytes within one histological cross-section of the ovary. The proportion of different stages of oocytes per sample was also recorded. Approximately 200 oocyte cells were counted from each of the slides per sample. From these 200 cells the proportion of the different stages of the oocytes from that sample was calculated.

Table 2.1: Microscopic ovary and oocyte stage criteria for female *Rhabdosargus globiceps*. Table adapted from Kokokiris *et al.* (2001) and Jackson & Sullivan, (1995).

Ovary Maturity Stage	Histological/biopsy appearance	Oocyte maturity Stage	Histological/biopsy appearance	Diameter range
Previtellogenic stage	Only previtellogenic oocytes are present. Previtellogenic oocytes have a densely stained cytoplasm when stained with haematoxylin and eosin.	Chromatin nucleolar (Stage 1)	Nucleus contains a single, large, basophilic nucleus.	30-60 μm
		Perinuclear (Stage 2)	Large, centrally located nucleus contains multiple, basophilic nucleoli along its periphery	30-75 μm
		Lipidic (Stage 3)	Unstained remnants of lipid droplets scattered throughout the cytoplasm	60-120 μm
		Cortical granule (Stage 4)	PAS-positive cortical granules evident in the peripheral cytoplasm	100-180 μm
Endogenous early vitellogenic stage	The cytoplasm of oocytes entering vitellogenesis was less basophilic, and the perinuclear zone was occupied by lipid globules and cortical alveoli.	Early vitellogenic (Stage 5)	Yolk and lipid globules evident in the peripheral ooplasm, indicating initial vitellogenesis	150-320 μm
Exogenous vitellogenesis stage A	Less than 50% of the histological cross-section area was occupied by vitellogenic oocytes, in which the central-peripheral zone of the ooplasm was colonised by small, spherical yolk globules.	Late vitellogenic (Stage 6)	Numerous, larger yolk globules and lipid droplets throughout the ooplasm. Well-developed follicle layers and chorion.	300-600 μm
Exogenous vitellogenesis stage B	More than 50% of the section was occupied by advanced vitellogenic oocytes. Large lipid and yolk globules that had started to coalesce occupied their ooplasm.			
Final maturation stage	Mature (hydrated) oocytes are present. The nucleus of these oocytes was absent. Ooplasm is occupied by large populations of yolk and lipid globules.	Final maturation (Stage 7)	Germinal vesicle migrating peripherally, marking resumption of meiosis; ooplasm undergoing lipid droplet coalescence	500-860 μm
Post-spawning stage		Atretic/overripe oocytes (Stage 8)	Oocytes highly vacuolated and collapsed inward: hypertrophied, phagocytic granulosa cells evident in follicles	500-860 μm

Assessment of reproduction under a compressed photothermal cycle

The experiments were conducted at the Department of Agriculture, Forestry and Fisheries (DAFF) Aquaculture Research Facility, in Seapoint, Cape Town, South Africa (33°55'14.27"S. 18°22'50.75"E).

Fish capture and husbandry

Mature *Rhabdosargus globiceps* (15 females and 4 males), were caught by rod and line in 2010 from Langebaan Lagoon, Western Cape, South Africa (33°05'46.94"S. 18°01'21.64"E) and transported to the Department of Agriculture, Forestry and Fisheries (DAFF) Aquaculture Research Facility, in Seapoint, Cape Town, South Africa (33°55'14.27"S. 18°22'50.75"E). All the fish were tagged with intramuscular numbered tags and the sex of the fish was determined by the colouration of the individuals during anaesthesia. The fish were divided and transferred into four indoor, circular, fully recirculating tanks in 2011 (Figure 2.2A & B). Three tanks had 5 females each, while one of the tanks held all the males (mean \pm standard error (SE) body weight, 1.17 ± 0.05 kg, $n=19$). As a diet, the broodstock were fed chopped pilchard and trout pellets (AQUANUTRO) every second day. With the onset of oocyte growth and maturation the amount of fresh feed, pilchard and mussel, was increased and the fish were fed daily. The amount of food added per tank was 2% of the total body mass.



Figure 2.2: A & B-Broodstock tanks used to control temperature and photoperiod in the compressed photothermal experiment.

Conditioning protocols

The indoor recirculating system allowed for the control of temperature and photoperiod. Four tanks were used to house the broodstock and simulate the change in seasons (Figure 2.2). Tank 2, 3 and 4 contained 5 females each. Two males were placed into Tank 2 and the other two were placed into Tank 3 and 4 respectively. These tanks were covered with a black plastic hood, with a central white light, which was used to control the photoperiod.

With the control of these two environmental parameters, the reproductive cycle of the captive broodstock was manipulated. Wild *Rhabdosargus globiceps* spawn at the start of spring in August to the end of summer in March (Griffiths *et al.* 2002; Attwood *et al.* 2010). Water temperature and photoperiod have been shown to greatly affect the progress of the reproductive cycle within the Sparidae family (Sheaves 2006; Pankhurst & Porter 2003; Morehead *et al.* 2000).

Over the period of 3 months (March to June) the temperature and photoperiod were changed from a winter setting to a summer setting, emulating that of Langebaan Lagoon. At the end of this conditioning period the female broodstock that were reproductively mature (oocytes >200 μm) and all the males were placed into tank 1. The female to male ratio was 1:1 (4 females and 4 males).

Temperature

Tank 1 and tank 2 were connected to the same recirculating system, while tank 3 and tank 4 were collected to another separate recirculating system. Temperature loggers were placed in tank 2 and tank 4 for the duration of the conditioning period. The water temperature was kept at the ambient incoming temperature until the 29/5/2012. At this time the temperature was increased in increments of 0.5°C until it reached 18°C. This was achieved by using heaters that were placed in the sumps which supplied the broodstock tanks with sea water. Digital controls were used to set the temperature in each sump. Water chillers were not used. Temperature profiles were created for both sets of tanks.

Photoperiod

The tanks were covered with a black plastic hood, with a central white light, which was used to control the photoperiod. The hoods and lights were used to increase the photoperiod from 9 to 12 hours of light before the hoods had to be taken off and the indoor lights of the facility used. This was done as the program that controlled the lights inside the hoods had a maximum photoperiod of 12 hours. The facility lights, which were positioned over the tanks, were used to increase the photoperiod up to 15 hours of useable light.

Broodstock Sampling

All broodstock were sampled every three weeks during the three month conditioning period. The intramuscular tags allowed for individual data for each fish to be recorded. They were caught and anaesthetised in a stretcher with a mixture of sea water and 2-Phenoxyethanol, at a concentration of 0.2 ml per litre of sea water (Vaughan *et al.* 2008) (Figure 2.3A). Their total length (TL) and weight were recorded to the nearest cm and gram, respectively.

Biopsy samples

Gonad biopsies were repeatedly conducted on all the broodstock five times over the three-month conditioning period. A cannula was used to extract ovarian tissue and when it was possible, sperm from the males (Figure 2.3B). This was inserted into the urogenital papilla and the oviduct. It was found that it was easier to check if the males were spermiating by pressing along their abdomen, instead of using the cannula. These biopsy samples provided a representation of the reproductive maturational stage of oocytes (Figure 2.10, 2.11). The extracted ovarian cells were analysed under a microscope (Olympus BX51 compound microscope). The oocytes were staged microscopically according to Jackson and Sullivan (1995) and Kokokiris *et al.* (2001) (Table 2.1). The diameters of 50 oocytes were measured from three different fields of view (FOV) for each biopsy sample using a Nikon Digital Sight Fi 1 digital camera and the NIS elements basic research software. The stage of ovarian maturation was determined by the mean oocyte diameter and the frequency of the different oocyte stages (Garcia, 1989) (Figure 2.11). Table 2.1 was used for both histology and biopsy assessment. The 4 stages of previtellogenic oocytes were clearly distinguishable in the

histologys. However, these stages in the biopsies were not clearly distinguishable, therefore they were grouped into one stage.

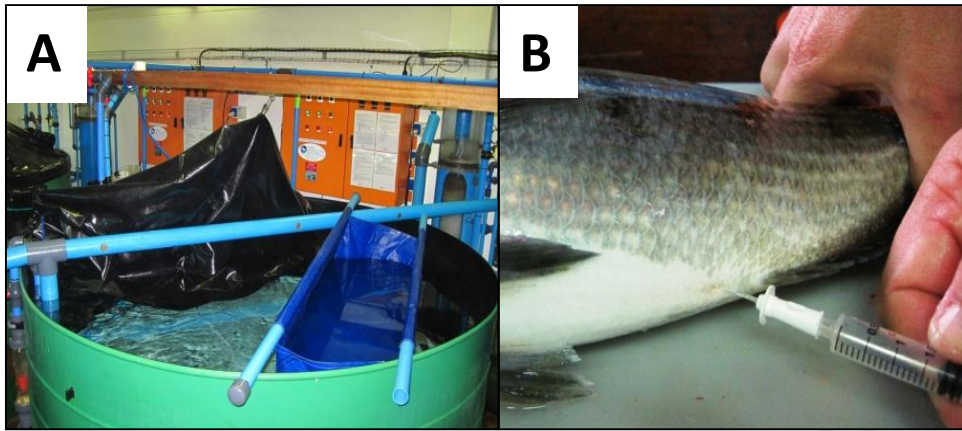


Figure 2.3: A-Broodstock tank with stretcher. B- Gonad biopsy sample being taken from the urogenital papilla of an anaesthetised fish.

Blood samples

A 0.5 ml blood sample was taken from the caudal vessel using a sterile 21 gauge needle and a 1 ml syringe (Figure 2.4). The blood samples were placed in heparinised blood collection tubes and kept on ice. After being centrifuged for 10 minutes at 4000 rpm at 4°C, the clear plasma was removed and put into a sterile 1.5 ml Ependorf tube. These samples were frozen and stored at -80°C for further analysis using the ELISA procedure to test for estradiol 17 β .



Figure 2.4: Blood sample being taken from the caudal vessel of *Rhabdosargus globiceps* during the broodstock conditioning experiment.

Hormone assay procedure

The assay was conducted to assess the concentration of estradiol 17 β , the major component of estrogen, in the blood plasma to examine the fluctuations brought about by the compressed photothermal cycle. The concentration was calculated to the nearest pg ml⁻¹. This allowed for the relationship between estradiol 17 β and the development of the oocytes to be examined.

The ELISA was optimised for the analysis of estradiol 17 β in the broodstock plasma samples. Dilution series were run using male broodstock blood plasma to assess any inhibition of the assay by the presence of other hormones. By doing so we could test where the concentration of estradiol 17 β could be read. No inhibition was found during the dilution series testing. Each plasma sample was run in triplicate. The manufacturer's procedure was followed and the absorbance values were obtained for standard and plasma samples. A standard curve was created by using the absorbance values. Using a 4 Parameter Logistics curve fit the concentration of the estradiol 17 β could be read from the corresponding standard curve.

Induction of spawning – out of season

The oocyte maturation of the fish from natural conditions suggested that the mean oocyte diameter is >200 μ m when the ovaries were reproductively mature. This diameter was apparent in September in fish that experienced the natural cycle, which is when the fish have been recorded to spawn.

The last sampling of the broodstock on the 28/6/2012 showed that four females had oocytes that had an average diameter of >200 μ m. These females were placed into the same tank as the four males. Two intramuscular injections of 1 μ g of LH-RH [LH-RHa des-Gly¹⁰ (D-Ala⁶) LH-RH ethylamide, acetate salt] per kg body mass were administered to each fish. There was a 24 hour interval between injections. The LH-RH injections were also administered to the 4 males.

Eggs were successfully spawned approximately 48 hours after the first injection. The eggs were given 24 hours to accumulate in the collecting net. This method of egg collection, and

not stripping, minimised the handling stress on the broodstock (Lahnsteiner & Patarnello 2004). After this time the total number of eggs and their quality was assessed.

Egg quality and quantity

Fertilisation success was evaluated as soon as the eggs were collected from the egg collectors (Mylonas *et al.* 2004). The approximate number of fertilised buoyant eggs and unfertilised non-buoyant eggs was estimated. Three subsamples were taken from the fertilised eggs and the percentage of good and low quality eggs was calculated. Good quality eggs were those that had a single oil globule, round shape and had a clear transparency (Figure 2.11). Bad eggs were those that had more than one oil droplet, were opaque or discoloured and had an irregular shape (Moretti *et al.* 1999) (Figure 2.11). The number of fertilised and unfertilised eggs was estimated by taking a 0.5 ml sample, counting the eggs present in this sample, and then multiplying it by the total volume of the beaker that they occupied. On average there were 390 eggs per 0.5 ml sample (780 eggs ml⁻¹).

Data analysis

To assess the change in mean oocyte diameter and estradiol 17 β concentration over time repeated measures ANOVA tests were run. A Levene's Test was run to test the assumption of normality of variance. A Repeated measures ANOVA was run to determine if there was any significant difference between the oocyte diameters and estradiol 17 β concentration. A Tukey HSD Post-Hoc test was then run to assess which sampling dates were significantly different to each other.

A regression analysis was used to assess the relationship between the estradiol 17 β concentration and the oocyte diameter. The linear relationship and its significance were recorded.

Where possible the measurements are given as the mean \pm standard error followed by the measured range and sample size in parentheses.

2.3. RESULTS

Natural environmental conditions

The water temperature and the photoperiod cycles show the seasonal fluctuations that are present in Langebaan Lagoon (Figure 2.5). The minimum temperature during winter was 9.4°C. At this time the average photoperiod length was 10.9 hours of sunlight. The increase in water temperature began at the beginning of August and reached its peak between December and February, where the maximum mean temperature recorded was 21°C. During December the photoperiod was at its maximum with an average of 15 hours of sunlight per day. Although photoperiod follows a smooth, predictable cycle, sea temperature along South Africa's west coast is affected by insolation and wind-induced upwelling. Temperature does not follow a uniform trajectory, but is rather interrupted by episodic upwelling events, which may be important in triggering spawning.

Mean oocyte diameter per month in relation to temperature and photoperiod

From April to July the mean oocyte diameter increased slowly from $74.43 \pm 3.47 \mu\text{m}$ (min-61.98 μm ; max-93.69 μm) ($n=11$) in April to $102.28 \pm 6.54 \mu\text{m}$ (min-76.63 μm ; max-128.15 μm) ($n=8$) in July (Figure 2.5). A substantial increase in oocyte diameter occurred in August. Peaks in mean oocyte diameter were recorded in October and February with values of $236.28 \pm 10 \mu\text{m}$ (min-181.18 μm ; max-278.8 μm) ($n=12$) and $195.72 \pm 10.74 \mu\text{m}$ (min-112.14 μm ; max-242.41 μm) ($n=13$) respectively. Between these peaks there was a fluctuation in size, with the fish sampled in December having the lowest mean oocyte diameter of $61.23 \pm 5.47 \mu\text{m}$ (min-50.5 μm ; max-86.8 μm) ($n=7$). This decrease in size occurred at the maximum temperature and photoperiod. The largest oocytes were hydrated and at the final maturation stage (stage 7) (Table 2.1). Hydrated oocytes had diameters that ranged from 600 – 850 μm .

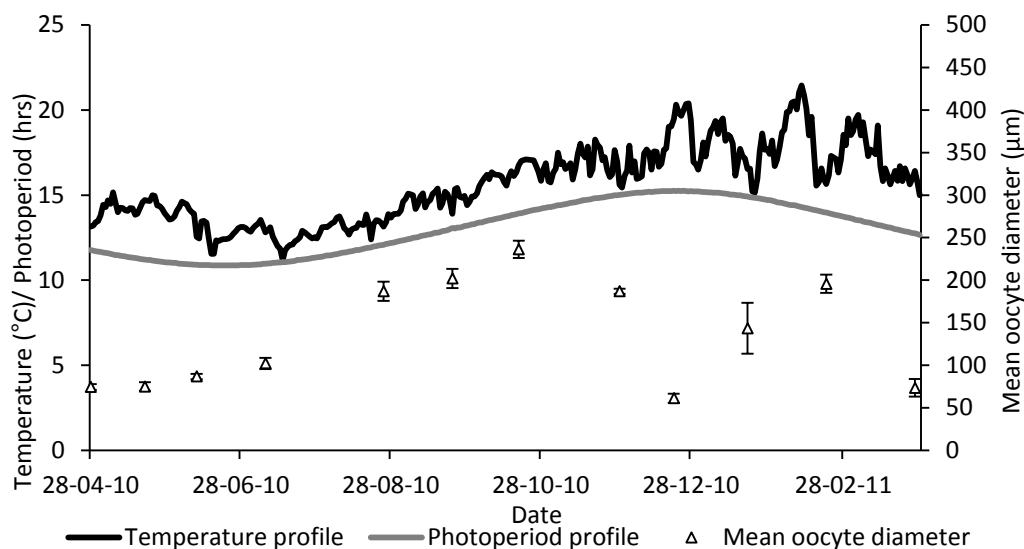


Figure 2.5: The relationship between mean oocyte diameter (\pm SE) of *Rhabdosargus globiceps* and natural water temperature and photoperiod over the 12-month sampling period in Langebaan Lagoon.

Mean oocyte diameter under a compressed photothermal cycle

The mean oocyte diameter increased with the increase in temperature and photoperiod (Figure 2.6 & 2.7). The smallest mean oocyte diameter (min-30 μ m; max-180 μ m) was recorded at the first sampling and the largest diameter (min-30 μ m; max-850 μ m) was recorded at the last sampling. All three groups of female broodstock showed that the greatest increase in oocyte diameter came between the second last and the last sampling time.

The Repeated Measures ANOVA test showed that there was a significant difference ($P < 0.001$; $F = 105.26$) between the oocyte diameters over the sampling dates. The mean oocyte diameter from the last sample was significantly different ($P < 0.05$; $F = 84.49$) to all the previous samples for the broodstock in Tank 2 and 3. Mean oocyte diameter in Tank 4 from the last sample was significantly different ($P < 0.05$) to the first and second sample, but not to the other samples. The female broodstock in tank 2 had the greatest mean oocyte diameter of $196.8 \pm 20.4 \mu$ m (min-140 μ m; max-248 μ m) ($n = 5$) at the last sample (Figure 2.6), while at this time the female broodstock in tank 3 and 4 had a mean oocyte diameter of $187.7 \pm 15.6 \mu$ m (min-143 μ m; max-215.5 μ m) ($n = 4$) and $162.6 \pm 12.9 \mu$ m (min-128.9 μ m; max-186.4 μ m)

($n=4$) respectively (Figure 2.7). Out of the fifteen female broodstock, only four matured with mean oocyte diameters ranging from 201 to 248 μm . Three fish from tank 2 and one fish from tank 3 were the females that were induced to spawn.

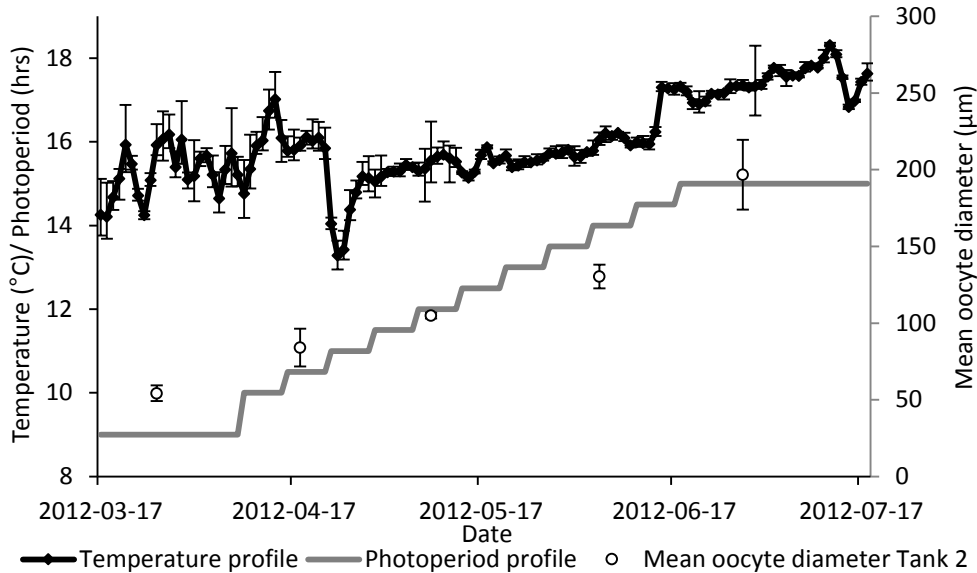


Figure 2.6: The relationship between mean oocyte diameter (\pm SE) of *Rhabdosargus globiceps* broodstock in tank 2 and the manipulated, compressed photothermal cycle over the 3 month conditioning period. Temperature values are mean \pm SE.

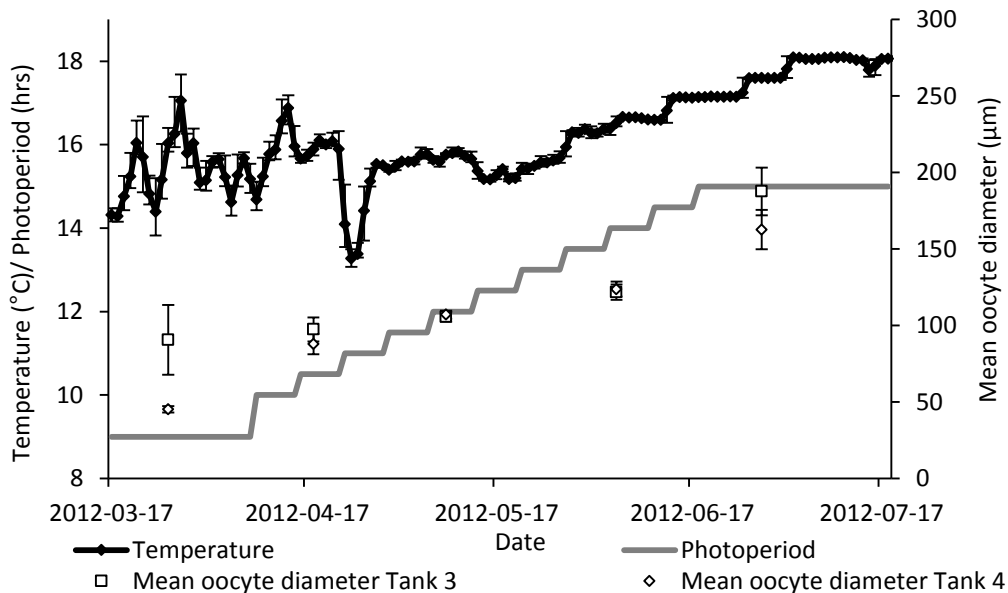


Figure 2.7: The relationship between mean oocyte diameter (\pm SE) of *Rhabdosargus globiceps* broodstock in tank 3 and 4, and the manipulated, compressed photothermal cycle over the 3 month conditioning period.

Ovarian development under natural conditions

Stage 2 previtellogenic oocytes made up the majority of the percentage composition from April to July, while from August to March the majority of the percentage composition was made up by stage 3 oocytes (Figure 2.8). The first early vitellogenic (stage 5) oocytes were present in the July sample. From August until November there was a high percentage of late vitellogenic (stage 6) oocytes, with a peak occurring in November. The December sample had a large decrease in oocytes above stage 3, however, there was another peak in stage 6 oocytes in February. The highest percentage of hydrated/final maturation (stage 7) oocytes was in October and January, with the first appearance of these oocytes in August. Atretic oocytes were present in the April, September, February and March samples (Figure 2.8).

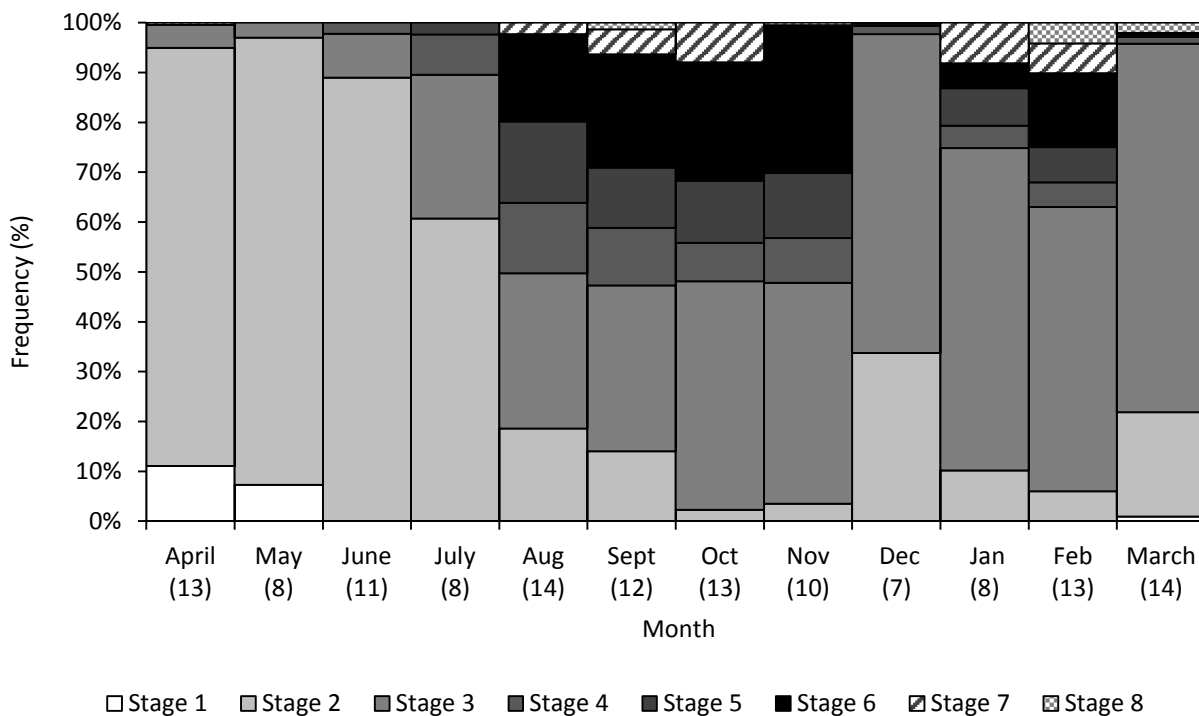


Figure 2.8: Seasonal cycle of oocyte development in *Rhabdosargus globiceps* from Langebaan lagoon. (n)= number of fish sampled. Ovaries were at a resting stage from April to June. Spawning occurred from August through to March.

Ovarian development under compressed photothermal cycle

Previtellogenic oocytes were the most prevalent stage in the ovaries in all of the samples. Overripe oocytes were present in the first sample. The prevalence of early vitellogenic (stage 5) oocytes increased over the conditioning period, with the last sample having the highest percentage (5.3%). The last sampling of the broodstock in June revealed that the females had late vitellogenic (stage 6) oocytes and oocytes going through final maturation (stage 7), but at a very low frequency of 4% and 0.3%, respectively (Figure 2.9). According to Moretti *et al.* (1999), when the ovary is at the exogenous vitellogenesis stage, where more than 50% of the field of view has late vitellogenic oocytes, the broodstock are ready to be induced to spawn.

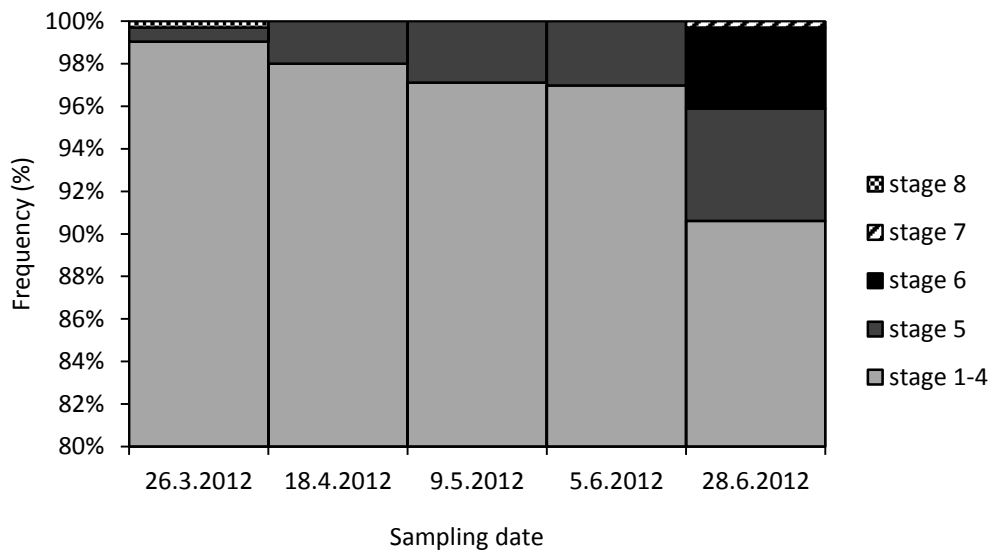


Figure 2.9: Change in oocyte composition of the ovaries during the compressed photothermal cycle. The four stages of previtellogenic oocytes were grouped. Broodstock were induced to spawn when stage 6 oocytes were present.

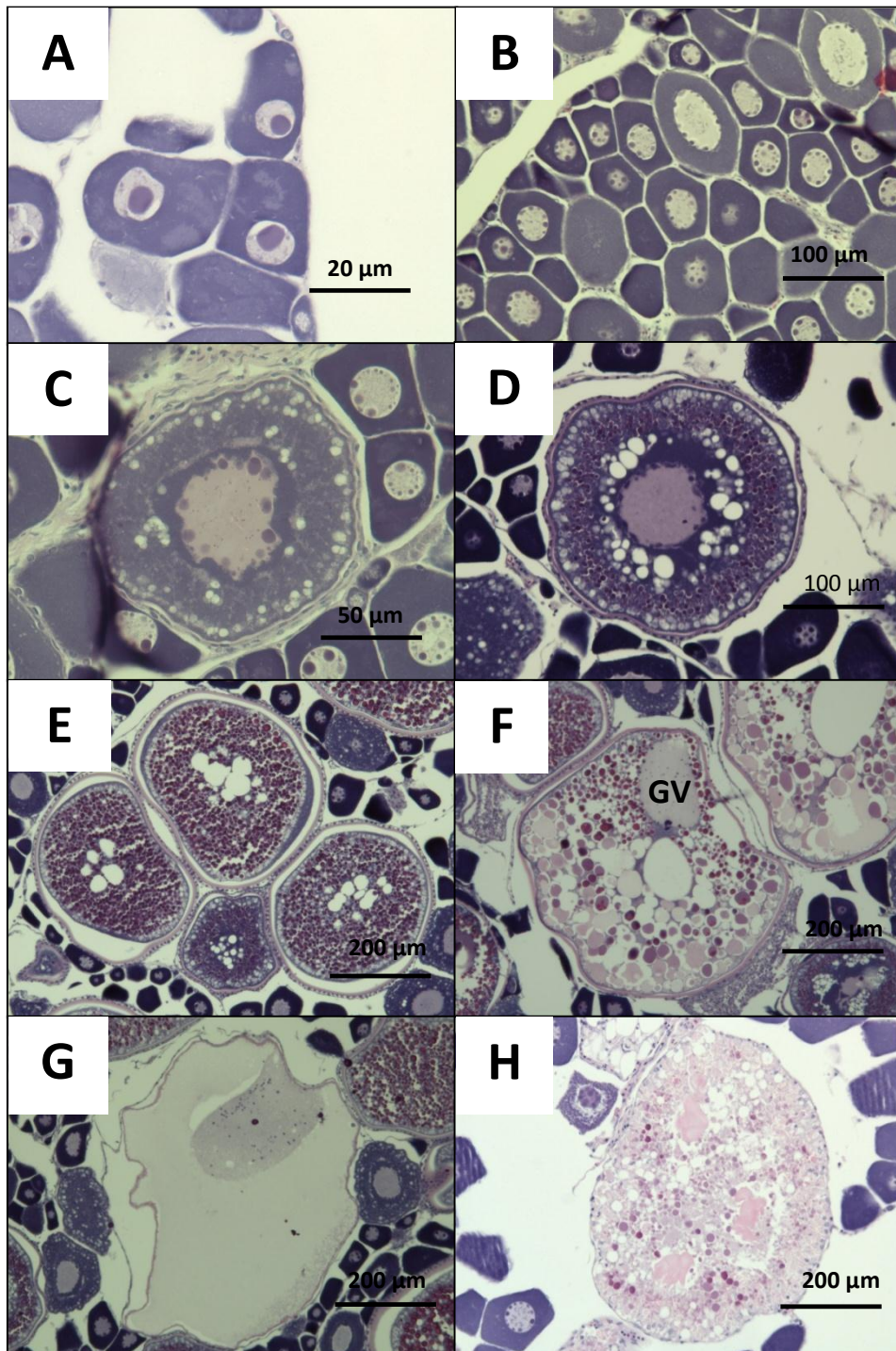


Figure 2.10: Stages of ovarian maturation in *Rhabdosargus globiceps*. A-Stage 1 (Chromatin nucleolar) oocytes with large nucleus. B-Immature ovary collected in May showing primary growth. 2 Perinuclear (stage 2) and Lipidic (stage 3) oocytes are present. C-Cortical granule oocytes (stage 4) with cortical granules and lipid droplets in the ooplasm. D-Early vitellogenic oocyte (stage 5). E-Late vitellogenic oocytes (stage 6). F-Stage 7 oocyte undergoing final maturation. Shows germinal vesicle (GV) movement to the periphery. G-Hydrated oocyte. H-Atretic oocyte, which are typical of *Rhabdosargus globiceps* ovaries collected just after spawning season. Magnification; A-100X, B and D-20X, C-40X, E to H-10X.

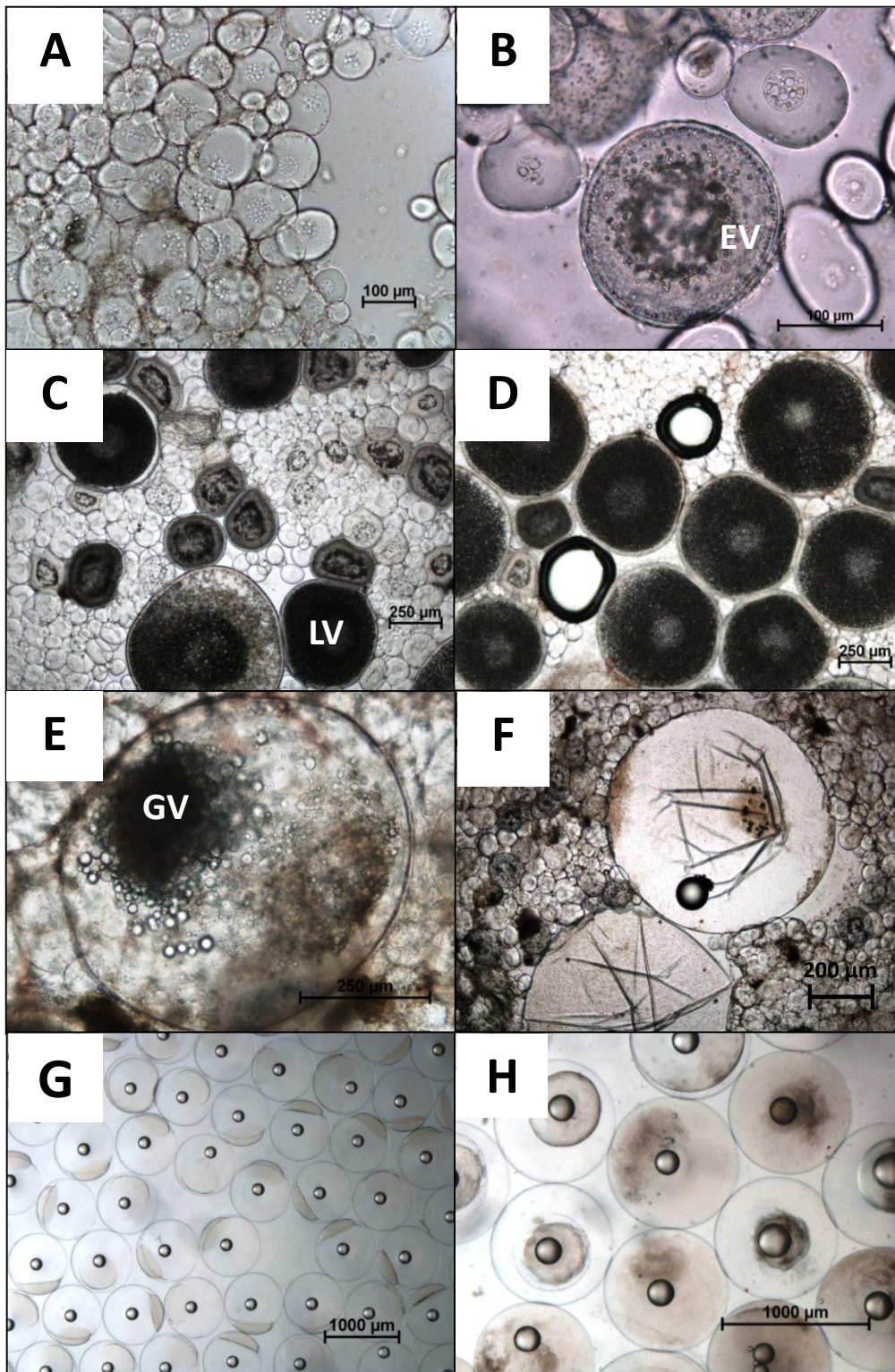


Figure 2.11: Stages of ovary and oocyte maturation shown from biopsy images. A–Previtellogenic ovary containing oocyte stages 1 to 4. B–Early vitellogenic oocyte. (EV). C–Ovary at the exogenous vitellogenic stage, containing oocytes of stage 1 to 6. Stage 6 oocytes are late vitellogenic (LV) and are large and black in colour. D–Proliferation of the stage 6 oocytes shows that the ovary was at the second stage of Exogenous vitellogenesis. E–Oocyte at stage 7 undergoing final maturation. Germinal vesicle (GV) moving to the periphery. F–Overripe hydrated oocytes, stage 8. G–Good quality, fertilised eggs. H–Low quality eggs.

Estradiol 17 β concentration

Under the compressed photothermal cycle, the mean concentration of estradiol 17 β increased for each broodstock tank (Figure 2.12). A significant increase ($P < 0.05$; $R^2 = 0.61$) in mean estradiol 17 β concentration were recorded at the last sampling date, with a mean concentration of 146.5 ± 23.9 pg ml $^{-1}$ (min-21.3 pg ml $^{-1}$; max-261.72 pg ml $^{-1}$) ($n=13$). At this time the highest concentration for an individual fish was recorded at 261.72 pg ml $^{-1}$. The lowest mean concentration was measured at the third sampling date with a concentration of 13.95 ± 2.42 pg ml $^{-1}$ (min-0 pg ml $^{-1}$; max-23.94 pg ml $^{-1}$) ($n=14$). This sampling date had the lowest mean estradiol 17 β concentration, but the two previous samples had very similar mean concentrations of 17.66 ± 1.98 pg ml $^{-1}$ (min-7.58 pg ml $^{-1}$; max-29.84 pg ml $^{-1}$) ($n=12$) and 15.26 ± 1.74 pg ml $^{-1}$ (min-7.84 pg ml $^{-1}$; max-23.8 pg ml $^{-1}$) ($n=14$), respectively.

The only significant difference between the mean estradiol 17 β concentrations came at the last sampling date for both Tank 2 and Tank 4 ($P < 0.05$; $F=76.4$), which showed that the concentration from this sample was significantly different to all previous samples. Although Tank 3 had a large increase in mean estradiol 17 β concentration at the last sampling date it was calculated as not being significantly different to the previous concentrations ($P > 0.05$).

The four female broodstock that were selected to be induced to spawn showed a constant estradiol concentration over the first three sampling dates, with an increase in concentration on the second last sampling date (Figure 2.13). The last sampling date saw the concentrations peak, with estradiol 17 β concentrations of 132.32 pg ml $^{-1}$, 187.5 pg ml $^{-1}$, 160.16 pg ml $^{-1}$ and 195.31 pg ml $^{-1}$ for the four individual female broodstock respectively.

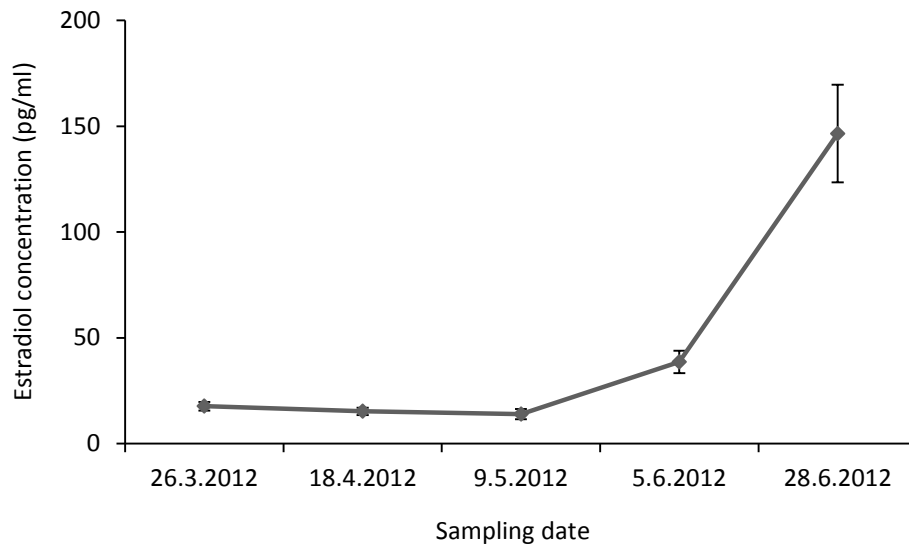


Figure 2.12: Mean (\pm SE) estradiol 17β concentration (pg ml^{-1}) over the duration of the compressed photothermal cycle.

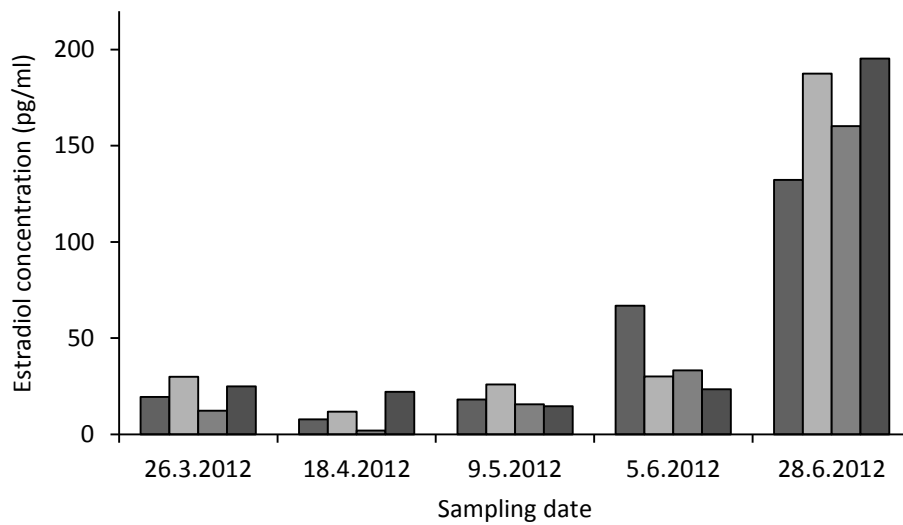


Figure 2.13: Estradiol 17β concentration (pg ml^{-1}) over time for the four female broodstock that were selected to be induced to spawn.

Estradiol 17 β concentration vs oocyte diameter

With the increase in estradiol 17 β concentration there was an increase in mean oocyte diameter (Figure 2.14). The positive correlation between estradiol 17 β concentration and mean oocyte diameter was significant ($R^2=0.55$, $P<0.05$). The highest estradiol 17 β concentrations was recorded with the appearance of the largest, most mature oocytes. The largest mean oocyte diameter recorded was 248 μm with a corresponding estradiol 17 β concentration of 187.5 pg m^{-1} . The mean oocyte diameter increased substantially when the estradiol 17 β concentration increased above 125 pg m^{-1} .

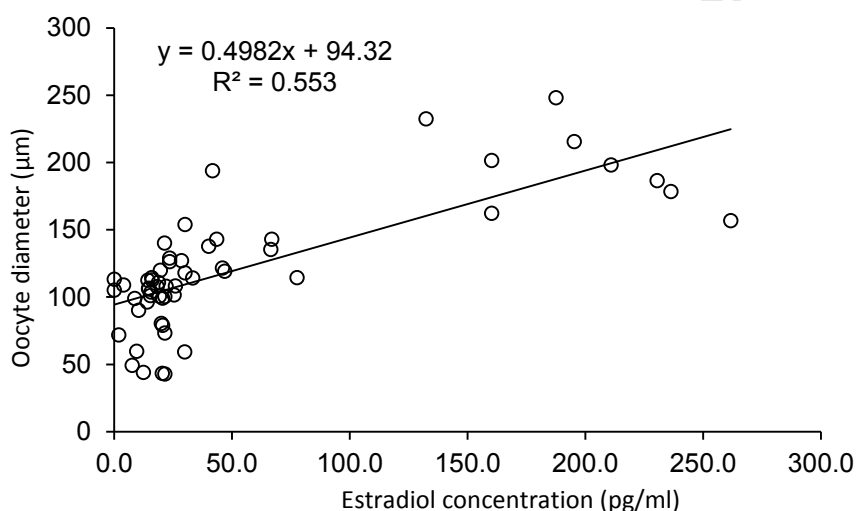


Figure 2.14: The relationship between the estradiol 17 β concentration and the mean oocyte diameter in the sampled fish over the compressed photothermal cycle. A regression analysis shows that the oocyte diameter increases with the increase in estradiol concentration and this is significant ($R^2=0.55$, $P<0.05$).

Quantity and quality of eggs

The total number of eggs and number of fertilised eggs in each batch increased for the first 72 hours of spawning (Figure 2.15). There was a sharp decrease in total egg numbers after the fourth 24 hour cycle. The highest number of spawned eggs was recorded after the third 24 hour cycle ($n=89200$ eggs). This batch had the greatest number of fertilised eggs (88000) and the lowest number of unfertilised eggs (1200). The fourth 24 hour cycle had the lowest

number of eggs. In total 281600 eggs were spawned over the sampling period with 24000 being unfertilised.

The egg quality of the fertilised eggs increased over the first 72 hours of spawning (Figure 2.16). The best egg quality was recorded in the third 24 hour cycle (93.1%). The lowest egg quality was recorded after the first 24 hour period (77.6%).

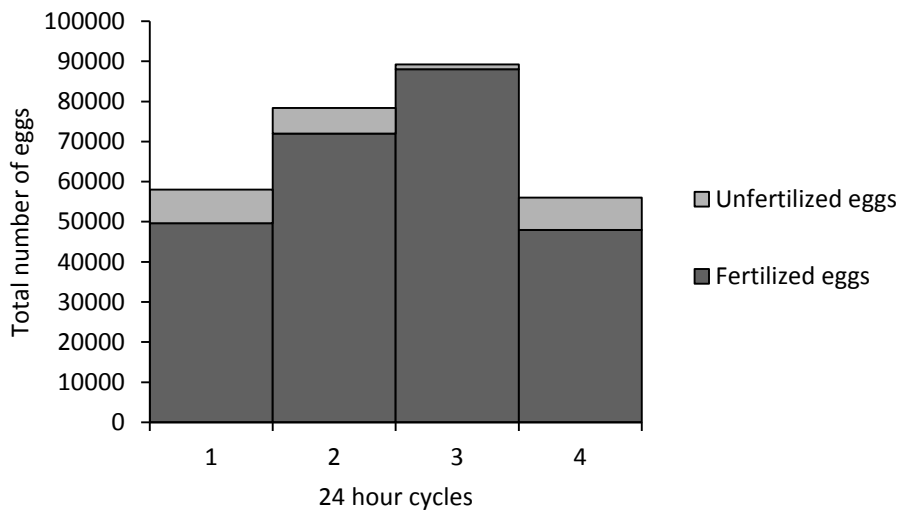


Figure 2.15: Total number of fertilised and unfertilised eggs collected at the four 24 hour spawning cycles.

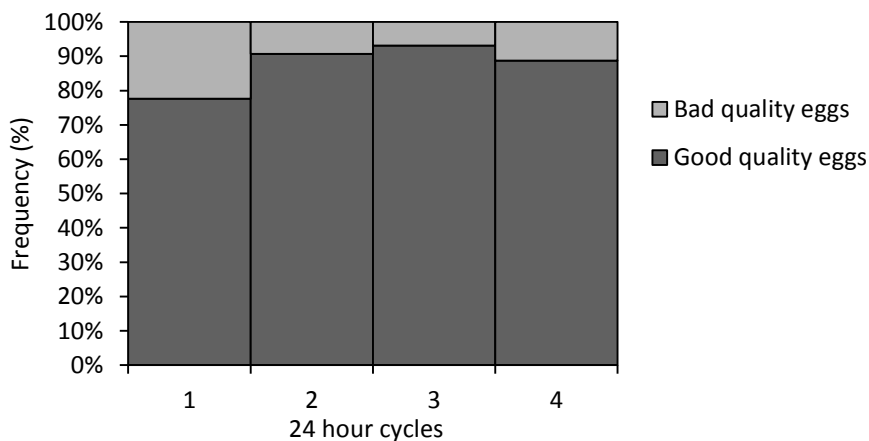


Figure 2.16: Frequency of the quality of eggs from the subsamples taken from the fertilised eggs after the four 24 hour spawning cycles.

2.4. DISCUSSION

For an environmental cue to control or phase reproductive development it must fit three criteria. It must be physiologically detectable, temporally predictable, and it must generate endocrine changes that control reproductive development of the fish (Pankhurst & Porter 2003).

The present research displays the effect of the seasonal as well as artificial effects of the change in temperature and photoperiod on the reproductive development of female *Rhabdosargus globiceps*. The effect has been described using the parameters of oocyte development and growth, and the change in estradiol 17 β concentration. Through the change in oocyte diameter and oocyte stage the effects of the natural and compressed photothermal cycles can be compared. The ability to display the estradiol 17 β concentration over the compressed cycle not only gives a more detailed account of the changes that occur in the *Rhabdosargus globiceps* endocrine system during ovary maturation, but also shows the success of the conditioning protocol to manipulate the reproductive development.

Rhabdosargus globiceps have been classed as an asynchronous spawner that has peaks in spawning and frequency of ripe ovaries in October and February (Attwood *et al.* 2010). It is at this time during the sampling period in Langebaan Lagoon that the largest oocytes, at the most mature stage (stage 6 and 7) were found, confirming the peak in spawning activity. The environmental conditions at this time are the most favourable. The female *Rhabdosargus globiceps* produces and releases multiple batches of hydrated eggs during the spawning period, which lasts from August to March (Murua & Saborido-Rey 2003; Attwood *et al.* 2010).

Comparable data concerning the development of the oocytes were gained during the conditioning period in which a compressed photothermal cycle was used to manipulate the reproductive maturation of the broodstock. This conditioning period allowed for the fish to mature within three months and some fish spawned out of season. This rapid development displays the effect of the photothermal cycle as a cue that not only initiates the timing of oocyte development, but also the rate of their development. This same effect of a compressed photothermal cycle was found on female striped trumpeter, *Latris lineata* (Forster, 1801) (Morehead *et al.* 2000). Being an asynchronous species the ovaries contain a

mixture of different stage oocytes, and before a batch of mature eggs is released this mixture is at its most diverse (Murua & Saborido-Rey 2003). The microscopic analysis of the histology and the biopsies showed the effects of batch spawning on the development of the oocytes.

Being able to accurately assess the maturation of the ovaries is a necessity for hormone-induced spawning to be a success (Mylonas *et al.* 2010). Hormone treatments administered to broodstock that are not reproductively mature will be ineffective or have an inefficient effect on maturation (Zohar & Mylonas 2001; Mylonas *et al.* 2010). The oocyte diameter and stage of maturation of the oocytes are used to determine ovarian maturation (Garcia 1989). In order to know whether the broodstock under the compressed photothermal cycle were maturing, the mean oocyte diameter and the composition of the biopsy, in terms of different oocyte stages, were compared to the oocyte diameter and the oocyte stages found in the fish sampled from Langebaan Lagoon under the natural cycle. This was done to determine the stage of ovary maturation.

Under the natural cycle vitellogenesis began in July when the first early vitellogenic (stage 5) oocytes were present. At the start of the natural spawning season in August the first late vitellogenic and final maturation stage oocytes were present. At this time the mean oocyte diameter was 187.1 μm , increasing to 236.28 μm at the peak of mature oocyte frequency in October. This showed that female *Rhabdosargus globiceps* entering the spawning season had a mean oocyte diameter of 187.1 μm or larger. Stage 6 and 7 oocytes appeared at the time of spawning for both the natural and compressed cycles. However, they were present at a lower temperature and photoperiod under the natural cycle (13.3°C, 12 hours) than under the compressed cycle (17.5°C, 15 hours). At the last sampling date under the compressed photothermal cycle early (stage 5), late vitellogenic (stage 6) and final maturation stage (stage 7) oocytes were present, even though they were produced at a far lower proportion of all oocytes. The mean oocyte diameter from this sampling date for Tank 2, 3 and 4 was 196.77 μm , 187.65 μm and 162.62 μm respectively, with the broodstock in Tank 2 having the greatest number of late vitellogenic oocytes. Both cycles showed the gradual increase in oocyte diameter as the ovaries matured, however under the compressed

cycle the oocytes showed the greatest increase in mean size between the second last and last sampling date.

The only parameters that were changed during the conditioning period were temperature and photoperiod. These environmental data from winter to summer in Langebaan Lagoon were used to bring the broodstock to reproductive maturity. These acted as the trigger for the female broodstock to start producing estradiol 17 β (Prat *et al.* 1999). The increase in concentration of estradiol 17 β then served as the trigger directly on the gonad to allow the fish to mature, leading to the growth in oocytes and the presence of early and late vitellogenic oocytes at the last sampling date (Mylonas & Zohar 2007).

The peak in estradiol 17 β on the last sampling date, three months after the conditioning period started, is directly correlated to the increase in oocyte diameter and oocyte maturity (Prat *et al.* 1999). Estradiol 17 β concentration does not increase gradually, but rather with a rapid increase (Morehead *et al.* 2000). A high concentration of estradiol 17 β was present as late vitellogenic oocytes appeared in the ovary (Prat *et al.* 1999). Fish species that spawn many times per season had high levels of estradiol 17 β before and during spawning (Mañanós *et al.* 1997). This advance in reproductive maturation under the shortened cycle is brought about when the brain is stimulated by external factors such as temperature and photoperiod. The ovaries produce estrogen, with estradiol 17 β as its main component (Mylonas *et al.* 2010). The combined presence of these three developmental factors displays the endogenous rhythm and shows that the manipulation of oocyte development and maturation was successful. Other studies on *Dicentrarchus labrax* have demonstrated that short conditioning periods advance spawning whereas long conditioning periods delay the onset of maturation (Zanuy *et al.* 1986; Mañanós *et al.* 1997; Prat *et al.* 1999). However, the long term effects on the productivity of the broodstock are not well studied.

Following the procedure described by Moretti *et al.* (1999) the most mature female broodstock, those with the highest frequency of late vitellogenic oocytes and with an average oocyte diameter of >200 μm , were placed into the same tank as the four males, which were spermiating. Only four of the fifteen females were shown to be at optimum maturity for induction, according to their oocyte diameter and stage. If the oocytes have not

reached the late vitellogenic stage the broodstock will not spawn when induced (Moretti *et al.* 1999).

All selected broodstock were induced to spawn with LH-RHa injections. This is the hormone secreted by the pituitary gland at the peak time of spawning, which initiates the final stage of ovary maturation and hydration of the oocytes (Mylonas *et al.* 2010).

Although only four females were selected to be induced to spawn, the analysis of the biopsies suggest that most of the females had ovaries that contained late vitellogenic oocytes. The change in mean estradiol 17 β concentration over the sampling period showed that the majority of females had similar timing to the increase in concentration. This trend rules out stress as the factor that inhibited the other females' reproductive capacity, as the level of estradiol 17 β would have been inhibited by the increase in cortisol in the blood (Haddy & Pankhurst 1999; Schreck *et al.* 2001). The environmental conditions would also not be an inhibiting factor as they were similar for all tanks. The different timing to ovary maturation could be due to varied endogenous rhythms in the females or a slight difference in light intensity or nutrition.

Egg quality has a profound effect on the success of rearing and eventually the profit produced via aquaculture and is viewed as one of the main constraints of production (Brooks *et al.* 1997; Lahnsteiner & Patarnello 2004). The viable, fertilised eggs are easily separated from the nonviable eggs, because of their buoyancy (Kjørsvik *et al.* 2003). The eggs produced in this experiment via the induction of spawning had a high rate of fertilisation and out of these eggs the majority were of good quality. In total over the spawning period 287600 eggs were produced, with only 9.32% being unfertilised. Assessing the fertilised eggs gave a good indication of their quality and an indication whether or not the female broodstock were conditioned. The overall quality of the fertilised eggs was high within each batch having >75% proportion of good eggs. In this first induction spawning experiment the third 24 hour cycle produced the best quality eggs.

Broodstock management of a species has direct consequences for the quality of eggs and larvae that are produced in aquaculture (Brooks *et al.* 1997; Lee 2003). The changes that occur in the endocrine system during reproductive maturation and oogenesis, and factors

such as handling stress, water quality and feed have been noted to affect egg quality to some extent (Brooks *et al.* 1997)

Fish fed on natural diets have been known to produce eggs of a better quality than those fish fed on commercially formulated diets (Brooks *et al.* 1997). The use of both pilchard and AQUANUTRO trout pellets could have provided the broodstock with a high quality of nutrition allowing for sufficient growth in abdominal fat. Both lipids and fat reserves have been shown to be used for vitellogenesis during egg production in both *Rhabdosargus globiceps* and *Pagrus pagrus* (Aristizabal 2007; Attwood *et al.* 2010). Aristizabal (2007) also states that dietary fat provides a crucial energy resource. Sufficient fat both from reserves and diet could be seen as a major factor that allowed the broodstock to produce viable eggs over such a short conditioning period. More research needs to be carried out on the effect of diet and fat reserves on spawning of captive *Rhabdosargus globiceps*.

The development that took place during the natural cycle served as a baseline for comparison to the development recorded during the compressed photothermal cycle. With these data, successful reproductive maturation under the compressed cycle could be examined. The presence of oocytes at late vitellogenic and final maturation stages and mean oocyte diameter of 187.1 μm or greater in the biopsies indicated that the compressed photothermal cycle had the same effect as the natural annual cycle. This comparison provided evidence for the successful reproductive maturation of *Rhabdosargus globiceps* broodstock under an artificial, compressed photothermal cycle. Further evidence of this was the estradiol 17 β concentration that strongly correlated to the increase in oocyte diameter and presence of mature oocytes. The combined results of the correct oocyte development, the estradiol 17 β and the high quality of fertilised eggs suggest that the conditioning and induction of spawning of *Rhabdosargus globiceps* was successful. However, whether this can be achieved repeatedly and on a greater scale needs to be assessed.

CHAPTER 3

First assessment of the early larval development and growth of the white sturgeon, *Rhabdosargus globiceps* (Teleostei: Sparidae).

ABSTRACT

I report on the first successful rearing of *Rhabdosargus globiceps* larvae in captivity. Eggs were obtained from broodstock that were conditioned to become reproductively mature via an appropriate photothermal cycle. Spawning was then induced with injections of LH-RHa. The stages of development from egg to 30 DPH (days post hatch) larvae were recorded, showing similar developmental stages as other sea bream aquaculture species. The larvae were reared from hatching (0 DPH) to 30 DPH at 20°C. The developmental stages were noted as being highly influenced by the food sources. Timing to first feeding, swimbladder inflation and flexion was recorded at 4 DPH, 6 DPH and 14 DPH respectively. Histologically, good proliferation of the internal organs with correct development of the digestive tract and liver 3 DPH were observed. Elevated mortalities were recorded during the transitional phase between endogenous food reserves and exogenous feeding and the growth pattern showed the same reduction in growth rate present for other sea bream larvae. Growth was constant and the larvae reached a mean total length of 11.14 ± 0.44 mm at the end of 30 DPH, whilst actively feeding on rotifers and *Artemia*.

3.1. INTRODUCTION

Knowledge of the growth and development and ontogeny of a species of fish is regarded as important information, not only for the data about the embryology and larval stages, but for fishery and aquaculture applications (Koumoundouros *et al.* 1999). Information regarding the growth and development of the larvae from hatching to 30 DPH represents the initial step in understanding and determining the species' capacity and the physiological capability of the larvae for rearing in captivity. With these data the techniques for growth and development of the species can be optimised to improve larval survival and growth rates (Koumoundouros *et al.* 1999; Sanchez-Amaya *et al.* 2007). Positive results with experimental species can lead to their use in aquaculture and the diversification of species in the industry.

Rhabdosargus globiceps, is a representative of the family Sparidae and is an endemic sea bream found off the West, South and South-East coast of South Africa (Griffiths *et al.* 2002). This species is popular in the recreational and commercial fishery, but unfortunately stocks have been heavily exploited in most areas of previous abundance (Griffiths 2000). Sea bream aquaculture has had great success on a global scale, yet in South Africa local sea bream species have not been considered as potential candidates for commercial aquaculture (DAFF 2011), and little is known about the early life stages of endemic sea bream species and how they behave in captivity. There has been major development with *Pagrus pagrus* and *Sparus aurata* aquaculture and new research is being carried out on biologically similar species (Hernandez-Cruz *et al.* 1999).

Fish larvae develop to adjust to the demands made by the environment. This can be described as dynamic morphology (Osse & van den Boogaart 1999). The body shape, behavior, feeding pattern and internal structures change dramatically over this time (Koumoundouros *et al.* 1999). This dynamic larval stage is recognised as the major bottleneck in the production of juveniles (Dhert *et al.* 1998; Crespo *et al.* 2001; Papandroulakis & Divanach 2002; Papandroulakis *et al.* 2004b; The Research Council of Norway, 2009). Certain key events, such as first feeding on exogenous food sources and swimbladder inflation are regarded as the most crucial during the larval phase and, if not initiated correctly, can have detrimental effects on the health of the larvae (Chatain 1989; Planas & Cunha 1999; Boglione *et al.* 2001). The rearing conditions in the tank also play a

significant role in creating the optimal environment for the correct development of the larvae (Papandroulakis & Divanach 2002; Papandroulakis *et al.* 2004a; The Research Council of Norway 2009), and there is debate around which rearing technique is best for potential new aquaculture candidate species due to the paucity of data about the optimal physical and biological parameters needed for successful larval growth and development (Divanach & Kentouri 2000; Papandroulakis *et al.* 2005).

The morphological developments of the larvae coincide with the physical actions that the larvae are capable of. Larvae have specific energy requirements and only when these are met can the larvae grow and develop successfully (Dhert *et al.* 1998; Verhaegen *et al.* 2007; The Research Council of Norway 2009). This drives the process of growth and differentiation of the larvae as they develop into juveniles (Koumoundouros *et al.* 1999). Larval development can be separated into distinct stages according to the morphological developments and the source of their food (Sarasquete *et al.* 1995; Elbal *et al.* 2004; Sanchez-Amaya *et al.* 2007).

The aim of this study was to determine and describe the early growth pattern and critical developmental stages of the *Rhabdosargus globiceps* larvae over the first 30 days post hatch. This period includes the physical changes of swimbladder inflation, notochord flexion and metamorphosis, as well as the critical phase of transition of the larvae from using endogenous food reserves to actively preying on exogenous food sources (Conides & Glamuzina 2001). The hypothesis regarding the ontogeny of the embryo and larvae was that growth and development would be very similar to that of other sea bream species, showing similar timing to hatching, first feeding, swimbladder inflation and notochord flexion. The null hypothesis was that the ontogeny of the larvae would not be similar to other sea bream species. This information considering the key developmental milestones will also allow for the comparison with other successfully cultured sea bream species or the development of *Rhabdosargus globiceps* larvae under different conditions. Good results with regard to growth and development of the *Rhabdosargus globiceps* larvae would suggest that the methods and techniques used to rear this species were appropriate. This research describes for the first time the morphological growth and development of *Rhabdosargus globiceps* larvae for the first month of their life.

3.2. METHODS

The experiment was conducted at the Department of Agriculture, Forestry and Fisheries (DAFF) Aquaculture Research Facility, in Seapoint, Cape Town, South Africa (33°55′14.27″S, 18°22′50.75″E).

Spawning of broodstock

Adult *Rhabdosargus globiceps*, three females and one male, were successfully spawned on the 24th August 2011. This was achieved with the manipulation of temperature and photoperiod over a period of 6 months, which initiated the development of gametes. Each broodstock tank system was set to increase to a higher temperature (20°C) and photoperiod (16 hours) at the peak of reproductive maturity. The cycles of temperature and photoperiod in captivity followed the natural cycles of temperature and photoperiod from Langebaan Lagoon from winter to summer. The females were not biopsied to check maturity, but the males were checked if they were spermiating. Two intramuscular hormone injections (1 µg per kilogram body mass), of LH-RHa (Luteinising hormone – Releasing hormone, D-Ala⁶-des-Gly¹⁰LHRH ethylamide, Syndel International INC.) were given to the fish with a 24 hour interval to initiate the final stage of egg maturation, full hydration and release (Moretti *et al.* 1999). The first injection was administered at 10 am. Approximately 48 hours after the first inducing injection the first batch of eggs was present in the tank. Spawning continued for 3 consecutive days with batches of eggs being produced throughout the day and night.

Egg collection

The eggs obtained from the induced spawning of the broodstock were collected in a 500 µm mesh net that covered the overflow from the surface of the tank. The eggs were then disinfected in a 10 L mixture of 0.1% formalin and 2 µm filtered sterile seawater for 5 minutes. This was expected to kill any bacteria and fungi present in the tank environment (Moretti *et al.* 1999). The eggs were then placed in 1 L beakers and left for 10 minutes. They separated into two distinct layers. Buoyant fertilised eggs floated to the top and dead, unfertilised eggs settled on the bottom of the beaker. The number of both the buoyant eggs and the non-buoyant eggs were estimated by taking a 0.5 ml sample, counting the eggs

present in this sample, and then multiplying it by the total volume they occupied. On average there were 390 eggs per 0.5 ml sample. Twenty fertilised eggs were examined. Their appearance was recorded and their mean diameter was measured to the nearest 0.01 μm using a Nikon Digital Sight Fi 1 digital camera and the NIS elements basic research software.

Incubation of eggs

For the incubation period the eggs were placed inside a 7.5 L container with a mesh bottom and port-holes, which was already floating in a 450 L circular tank (Figure 3.1A). The mesh bottom and port-holes allowed water flow into the container. The water was kept at the spawning temperature of 20°C. The eggs were stocked at the same density that was needed for the 450 L tank. The ideal density is 100-200 eggs L^{-1} for the intensive rearing of sea bream (Moretti *et al.* 1999; Divanach & Kentouri, 2000), but because of the small number of eggs received from the broodstock, the density was 67 eggs L^{-1} .

An air supply was placed in the centre of the container with a light flow when the eggs were still in the early stages of development. This allowed them to be kept in suspension. The air supply was turned off when the larvae were fully visible in the eggs. Once the larvae had hatched they were carefully released into the larger tank and the dead eggs and egg debris were removed with the container (Figure 3.1B). The day of hatching was noted as 0 DPH (days post hatch).

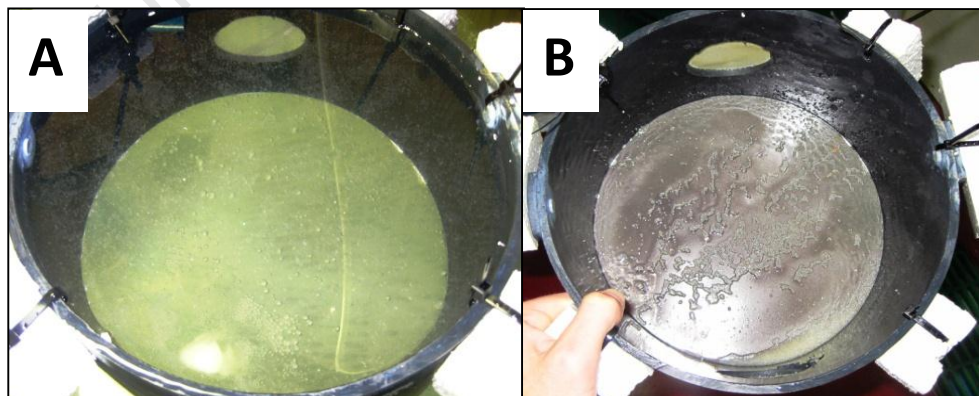


Figure 3.1: A - Photograph of the 7.5 l container in which the eggs were incubated. The eggs are visible on the surface of the water. B - The dead/unhatched eggs were removed after releasing the hatched larvae.

Larval rearing and feeding

An intensive rearing method was used for the *Rhabdosargus globiceps* larvae. The environmental conditions in the 450 L tank were kept as stable as possible. The temperature in the tank was kept at $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and the photoperiod was kept at 15 hours light, 9 hours dark. Light was provided by fluorescent lamps, which were positioned directly above the rearing tank. The light intensity at the water surface was 1000 to 1200 lux. Salinity varied between 33.8 and 35.2‰. There was no water turnover for the first 3 days. Water turnover began on the 3 DPH (80-100% daily). The tank was set up on an open system and the water emptied out the tank via a banjo-sieve fitted with 250 μm mesh. When the larvae grew large enough (14 DPH) the 250 μm mesh banjo sieve was replaced with a 500 μm mesh banjo sieve for the night.

During the day the turnover of the tank was kept very low, while at night it was increased to have one full turnover. This would allow the larvae to feed in the day without being impeded by the current. The water turnover was increased as the larvae grew older. From 20 DPH to 30 DPH the water turnover was at two total turnovers daily. A polystyrene surface skimmer was placed in the tank at 7 DPH, but with the use of a banjo-sieve that emptied the tank via the surface, and having a very low larvae density, no oily-layer accumulated to be detrimental to the larvae's health. This allowed the larvae to successfully initiate swimbladder inflation.

The 'pseudo-green water' technique was used during the larval rearing period (Papandroulakis & Divanach 2002; Papandroulakis *et al.* 2002). The biological performance of sea bream has been shown to be improved by green water conditions *Nannochloropsis oculata* (Hibberd, 1981) was added to the rearing tank once in the morning and once in the afternoon to keep the algal concentration at approximately $6\text{-}12 \times 10^6$ cells per ml. The microalgae was added to the rearing tank to help to control the water quality, and to become supplementary food for the rotifers in circulation (Yoshimatsu *et al.* 1995; Planas & Cunha 1999; Hernandez-Cruz *et al.* 1999; Mihelakakis *et al.* 2001). On 4 DPH (mouth opening) the larvae were fed rotifers, *Brachionus plicatilis*, which had been enriched with the unicellular algae, *Nannochloropsis oculata* (Table 3.1).

The rotifers were added twice daily (9am and 3pm) to the rearing tank to keep the density at 10 individuals ml⁻¹. Rotifers were fed to the larvae from 4 DPH to 25 DPH. Instar I *Artemia* nauplii were fed to the larvae from 17 DPH to 60 DPH when their mouths had grown large enough to ingest them. Instar II *Artemia* nauplii were added from 20 DPH. The concentration of *Artemia* was 3-5 individuals ml⁻¹. They were fed to the larvae once in the morning. In the afternoon the remaining numbers were assessed and an extra batch was added if the concentration was below what was recommended. The *Artemia* used in this experiment were not enriched. The number of *Artemia* added to the rearing tanks was increased over the rearing period to maintain optimal feeding conditions. From 27 DPH a fine particle artificial feed was added. All feed and phytoplankton was added by hand to the rearing tank (Table 3.1).

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Table 3.1: Feeding scheme for *Rhabdosargus globiceps* larvae. The concentrations listed below are used for larval rearing in a 400 litre tank with a larval density of 100-200 individuals per tank. Estimations of the number of rotifers and *Artemia* in the rearing tank were calculated before addition to the tank.

400 litre tank						
Age (Days Post Hatching)	Photoperiod (hours)	Rotifer concentration (ml ⁻¹)	Algal concentration (ml ⁻¹)	<i>Artemia</i> nauplii - Instar I (thousands)	<i>Artemia</i> nauplii – Instar II (thousands)	Larval feed: 80-200 um (g)
0-3	15					
4-16	15	5-10	12x10 ⁶			
17-19	15	5-10	6x10 ⁶	40-100		
20-23	15	5-7	6x10 ⁶	200-400	120-20	
24-30	15	1-3	6x10 ⁶	400-600	400 - 1 million	1-4

Observations, sampling and measurement

To assess the development of the eggs before hatching a batch of 48 eggs was divided among four well-plates and twelve single wells and placed in an incubator at 20°C. The development of the eggs was assessed hourly for the first 24 hours, then every 6 hours, until the eggs hatched. The timing to the different cell divisions was noted as well as the developmental state of the larvae.

To assess the development of the larvae, twenty individuals were randomly sampled using a beaker and a pipette every 24 hours at 12pm starting at the time of hatching (0 DPH). These were photographed under a microscope (Nikon SMZ1500, Lense HR Plan Apo (1X) WD 54) using the imaging software NIS Elements Basic Research 3.2. The following measurements were taken: total length (*TL*), from the furthest point of the lower jaw to the posterior margin of the caudal fin; both the yolk-sac axes (*L* - major length, *H* - minor length); oil globule diameter (*d*); body height (*BH*), from the anus to the upper limit of the myomere excluding the dorsal fin; and eye diameter (*ED*) (Mihelakakis *et al.* 2001). By examining the photographs the percentage frequency of repletion rate (food in gut), flexion and swimbladder inflation were also recorded.

Measurements were all recorded to the nearest 0.01 mm and they were all performed on live specimens. Illustrations were done of the larvae to show the detail of development from hatching to 30 DPH. The photomicrographs of the larvae were enlarged and the body details were traced. These were then scanned into a computer and minimised to the preferred size.

Mortality was not measured as it is very difficult to count the larvae. Their bodies are extremely fragile and decompose very quickly. It was found that using a syphon to extract the dead larvae was detrimental to the living larvae.

Using the equations suggested by Blaxter and Hempel (1966) and Cetta and Capuzzo (1982), the yolk-sac volume (*VYS*) and the oil globule volume (*VOG*) were calculated to the nearest cubic millimeter.

To find the volume of the yolk-sac (VYS), both the lengths of the axes of the spheroid yolk-sac were measured and used into equation 1:

$$VYS = 4/3\pi(L/2)(H/2)^2 \quad \text{Equation 1}$$

To find the volume of the oil globule (VOG), the diameter was measured and used into equation 2:

$$VOG = 4/3\pi(d/2)^2 \quad \text{Equation 2}$$

Specific growth rate (SGR) was calculated using equation 3:

$$SGR (\%) = 100 \times \left(\frac{\ln TL_2 - \ln TL_1}{\text{time}} \right) \quad \text{Equation 3}$$

where TL_1 and TL_2 indicate the initial and final total length (mm), respectively (Koumoundouros *et al.* 1999; Biswas *et al.* 2005). Time refers to the number of days between TL_1 and TL_2 .

Histological examination

Fifty larvae were sampled every 2nd day for the 30-day period. They were fixed in 10% buffered formalin. The samples were sectioned at 5 μ m and stained with haematoxylin and eosin. Samples were analysed using the 20X, 40X and 100X objectives of the microscope (Olympus BX51). The small size and the fragility of the larvae at the early stages of the rearing period made it difficult to get suitably fixed and orientated larvae for histological analysis.

Data analysis

The statistical analysis package STATISTICA 10 was used for the statistical analyses in this chapter. Regression analyses were performed to calculate the growth equation for total

length, body height and eye diameter. Using box-and-whisker plots the development of eye diameter and body height was described. All measurements are given as the mean \pm standard error followed by the range and sample size in parentheses.

3.3. RESULTS

Description of eggs and hatching

The fertilised eggs were spherical, transparent and buoyant with the oil globule situated in the centre of the egg. They had a mean diameter of $859.14 \pm 2.65 \mu\text{m}$ (min-828.79 μm ; max-881.78 μm) ($n=20$). The oil globule inside the buoyant eggs had a mean diameter of $156.79 \pm 0.98 \mu\text{m}$ (min-148.72 μm ; max-166.81 μm) ($n=20$). At the time of collection the batch of eggs were composed of different developmental stages. The development stage with the highest frequency in the batch was noted as the developmental stage for that specific time.

Four stages of egg development were recorded: the cleavage stage, blastula stage, gastrula stage and the embryo stage. The change from 1 cell to morula stage took 3 hours at 20°C. The longest time for a single stage was 6 hours between the first appearance of the gastrula stage and the first appearance of the embryo. It took 22 hours for the embryo to mature to the hatching stage, going through the stages of formation of optic vesicles, formation of the rudimentary heart, elongation of the tail and movement of the embryo. When the larvae were ready to hatch they moved inside the egg with full movement of the tail. The yolk-sac and oil globule were clearly visible.

Hatching occurred 36 hours after spawning at 20°C. At hatching the larvae could not actively move and were found in groups on the surface. Their eyes were not pigmented, the mouth and anus were closed and the digestive system had not developed.

Table 3.2: The chronological (in hours) embryonic development of the *Rhabdosargus globiceps* eggs at 20°C.

Developmental stage	Time to stage (hours after fertilisation)	Description
Cleavage stage		
1 cell Figure 3.2A	-	At the time of collection, many of the eggs were at the one cell stage. The eggs that had not developed were examined. The time taken to develop to 1 cell from fertilisation was approximately 30-40 minutes. The chorion separated from the cell membrane and a thin perivitelline space was observed.
2 cell Figure 3.2B	1	The first division occurred 1 hour after fertilisation. Two cells were visible at the outer edge of the egg. This stage lasted for an hour before the first eggs with four cells were noticed.
4 cell Figure 3.2C	2	Two hours after fertilisation the second division occurred and four cells were visible.
8 cell Figure 3.2D	2	The third division occurred shortly after the second. Eggs containing eight cells were apparent after 2 hours.
16 cell Figure 3.2E	3	Three hours after fertilisation sixteen cells formed.
32 cell Figure 3.2F	3	Within the third hour after fertilisation the fifth division also took place.
Morula stage Figure 3.2G	3	The first eggs at the morula stage were apparent at 3 hours after fertilisation. This stage was characterised by a ball of cells, greater than 32 cells, at the back of the egg.
Blastula stage Figure 3.2H	3.5	The blastula was apparent at three and a half hours after fertilisation
Gastrula stage Figure 3.2I	8	The gastrula appeared eight hours after fertilisation. The stage is distinguished by a half circle inside the egg.
Embryo stage		The embryo development started when the embryo was visible for the first time. The embryo went through changes, while organogenesis occurred. At 20°C the embryo stage lasted 22 hours and hatching occurred at 36 hours.

Appearance of embryo Figure 3.2J	14	Fourteen hours after fertilisation the embryo was distinguishable. A faint elongated shape had formed behind the oil globule.
Formation of Optic vesicles Figure 3.2K	15	Fifteen hours after fertilisation the optic vesicles were visible. These were positioned at the ends of the 'T-shape' that the embryo had taken and will later become the eyes.
Formation of rudimentary heart Figure 3.2L	18	The rudimentary heart was visible and beating eighteen hours after fertilisation. It was visible just below the head of the embryo in the coelomic cavity (Sanchez-Amaya et al. 2007).
Elongation of the tail Figure 3.2M	24	Twenty four hours after fertilisation the tail became elongated and met the oil globule. Pigmentation of the embryo and oil globule was visible.
Movement of the embryo Figure 3.2N	30	The embryo made its first movements thirty hours after fertilisation. This was characterised by bending motions of the entire body. The pigmentation of the embryo and the oil globule increased and more xanthophores and melanophores were visible (Radonic et al. 2005).
Ready for hatching Figure 3.2O	36	Thirty six hours after fertilisation the embryo was ready to hatch. The embryo was ready to hatch when it began to move more aggressively and frequently. The oil globule was attached to the middle area of the embryo where it linked to the digestive tract. The yolk-sac was also visible when the embryo moved.
Hatching Figure 3.2P	36	Within the same hour of the embryos becoming ready to hatch the first larvae hatched. The large yolk-sac and oil globule started to be used as soon as the larvae hatch, as the mouth and anus are not open and the larvae cannot feed.

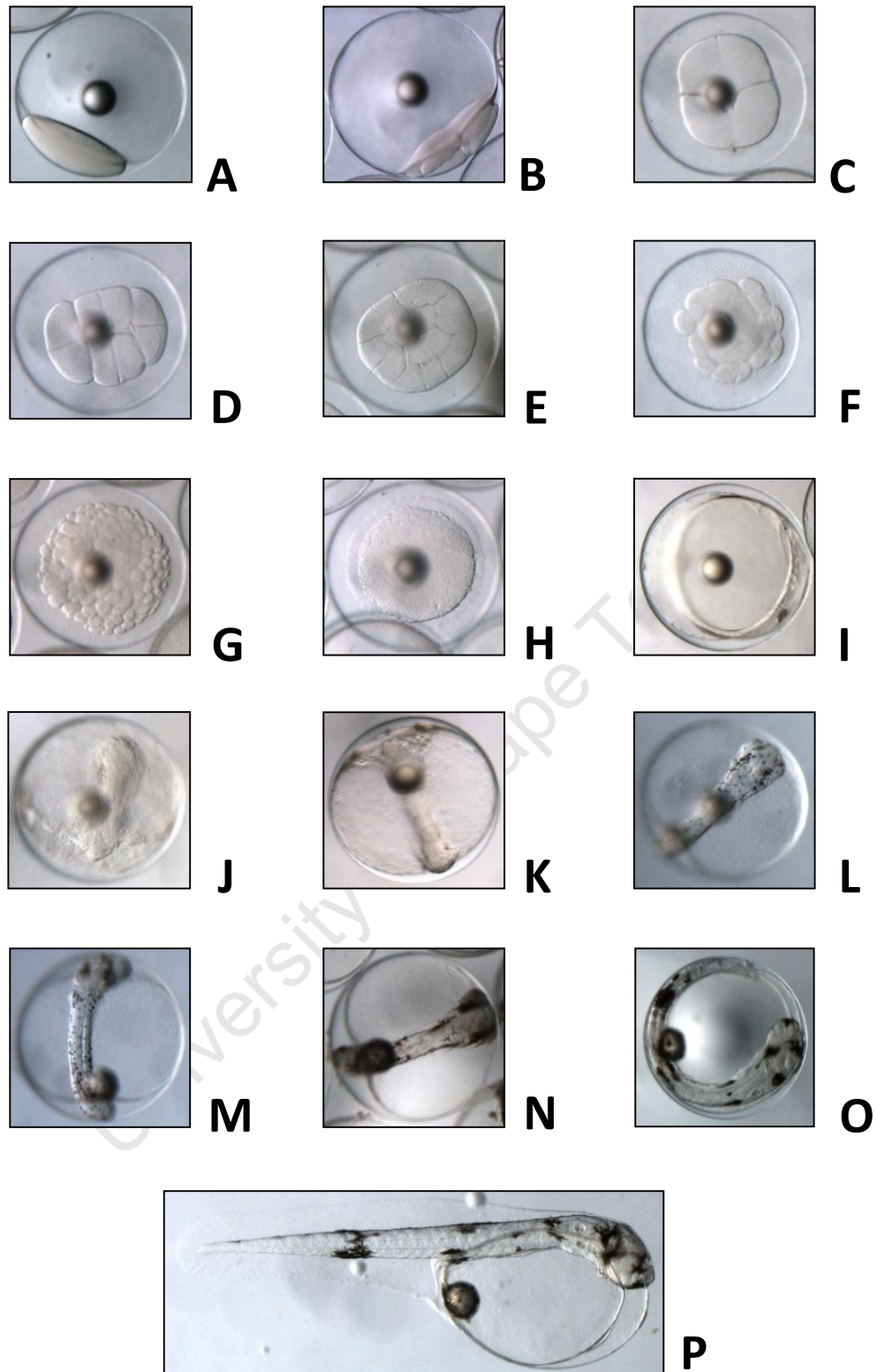


Figure 3.2: Photomicrographs of the embryonic development of *Rhabdosargus globiceps* at 20°C. A-1 cell; B-2 cells; C-4 cells; D-8 cells; E-16 cells; F-32 cells; G-Morula; H-Blastula; I-Gastrula; J- Appearance of embryo; K-Formation of Optic vesicles; L- Formation of the heart; M- Elongation of the tail; N-Movement of the embryo; O-Ready to hatch; P-Hatched larva.

General morphology and organ development

Stage 1 (0-3 DPH)

At hatching the larvae were 2.278 ± 0.022 mm (min-2.065 mm; max-2.395 mm) ($n=20$) in total length and lacked eye pigmentation, a mouth and anus, and a functional digestive system (Figure 3.3A). The body of the larvae was surrounded by a primordial finfold (Santamaría *et al.* 2004; Conides & Glamuzina 2001). The yolk-sac and the oil globule had mean diameters of 792.91 ± 3.79 μ m (min-754.25 μ m; max-822.17) ($n=20$) and 157.48 ± 0.831 μ m (min-150.62 μ m; max-166.08 μ m) ($n=20$), respectively. The yolk-sac in sea bream species has been described as being homogeneously acidophilic (Sarasquete *et al.* 1995). At 3 DPH the yolk-sac had been depleted, the oil globule had been greatly reduced, the larvae eyes were pigmented and a simple digestive tract had developed as a single long tube (Table 3.3).

Stage 2 (4-9 DPH)

At 4 DPH the mouth and anus opened, and stomach folds appeared, as the larvae were actively feeding on exogenous food sources (Figure 3.5B, 3.6, 3.11A). Over this time the digestive tract developed into a functional system and was comprised of the oesophagus, stomach and intestine. Lipid cells were visible in the liver and the folds of the digestive tract (stomach and intestine) were also visible (Crespo *et al.* 2001) (Table 3.3). At 5 DPH the oil globule was depleted. The beginning of swimbladder development was visible at this stage (Figure 3.6, 3.11B). At 4 DPH none of the larvae examined had a swimbladder ($n=20$). At 9 DPH the prevalence of swimbladders had risen to 45% ($n=20$) (Figure 3.10).

Stage 3 (10 – 14 DPH)

The swimbladder in the sampled larvae was larger and more visible (Figure 3.3B, 3.4B, 3.5D). From 10 DPH to 14 DPH the average presence of a swimbladder in the examined larvae was 90% ($n=97$) (Figure 3.10). Larvae became more pigmented. The primordial fin was still present and the gill filaments were developing (Table 3.3) (Figure 3.3B). The gill's function at this early stage is the regulation of ions and acid-base balance instead of respiratory gas

exchange (Sanchez-Amaya *et al.* 2007; Rombough 2007). Flexion of the notochord was first noticed at 14 DPH (Figure 3.6, 3.10, 3.11E).

Stage 4 (15-30 DPH)

Flexion of the notochord was first recorded during this stage. This enabled the caudal fin to become functional (Figure 3.11E, F, G). The gill filaments had developed and now had a red colouration, indicating the presence of haemoglobin (Figure 3.3C). The primordial fin had been reabsorbed by 20 DPH and the dorsal, anal, pectoral and caudal fins were developing (Figure 3.3B, 3.3C, 3.4B, 3.4C). From 40 DPH the larvae started to resemble a small fish (Figure 3.3E, 3.4E).

Table 3.3: Main morphological observations during the four stages of development of the *Rhabdosargus globiceps* larvae.

Stage	Morphological observations	Days Post Hatch (DPH)	Food sources
1	Endogenous food reserves. Eyes not pigmented, until 3 DPH, and digestive system not developed. Yolk-sac fully depleted at 3 DPH.	0 – 3	Endogenous
2	First feeding on endogenous food sources. Digestive system became active and formed a loop structure. Liver is visible. Oil globule depleted at 5 DPH. First appearance of swimbladders at 6 DPH.	4 – 9	Endo/exogenous
3	High frequency of swimbladders. Larvae became more pigmented. Notochord flexion starts.	10 – 14	Exogenous
4	Flexion is more visible. Primordial finfold is resorbed. Fins have developed and larvae are more pigmented. Appearance and proliferation of organs.	15 – 30	Exogenous

Larval behaviour and reaction to rearing conditions

As with all small pelagic fish larvae there was a high rate of mortality through the early life stages. Hatching success was not quantified. From observations it was found that the highest rate of mortality was at the first feeding stage (4 DPH to 8 DPH). Visible numbers of the larvae decreased significantly at this time. It was also found that the larvae tended to move down in the water column and could settle on the bottom of the tank. This behaviour began at 5 DPH. Movement of the larvae at the surface continued as they 'gulped' air to inflate their swimbladders. At the same time the larvae showed a response to foreign bodies in the water and moved away quickly, often with one rapid movement. When the larvae were 10-20 DPH they are predominantly found in the water column, however, some were found to be feeding and swimming at the water surface at the edge of the tank. As the larvae got bigger they moved away from the edge of the tank.

Table 3.4: Major events during the growth and development of *Rhabdosargus globiceps* larvae over 30 DPH.

Event	Time	Mean Total Length (mm \pm SE)
Hatching at 20°C	36 hours after fertilisation	2.278 \pm 0.022
Eye pigmentation	3 DPH	2.901 \pm 0.022
Yolk depletion	3 DPH	2.901 \pm 0.022
Oil globule depletion	5 DPH	2.874 \pm 0.030
First feeding	4 DPH	2.905 \pm 0.020
Swimbladder inflation	6 DPH	2.964 \pm 0.021
Start of flexion	14 DPH	4.904 \pm 0.092
Feeding with Artemia	17 DPH	5.687 \pm 0.158
Feeding artificial feed	24 DP	6.702 \pm 0.174

First feeding represents the addition of microalgae and rotifers.

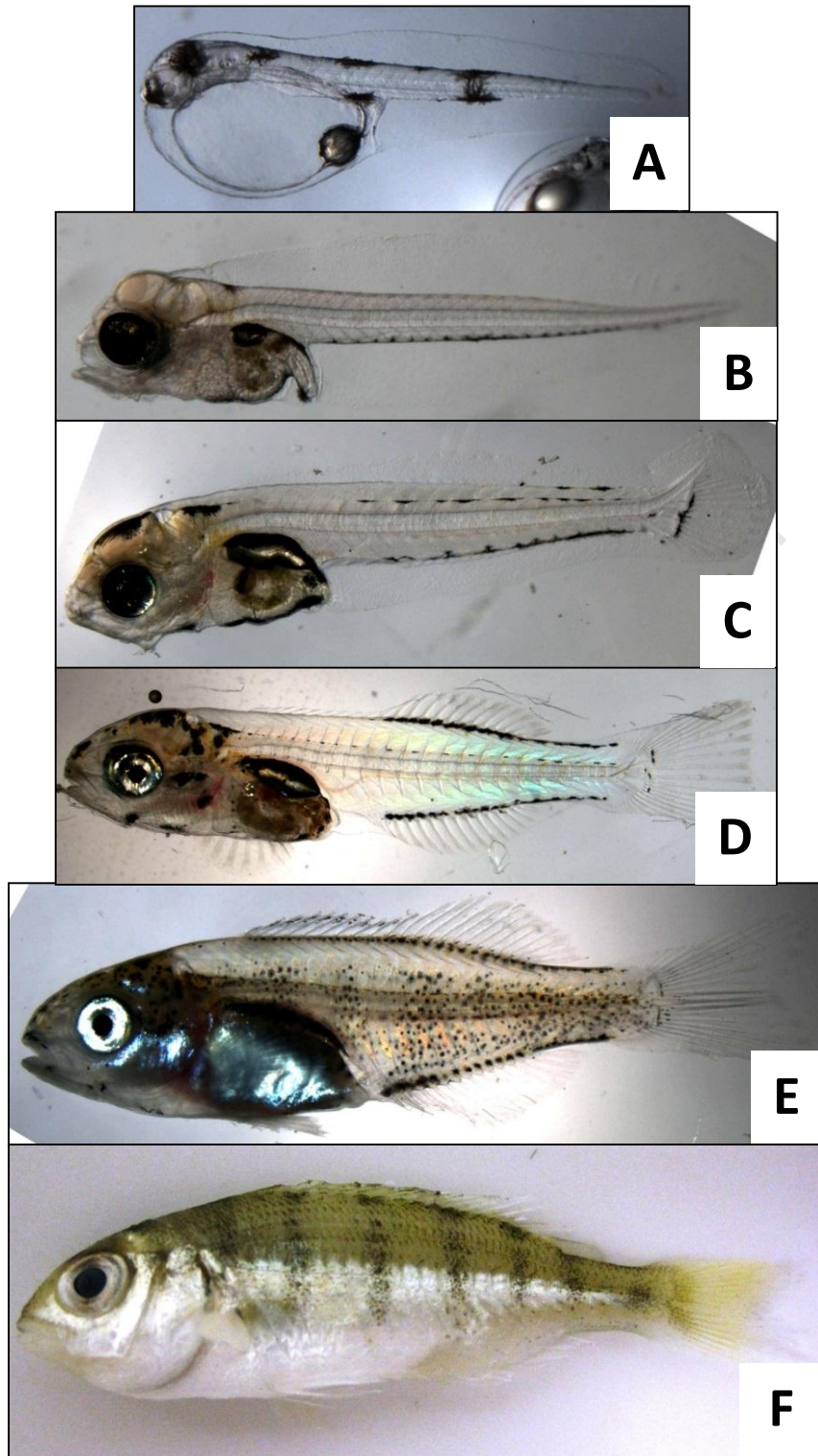


Figure 3.3: Photomicrographs of the larval development of *Rhabdosargus globiceps*. The mean total length and SE are shown. A-At hatching 0 DPH (2.278 ± 0.022 mm). B-10 DPH (3.791 ± 0.056 mm). C-20 DPH (6.79 ± 0.171 mm). D-30 DPH (11.136 ± 0.442 mm). E-40 DPH (15.75 ± 0.689 mm). F-90 DPH (5 cm).

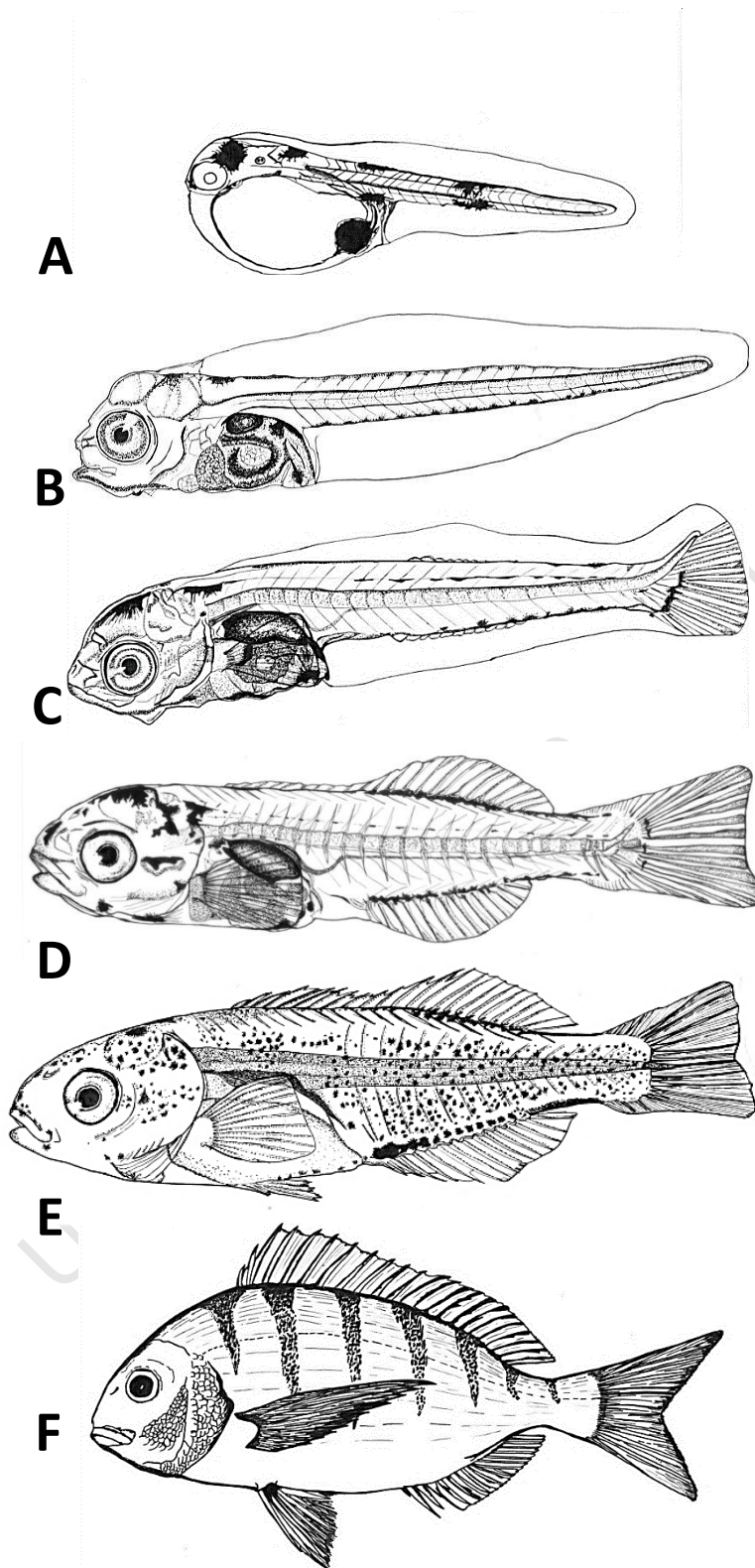
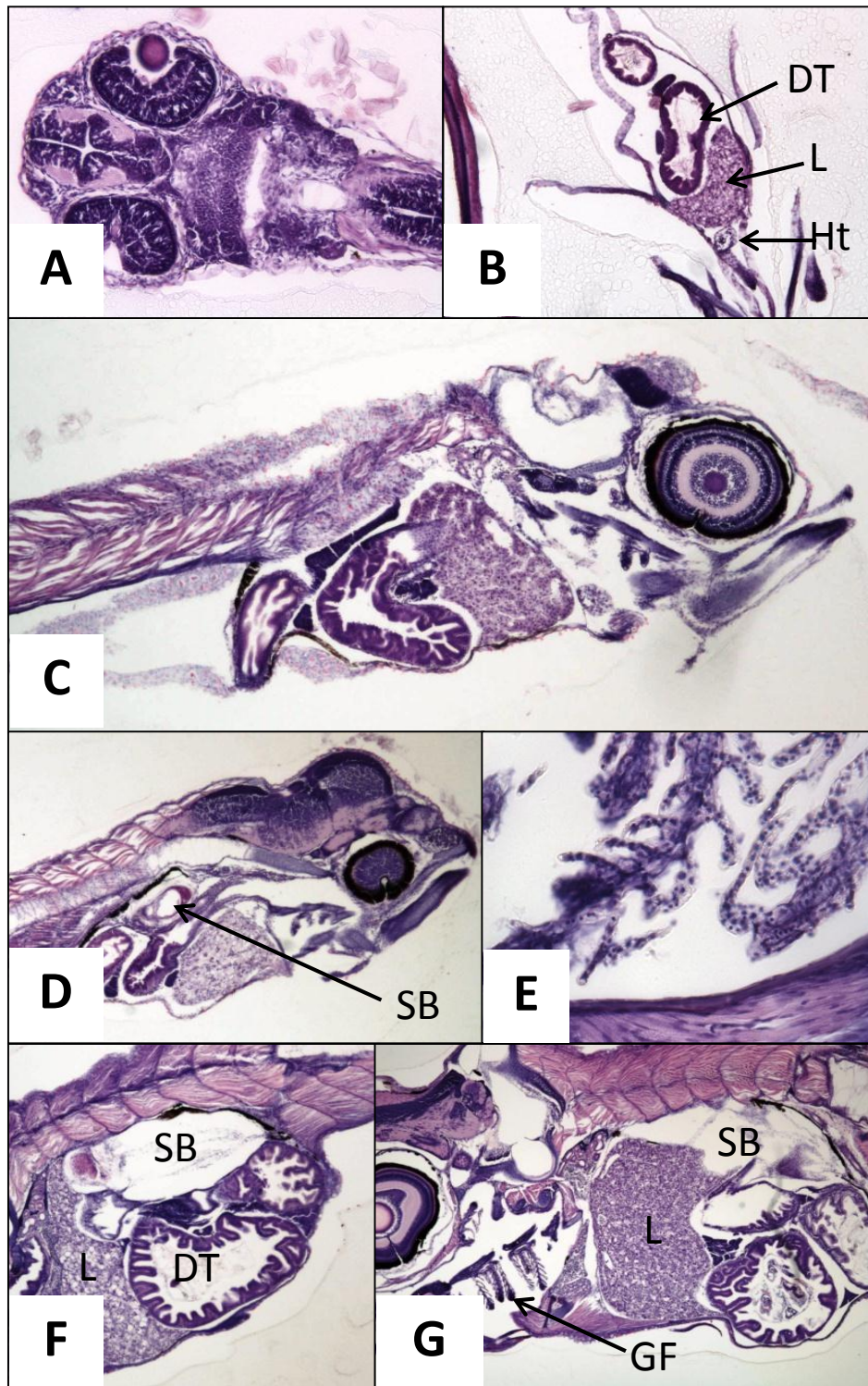


Figure 3.4: Detailed illustrations of the larval development of *Rhabdosargus globiceps*. The mean total length and SE are shown. A-At hatching 0 DPH (2.278 ± 0.022 mm). B-10 DPH (3.791 ± 0.056 mm). C-20 DPH (6.79 ± 0.171 mm). D-30DPH (11.136 ± 0.442 mm). E-40 DPH (15.75 ± 0.689 mm). F-Adult (25 cm). Original drawings by author.



*Figure 3.5: Photomicrographs of Rhabdosargus globiceps larvae. A-Head of larvae at 1DPH. B-The organs present in an 8 DPH larvae. The heart (Ht), accessory glands of liver (L) and digestive tract (DT). Rotifers are visible in the gut. C-Cross section of 12 DPH larvae. D-Cross section of a 12 DPH larvae showing the swimbladder (SB). E-Gill filaments. F-Organ enlargement and development in a 26 DPH larvae. G-This micrograph shows the gill filaments (GF) and the presence of food, possibly *Artemia*, in the digestive tract of a 27 DPH larvae (Sanchez-Amaya et al. 2007; Navarro & C. Sarasquete 1998).*

Growth

Total Length

The initial growth from 0 DPH to 3 DPH was rapid, with an SGR of 8.06%, growing from 2.278 mm to 2.901 mm (Figure 3.6). Over the next 6 days the SGR decreased to 2.40% and at 9 DPH the larvae were 3.276 mm. The SGR for stages 1-4 was 8.06%, 2.40%, 6.43% and 5.28%, respectively. The mean length of the larvae at 0 DPH was 2.278 ± 0.022 mm (min-2.061 mm; max-2.395 mm) ($n=20$). At 30 DPH the mean length of the larvae was 11.14 ± 0.44 mm (min-7.627 mm; max-15.187 mm) ($n=20$). As the larvae got older the heterogeneity in total length within the sample increased. The mean daily growth over the 30 day rearing period was 0.295 mm day⁻¹. The exponential growth in total length for stages 1-4 is represented by the following equations; 1. $y = 2.154e^{0.0806x}$, ($R^2 = 0.93$, $n = 80$), 2. $y = 2.783e^{0.0207x}$, ($R^2 = 0.69$, $n = 120$), 3. $y = 3.5715e^{0.0612x}$, ($R^2 = 0.99$, $n = 97$), 4. $y = 4.8568e^{0.0511x}$, ($R^2 = 0.98$, $n = 269$).

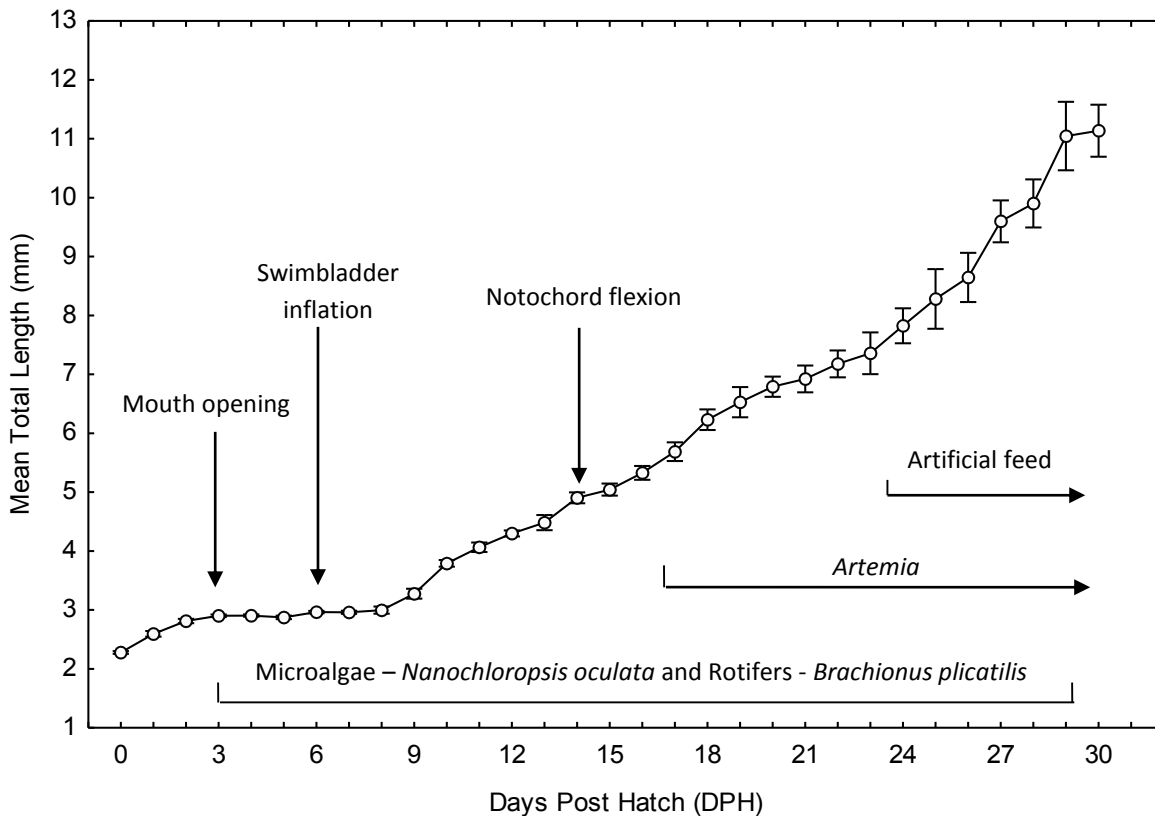


Figure 3.6: Total length (mean \pm SE) of *Rhabdosargus globiceps* larvae from 0 DPH to 30 DPH. The feeding scheme is shown at the days it began and finished. Time to first appearance of mouth opening, swimbladder inflation and notochord flexion is displayed.

Body height

The body height over the first 3 DPH saw increased growth, which levelled off for the next 5 days (Figure 3.7). The SGR from 0 DPH to 3 DPH was 8.89%. An increase in body height began at 8-9 DPH. The exponential growth of the larvae continued for the 30 day rearing period, increasing in heterogeneity within the sample from hatching to 30 DPH. The mean body height at 0 DPH was $502.89 \pm 10.6 \mu\text{m}$ (min-385.27 μm ; max-577.86 μm) ($n=20$). The mean body height at 30 DPH was $2123.01 \pm 131.23 \mu\text{m}$ (min-1406.59 μm ; 3448.36 μm) ($n=20$). The SGR of the body height for the stage 1-4 was 8.90%, 2.22%, 4.17% and 5.05% respectively. The mean daily growth of the body height over the 30 day rearing period was $54 \mu\text{m day}^{-1}$. The exponential growth in body height for stage 1-4 is represented by the following equations; 1. $y = 450.28e^{0.0865x}$, ($R^2 = 0.92$, $n = 80$), 2. $y = 586.17e^{0.0229x}$, ($R^2 = 0.94$, $n = 120$), 3. $y = 711.3e^{0.0519x}$, ($R^2 = 0.80$, $n = 97$), 4. $y = 966.87e^{0.0459x}$, ($R^2 = 0.95$, $n = 269$).

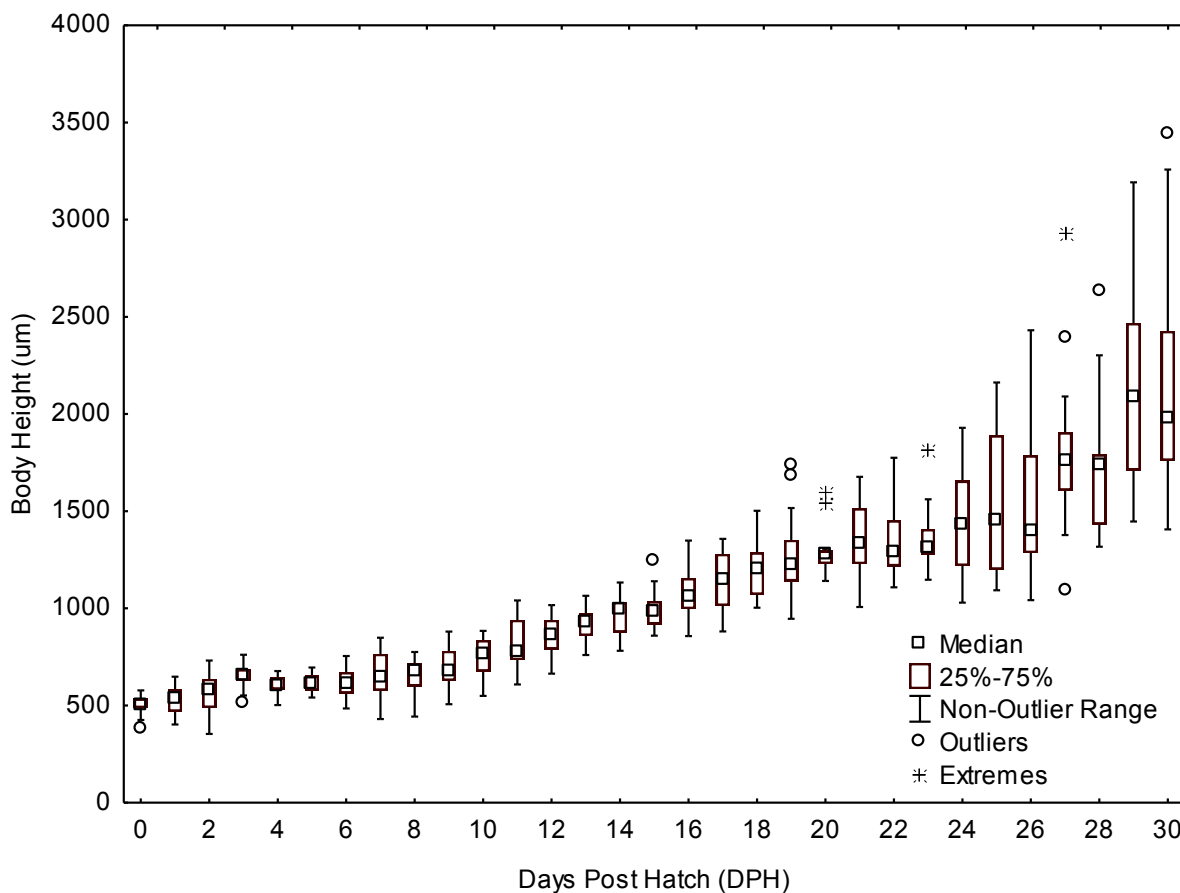


Figure 3.7: Box and Whisker plot illustrating the change in body height of *Rhabdosargus globiceps* larvae from 0 DPH to 30 DPH.

Eye diameter

This growth followed a similar pattern to that of body height and total length. There was a period of fast growth from 0 DPH to 3 DPH, followed by a period of little to no growth until 7 DPH (Figure 3.8). From 8 DPH until 30 DPH there was rapid growth and an increase in heterogeneity within the sample. The mean eye diameter at 0 DPH was $168.68 \pm 4.53 \mu\text{m}$ (min-122.54 μm ; max-202.1 μm) ($n=20$). At 30 DPH the mean eye diameter was $806.24 \pm 38.58 \mu\text{m}$ (min-517.01 μm ; max-1107.04 μm) ($n=20$). The SGR of the eye diameter for the stages 1-4 was 10.79%, 3.57%, 4.48% and 5.31% respectively. The mean daily growth of the eye diameter over the 30 day rearing period was $21.25 \mu\text{m day}^{-1}$. The exponential growth in body height for stages 1-4 is represented by the following equations; 1. $y = 143.72e^{0.1228x}$, ($R^2 = 0.87$, $n = 80$), 2. $y = 218.16e^{0.0354x}$, ($R^2 = 0.96$, $n = 120$), 3. $y = 279.96e^{0.0457x}$, ($R^2 = 0.96$, $n = 97$), 4. $y = 361.79e^{0.0474x}$, ($R^2 = 0.97$, $n = 269$).

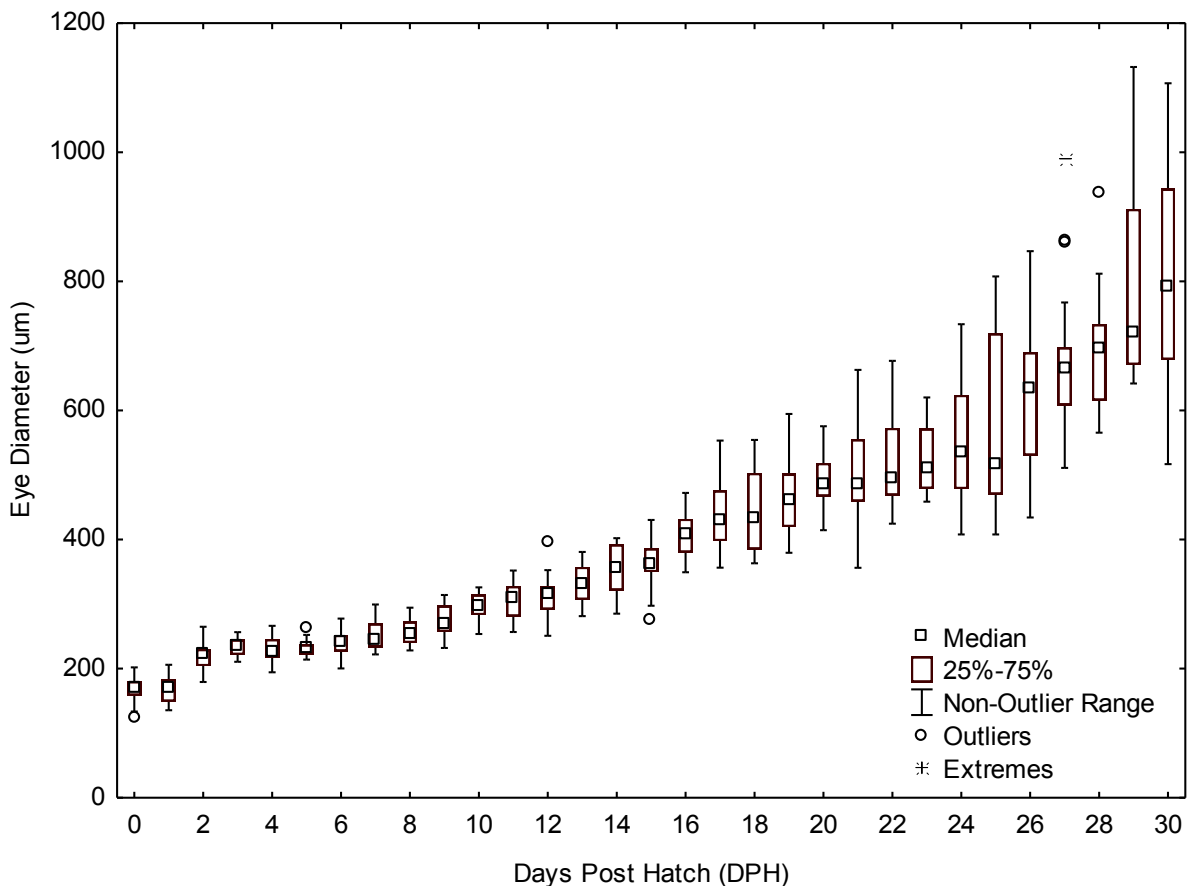


Figure 3.8: Box and Whisker plot illustrating the change in eye diameter of *Rhabdosargus globiceps* larvae from 0 DPH to 30 DPH.

Yolk-sac and oil globule depletion

Yolk consumption started almost immediately after hatching, while the oil globule is used at a slower rate (Figure 3.9). Total adsorption of the yolk-sac occurred at 3 DPH, while the oil globule is used up by 5 DPH (Table 3.5) (Figure 3.9). The initial absorption of the yolk from hatching to 1 DPH is large and accounted for 68% ($n=20$ per day) of the total volume from the initial volume at 0 DPH. This rapid absorption of the yolk-sac showed its effect in the change in total length from 0 DPH to 1 DPH, which was the greatest over the first 5 DPH, with a SGR of 12.99%. The greatest absorption of the oil globule occurred between 2 DPH and 3 DPH. The oil globule at 4 DPH was extremely small and was only visible in 10% of the selected larvae ($n=20$).

Table 3.5: Changes in the total length, yolk-sac volume, oil globule volume and SGR over the first 5 days post hatch in larvae of *Rhabdosargus globiceps*.

Days post hatch	Total length (mm)	Yolk volume (mm ³)	Oil globule volume (mm ³)	SGR (% increase day ⁻¹)
0	2.278 ± 0.022	0.121 ± 0.006	0.026 ± 0.0003	
1	2.595 ± 0.048	0.039 ± 0.0022	0.023 ± 0.0005	12.99
2	2.812 ± 0.036	0.008 ± 0.0016	0.013 ± 0.0007	8.04
3	2.901 ± 0.022	0	0.0014 ± 0.0003	3.14
4	2.905 ± 0.02	0	0.0003 ± 0.00007	0.13
5	2.873 ± 0.03	0	0	-1.08

Data shown as mean ± standard error. ($n=20$ per day). SGR calculated between days.

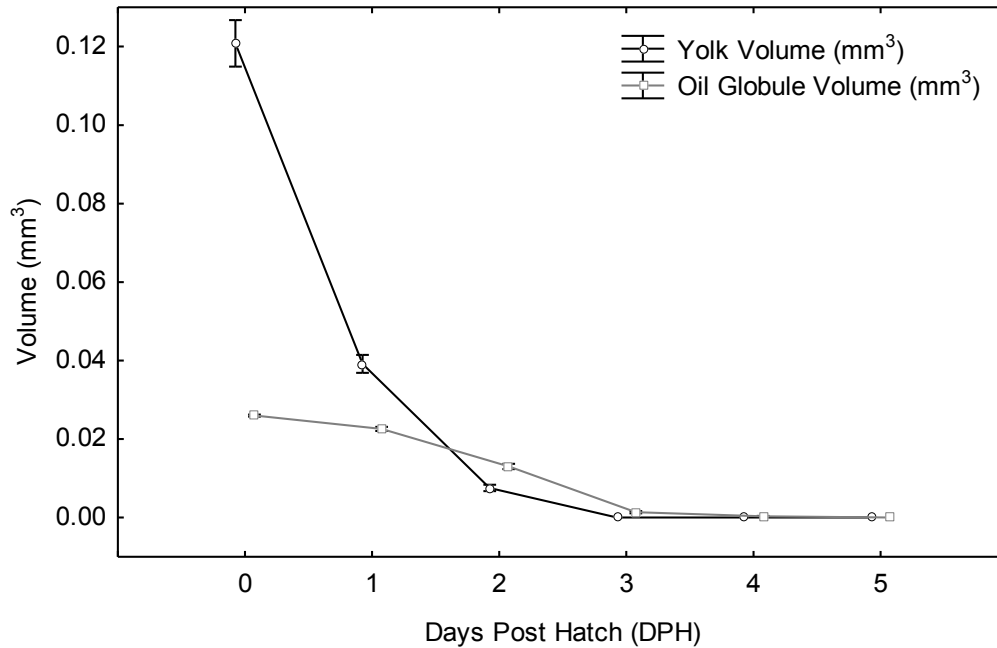


Figure 3.9: Depletion of the yolk-sac and the oil globule (mean \pm SE) over the first 5 DPH.

First feeding, swimbladder inflation and flexion

Feeding first occurred at 4 DPH, when the larvae had a mean total length of 2.905 ± 0.019 mm (min-2.724 mm; max-3.02 mm) ($n=20$). On the day of first feeding, 65% of the sampled larvae had microalgae and rotifers in their stomach. The frequency of feeding fluctuated between 80% and 100% until 16 DPH (Figure 3.10). From 4 DPH onwards the larvae were seen actively preying. Feeding became more active from the time *Artemia* nauplii were added at 17 DPH. The eyes became pigmented at 3–4 DPH, which coincided with first feeding (Figure 3.11A).

Swimbladder inflation began at 6 DPH, when the larvae had a mean total length of 2.96 ± 0.021 mm (min-2.748 mm; max-3.142 mm) ($n=20$) (Figure 3.11B). Only 5% of the sampled larvae had a clearly visible swimbladder at this time (Figure 3.10). Under the microscope the swimbladder appeared as a small circular silver bubble in the dorsal area of the abdomen between the digestive tract and the prehemal vertebrae (Figure 3.11B, D, E) (Koumoundouros *et al.* 2000). As the larvae grew the swimbladder enlarged and extended posteriorly along the vertebral column, to the level of the anus (Figure 3.5D, F, G). There

was a high rate of fluctuation in frequency of fish with swimbladder inflation from 13 DPH to 30 DPH (Figure 3.10). The lowest frequency of swimbladder inflation in this period was 67% at 17 DPH. At 30 DPH the frequency of fish with inflated swimbladders was 95%.

Flexion of the notochord was first present in the larvae sampled on 14 DPH at a mean total length of 4.90 ± 0.09 mm (min-4.20 mm; max-5.482 mm) ($n=20$). This morphological development is represented by a clouded area at the ventral side of the tail (Figure 3.11E). The previously straight notochord curves upward at its posterior part to later form the caudal fin (Figure 3.11F, G) (Koumoundouros et al. 1997). On 15 DPH the prevalence of flexion in the sampled larvae rose to reach a frequency of 87.5% (Figure 3.10). All sampled larvae from 16 DPH to 30 DPH went through notochord flexion. Completed flexion was apparent in the larvae at 30-36 DPH (Figure 3.3D, E).

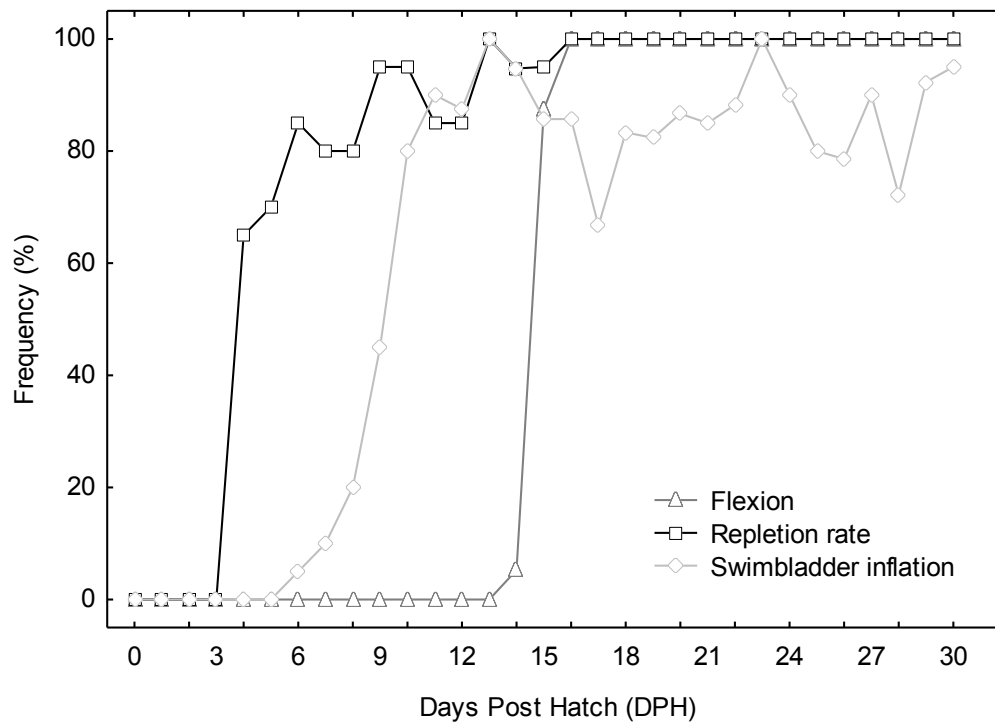


Figure 3.10: Frequency of repletion rate (food in gut), swimbladder inflation and flexion in *Rhabdosargus globiceps* larvae from 0 DPH to 30 DPH.

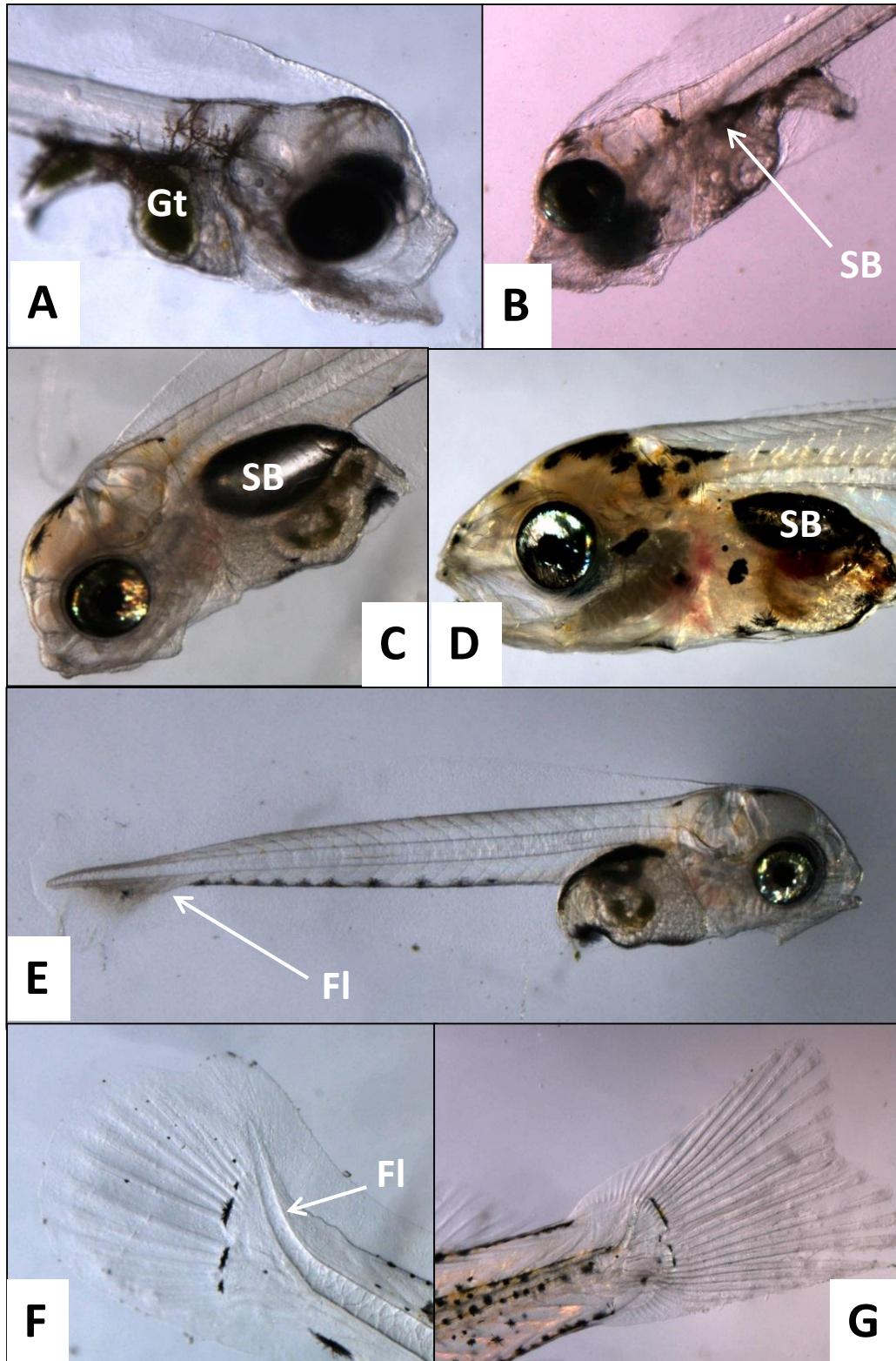


Figure 3.11: Photomicrographs of the development of *R. globiceps* larvae. A-First feeding, 4 DPH: Microalgae and rotifers present in the gut (Gt). B-First inflation of the swimbladder (SB), 6 DPH. C-Overinflated swimbladder, 18 DPH. D-Larvae with perfectly inflated swimbladder and gut containing *Artemia*, 28 DPH. E-Beginning of notochord flexion (FI), 14 DPH. F- Flexion of the notochord at 20 DPH. G-Completed flexion at 36 DPH.

3.4. DISCUSSION

The lack of biological information regarding the early life stages of *Rhabdosargus globiceps*, considering their fragility, size and similarity to other sea bream species at this stage caused certain challenges. By adapting methods for rearing of *Sparus aurata* and *Pagrus pagrus*, which have been shown to have similar biological requirements as they come from the same family, *Rhabdosargus globiceps* larvae were successfully reared to 30 DPH under intensive larval rearing conditions using the pseudo-green water technique.

The external and the internal anatomy could be assessed from the histological examination and photomicrographs of the *Rhabdosargus globiceps* embryos and larvae. This allows for an understanding of the functional status of the embryo and larvae at specific stages of their life cycle (Koumoundouros *et al.* 1999; Sanchez-Amaya *et al.* 2007)

Rhabdosargus globiceps larvae share the same development stages with many other sea bream species and teleosts (Moretti *et al.* 1999; Koumoundouros *et al.* 1999; Santamaría *et al.* 2004; Radonic *et al.* 2005; Bjelland & Skiftesvik 2006; El-Gharabawy & Assem 2006; Sanchez-Amaya *et al.* 2007). Although this is common and the stages are well known, the timing and duration of these stages is variable among sea bream species (Sanchez-Amaya *et al.* 2007). It took 36 hours for the embryos to develop to the stage at which they could hatch. Over this time the embryo went through four main development stages. In the cleavage stage the cells divided five times, to go from a single cell to 32 cells. The morula stage, blastula stage and gastrula stage saw the developing embryo's cells change shape from a ball of cells to a half circle inside the egg. The embryo stage had the most noticeable development when the larvae first appeared as a faint elongated shape behind the oil globule. Over the next 22 hours the optical vesicles and rudimentary heart had formed, the tail had elongated and the embryo was moving and ready to hatch. Formation of the optical vesicles and movement were first recorded at 15 and 30 hours, respectively. The timing to these stages at 20°C was similar to that of *Pagrus pagrus*, which hatched 37 hours post fertilisation, also at 20°C (Radonic *et al.* 2005). The rearing water temperature is one of the most influential environmental variables, affecting the incubation time and rate of development of the embryo (Kucharczyk *et al.* 1997; Koumoundouros *et al.* 2001; Klimogianni *et al.* 2004).

From the time of hatching until 30 DPH the larvae go through intensive morphological changes and organogenesis. These morphological developments are described in Table 3.3. The stages include the most important morphological developments, first feeding, swimbladder inflation and flexion of the notochord. Apart from the development of notochord flexion, stage 4 is recognised as a development stage where pre-existing structures are modified (Santamaría *et al.* 2004).

Other studies have described the changes in morphology with more detail using the source of food and the morphological features to stage them (Elbal *et al.* 2004; Sanchez-Amaya *et al.* 2007). The stages of development have been described as lecithotrophic (newly hatched larvae), lecithoexotrophic (larvae feeding on endogenous and exogenous food sources) and exotrophic (larvae feeding on exogenous food sources) (Sarasquete *et al.* 1995; Elbal *et al.* 2004; Sanchez-Amaya *et al.* 2007). This information separates the development of the larvae into two distinct phases, larvae feeding on endogenous food reserves (endotrophic) and larvae feeding on exogenous food resources (exotrophic).

Endotrophic phase

Rhabdosargus globiceps larvae were 2.278 mm in mean total length at hatching. This is smaller than other sea bream species currently exploited as aquaculture candidates. *Pagrus pagrus* have a mean total length of approximately 3.02 mm and only hatch after 50 hours at 18°C (Conides & Glamuzina 2001). *Sparus aurata*, were 2.5-2.95 mm at hatching (Parra & Yufero 2000; Conides & Glamuzina 2001; Elbal *et al.* 2004). All sea bream larvae feed solely on their endogenous food reserves for the first 3 DPH and a mixture of endogenous and exogenous food sources from 4-5 DPH. *Rhabdosargus globiceps* the yolk-sac was depleted by 3 DPH, while the oil globule lasts until 5 DPH. The growth variables of total length, body height and eye diameter are shown to have rapid growth from 0 DPH to 3 DPH, with SGRs of 8.06%, 8.90% and 10.79%, respectively. This high growth rate is explained by the significant decrease in yolk volume. The yolk-sac lost 68% of its total volume from 0 DPH to 1 DPH. This had a direct effect on the growth of the larvae with the greatest total length SGR in the first 5 DPH occurring between 0 DPH and 1 DPH. This shows the nutritional effect of the

endogenous reserves, which is used with varying efficiencies by each species (Parra & Yúfera 2001; El-Gharabawy & Assem 2006).

The absorption rate of the yolk-sac and the oil globule is affected by other factors, such as size of the yolk-sac itself due to the maternal condition of the broodstock at spawning, and the temperature of the water (Buckley *et al.* 2000; Yufera & Darias 2007). These factors determine the growth rate and health of the larvae, which in turn allows the larvae to become ready for the exotrophic phase.

Exotrophic phase

First-feeding on the exogenous food sources of *Rhabdosargus globiceps* occurred at 4 DPH. This follows the same trend as with other cultured sea bream such as *Pagrus pagrus*, *Sparus aurata* and *Dentex dentex* (Koumoundouros *et al.* 1999; Papandroulakis *et al.* 2004a; Elbal *et al.* 2004). At first-feeding the *Rhabdosargus globiceps* larvae measured 2.905 ± 0.020 mm total length, while *Dentex dentex* was 3.6 ± 0.1 mm at this stage (Koumoundouros *et al.* 1999). At first-feeding the larvae still had a small percentage of their oil globule left, which shows that the *Rhabdosargus globiceps* larvae have a transitional phase (lecitoexotrophic phase) of endogenous and exogenous feeding. The oil globule was completely depleted by 5 DPH and by this time the larvae were feeding on rotifers. A period of transition between endogenous and exogenous feeding provides the larvae a time of resistance to starvation and gives it a better chance to start actively feeding (Yufera & Darias 2007).

In this experiment a larger strain of rotifers was used, *Brachionus plicatilis*. When the small size of the larvae and the large size of the rotifers are coupled together this could mean that their ability to feed at 4 DPH is impeded, leading to a reduced growth rate and possibly to increased mortality (Cunha & Planas 1999). At 4 DPH 65% of the sampled larvae had successfully fed. The use of different strains and sizes of rotifers should be considered as this would allow for the larvae to feed on prey of the optimal size and obtain the optimal growth and survival (Oie & Olsen 1997; Planas & Cunha 1999). This information corresponds well with the results from this study. The growth data for total length, body height and eye diameter describes a reduction in growth rate from 4 DPH to around 9 DPH. The SGR for the larvae was the lowest in stage 2 for total length, body height and eye diameter. This could

indicate that the larvae struggled to ingest the rotifers during the transition period between endogenous and exogenous food sources, subsequently limiting the amount of energy used for growth (Polo *et al.* 1992).

Yufera & Darius (2007) describe a period in the early development of the larvae called the point of no return (PNR), where the larvae suffer mass mortalities due to starvation, after the time of first-feeding. The organs fail before the PNR is reached (Gisbert *et al.* 2004; Yufera & Darias 2007). Slow growth as well as elevated mortalities were noted at the time of first-feeding in stage 2, indicating that a large number of larvae may have reached the PNR. Nutrition limitation has obvious consequences for organisms at any life stage. These mass mortalities and the reduction in growth rate at the larval stage would be recognised as obstacles for its potential use in aquaculture. However, similar results have been recorded with successful candidates such as *Pagrus pagrus*, *Dentex dentex* and *Pagrus auratus* (Battaglione & Talbot 1992; Conides & Glamuzina 2001; Crespo *et al.* 2001). Conides & Glamuzina (2001) state that a 'low growth phase', in terms of length, starts at the completion of the endogenous food reserves in *Pagrus pagrus*. This occurs even though the larvae are larger than *Rhabdosargus globiceps* larvae suggesting that this is a normal characteristic of sea bream larval growth.

The use of smaller organisms like small strain rotifers, copepod nauplii, and more natural prey such as *Tisbae* sp. would have helped to initiate more successful first-feeding, nullifying the slow growth phase and potentially reducing the mortalities (Polo *et al.* 1992). The introduction of smaller sized prey has improved the first-feeding phase in both turbot larvae and sea bream larvae (Polo *et al.* 1992; Cunha & Planas 1999).

From 14 to 30 DPH the histological examinations show that the organs were healthy, developing and proliferating inside the larvae, with the liver and digestive tract showing the most growth and differentiation. All larvae were feeding and had undergone successful notochord flexion. However, not all larvae had swimbladders. The absence of swimbladders is a common problem in the rearing of sea beam and sea bass species (Planas & Cunha 1999) and is brought about by physostomous larvae not being able to perform the essential requirement of gulping air from the surface (Chatain 1989; Trotter *et al.* 2001). This reduces the condition of the larva and its ability to grow at an optimal rate.

The slow growth rate at stage 2 could therefore also be due to larvae not being able to initiate swimbladder inflation, as the larvae cannot maintain their buoyancy resulting in high energy usage in order to maintain positive buoyancy (Chatain 1989). The incidence of swimbladders at the early stages of development was low with only 5% at 6 DPH and 20% at 8 DPH. However, this shows that the timing to first appearance of swimbladders is correct, as initial swimbladder inflation occurs after the endogenous food reserves have been depleted (Trotter *et al.* 2001). The oil globule was fully depleted by 5 DPH meaning that there was only a 24 hour period before the first larvae with swimbladders were recorded. After 10 DPH the frequency was 80% and although the percentage fluctuated over the next twenty days of sampling the majority had a greater than 80% occurrence of a swimbladder, with 95% of the sampled larvae at 30 DPH having swimbladders. The main reason for the lack of initiation of swimbladder inflation is the presence of an oily layer on the water surface (Planas & Cunha 1999). The film impedes the intake of air by the larvae. An oily layer was not noticed during the larval rearing of the *Rhabdosargus globiceps*, as the water overflowed via a banjo sieve at the surface. Ways to combat the oily layer is to use surface skimmers that blow air onto the surface to accumulate the oily layer, so it can be removed later (Moretti *et al.* 1999; Koumoundouros *et al.* 2000; Fielder *et al.* 2005). Without a swimbladder the chance of malformation and deformity is high and the market value of the fish is detrimentally affected (Planas & Cunha 1999; Koumoundouros *et al.* 2000).

At the end of stage 2 the growth of the larvae increased substantially through stages 3 and 4. This increased growth brought about a substantial increase in variability in the total length, body height and eye diameter. This is attributed to the timing of first feeding and swimbladder inflation by the larvae (Battaglione & Talbot 1992). The larger the larvae are, the better they can feed and the earlier they can move onto larger prey, such as *Artemia*, that have a greater nutritional content. The larvae first accepted *Artemia* at 17 DPH when they had a mean total length of 5.687 mm. The high concentration of *Artemia* added at this stage allowed for constant feeding.

The increase in heterogeneity in body size of fish larvae favoured cannibalism and has been recorded during the rearing of *Pagrus auratus* and *Sparus auratus* (Battaglione & Talbot 1992; Agnese *et al.* 1995; Papandroulakis & Divanach 2002). There was large heterogeneity

of body size in the population of reared *Rhabdosargus globiceps*, but no cannibalism was observed over the 30 day rearing period. The larvae reached a mean total length of 11.14 ± 0.44 mm at 30 DPH. *Pagrus pagrus*, which is recognised as a good candidate for aquaculture in the Mediterranean has a mean total length of approximately 10.8-11 mm at 30 DPH (Mihelakakis *et al.* 2001; Conides & Glamuzina 2001), and *Sparus aurata*, which has been used for many years as an aquaculture species, obtains a length of approximately 9-10 mm at 20 DPH (Papandroulakis & Divanach 2002), while *Rhabdosargus globiceps* had a mean total length of 6.79 mm at this time.

Another aspect of the larval stage that is also affected by the food source is movement. The settlement of the larvae near the bottom of the tank at the start of feeding could be due to rotifers and *Tisbae* sp. also settling on the bottom (Stottrup 2000). These copepods provide the larvae with greater nutrition than rotifers and *Artemia* and allow for fish larvae to prey on a more natural food source (Stottrup 2000). This also allows for the normal development of the larvae and other important benefits for the larvae and the hygiene conditions of the tank environment (Stottrup 2000).

The results of the experiment suggest that *Rhabdosargus globiceps* larvae share very similar characteristics with successful culture sea bream species, with the growth phases occurring according to the food source (Sarasquete *et al.* 1995; Mihelakakis *et al.* 2001; Santamaría *et al.* 2004; Elbal *et al.* 2004; Sanchez-Amaya *et al.* 2007). However, *Rhabdosargus globiceps* was smaller than most other sea bream larvae. The most important aspect of this life stage is the larvae's ability to make a successful transition from endogenous food sources to exogenous food sources (Papandroulakis & Divanach 2002). The more emphasis that is put on this stage to create the correct water conditions and provide the appropriate food source, the more growth, development and survival of the larvae could be increased. *Rhabdosargus globiceps* larvae accepted exogenous food, a significant number of larvae initiated swimbladder inflation successful and notochord flexion occurred in all sampled larvae post 16DPH.

CHAPTER 4

Effect of water temperature on the embryonic and larval growth and development of white sturgeon, *Rhabdosargus globiceps* (Teleostei: Sparidae).

ABSTRACT

This research aimed to determine the optimal temperature range for successful embryonic development and rearing of larvae from egg to 30 DPH (days post hatch). To assess the effect of temperature on embryonic development, eggs were placed at three different incubation temperatures (25°C, 20°C and 15°C) and the timing to the developmental stages was recorded. Eggs placed at 25°C hatched 30 hours after fertilisation, while eggs at 20°C and 15°C hatched after 36 and 48 hours respectively. Three-thousand-eight-hundred fertilised eggs were placed in the four replicate tanks of each temperature treatment (25°C, 20°C and 15°C). Five to ten larvae were sampled from each tank every 3 days starting from hatching (0 DPH). Morphological measurements were made with assessments of feeding, swimbladder inflation and notochord flexion also being conducted. Final survival and wet weight were measured at 30 DPH. The larvae were reared under intensive conditions with a photoperiod of 15 hours. Temperature, salinity, pH and dissolved oxygen were similar between treatments and within replicate tanks. Larvae reared at 25°C died 6 DPH and were not used in the overall assessment of growth and development. The endogenous food reserves of yolk-sac and oil globule were depleted at a faster rate at a higher temperature. In terms of growth the larvae reared at 20°C performed significantly better than those reared at 15°C ($P < 0.05$), with larvae having a mean total length of 11.87 ± 0.34 mm. Larvae reared at 15°C reached a mean total length of 9.01 ± 0.23 mm at 30 DPH. Larvae reared at 20°C had significantly earlier indications and higher percentages of feeding ($\chi^2 = 7.37$, $df = 2$, $P = 0.025$), swimbladder inflation ($\chi^2 = 28.86$, $df = 3$, $P = 0.000$) and notochord flexion ($\chi^2 = 33.44$, $df = 2$, $P = 0.000$) than at 15°C. The results suggest that the larvae with the best condition were achieved at a rearing temperature of 20°C.

4.1. INTRODUCTION

Temperature is recognised as the principle abiotic factor governing crucial physiological, biochemical and life history processes in fish (Beitinger & Fitzpatrick 1979). Temperature has direct control over biological reactions that occur inside fish larvae, which has great influence on hatching size, yolk-sac and oil globule absorption rate, growth, digestion rates and metabolic demand (Van der Kraak & Pankhurst 1996; Jobling, 1997; van Maaren & Daniels 2001; Choa *et al.* 2010). Growth and development rates are increased with an increase in temperature (Magnuson *et al.* 1979; Fielder *et al.* 2005). However, there will be a maximum tolerable limit. At this limit the enzymatic reaction starts to decrease reducing the rate of metabolism, which leads to death (van Maaren & Daniels 2001). For an aquaculture candidate to be successfully cultured, the minimum and maximum environmental limits must be known. With this information the larvae and fish can be cultured in a manner that is conducive to fast growth, while maximising survival and condition of fingerlings. These results would make it possible to reduce the amount of time needed to produce fish of a market size, therefore minimizing costs of production (Moretti *et al.* 1999).

Growth of each species of fish is maximized at a temperature within its thermal tolerance range. At the optimum temperature the growth rates in fish are known to increase up to a 'species-specific maximum' (Jobling 1997). The 'species-specific maximum' is the maximum growth rate a fish species can have. If a species is going to be considered as a candidate for aquaculture, the optimal rearing conditions must be known. The environmental tolerance data are vital in being able to establish appropriate, if not optimum, conditions for the artificial culture of the species.

Rhabdosargus globiceps inhabits an extensive geographic range around the South African coast, from the Kei River on the south coast to Angola on the west coast (Attwood *et al.* 2010). This sea bream species is endemic to South African waters (Griffiths *et al.* 2002). The temperatures found throughout this distribution range describe the species-specific 'zone of thermal tolerance' (Katersky & Carter 2005). This species' eggs are pelagic and the juveniles are found in small sheltered bays. In general there is a preference for estuarine nursery

grounds (Griffiths *et al.* 2002). This displays *Rhabdosargus globiceps*' tolerance for varied environmental conditions during their early life stages.

This research would allow for the understanding of how temperature affects survival, growth, and development of the embryo and larval stage of *Rhabdosargus globiceps* in a culture environment. These quantitative results are regarded by hatchery managers as extremely useful tools, as they can be used to conduct a cost benefit analysis (van Maaren & Daniels 2001). These analyses would determine if a hatchery would profit from spending money to heat water during the cold winter months in order to create the optimum conditions.

Sea bream larvae, such as *Pagellus erythrinus* and *Pagrus auratus*, have exhibited physiological compensation when reared at different temperatures (Klimogianni *et al.* 2004; Fielder *et al.* 2005). This can lead to feeding, swimbladder inflation and notochord flexion all being affected, which reduces the condition of the larvae and consequently the quality of the fingerlings. Mass mortalities are common when the larvae cannot feed (Conides & Glamuzina 2001). Failure to initiate swimbladder inflation and notochord flexion can result in mortalities, skeletal abnormalities and severe reduction in larval growth (Chatain 1989; Leu 1996). Assessing these factors will identify the temperature range that produces the larvae with the best condition. There is an increasing demand for aquaculture products, which has stimulated the industry to produce higher quality juveniles (Lee 2003). The technology and rearing techniques must be constantly improved so that production of high quality juveniles, possessing the preferred characteristics, is increased.

The aim of the experiment was to assess the effect of temperature on the growth, survival and overall condition of *Rhabdosargus globiceps* eggs and larvae from fertilisation to 30 DPH (days post hatch). By examining these factors at the three temperatures, the time taken to reach the specific stages in embryo and larval development could be estimated. The hypothesis tested in this experiment is that there would be an increase in growth and development with an increase in temperature, for both the embryo and larval stage. The null hypothesis is that there would not be an increase in growth and development with an increase in temperature. The effect of the temperature on the survival and overall condition

of the larvae will also be assessed by comparing the success of feeding, swimbladder inflation and notochord flexion.

4.2. METHODS

The experiments were conducted at the Department of Agriculture, Forestry and Fisheries (DAFF) Aquaculture Research Facility, in Seapoint, Cape Town, South Africa (33°55'14.27"S. 18°22'50.75"E).

Source of eggs

Fertilised eggs were obtained from four *Rhabdosargus globiceps* female broodstock (1225.3 ± 152.5 g; min-835 g; max-1573 g) ($n=4$) that were induced to spawn via environmental manipulation and hormone injections as described in Chapter 2. The females were biopsied and eggs at a late stage of maturation (late vitellogenic) were present after a 3-month photothermal cycle. Spawning was induced according to the protocol described by (Moretti *et al.* 1999) for *Sparus aurata*. Two intramuscular hormone injections (1 µg per kilogram body mass), of LH-RHa (Luteinising hormone–Releasing hormone; D-Ala⁶-des-Gly¹⁰LHRH ethylamide; Syndel International INC.) were given to the fish within a 24 hour interval to initiate the final stage of egg maturation, full hydration and release of eggs. The sex ratio of the broodstock was 1:1, four females to four males. The broodstock spawned for four consecutive 24 hour periods.

Newly fertilised/buoyant eggs from the water column were used in the experiment to assess the effect of temperature on the embryonic development. The eggs from the first batch that were collected over the first 24 hours were used in the experiment that assessed the effect of temperature on larval performance. This ensured that eggs of similar quality were used. After spawning the fertilised eggs were collected in a 500 µm mesh net that was present at the overflow of the broodstock tank. This net and the eggs were then disinfected in a 10 L mixture of 0.1% formalin and 2 µm filtered sterile seawater for 5 minutes. The eggs were then placed in 1 L beakers of sterile, filtered seawater and left for 10 minutes. They separated into two distinct layers. Buoyant fertilised eggs floated to the top and dead, unfertilised eggs settled on the bottom of the beaker. The number of buoyant eggs was

estimated by taking a 0.5 ml sample, counting the eggs present in this sample, and then multiplying it by the total volume that they occupied. On average there were 390 eggs per 0.5 ml sample (780 eggs ml⁻¹). The buoyant eggs were added to the rearing tanks after they were brought to the temperature of the rearing tank whilst kept in a 1 L beaker. A sample of the eggs was used to test the effect of temperature on the embryonic development.

Experimental design

Embryonic development

Four multiwell tissue culture plates, each consisting of twelve wells, were each placed in three different temperature controlled incubators ($n=48$ at 15°C, 20°C, 25°C). A single fertilised egg was placed in each well. Over a 72 hour period the timing to the specific stages was noted using a stereomicroscope (Nikon SMZ1500, fitted with a HR Plan Apo (1X) WD 54 lens). The eggs were assessed hourly for the first 12 hours, then every 6 hours, until hatching. The percentage of eggs at a specific stage and the mortalities at each stage were recorded.

Larval development

Approximately 3800 fertilised eggs were added to each 50 l rearing tank (76 larvae l⁻¹). This was achieved by aliquoting approximately 5 ml of eggs (780 eggs ml⁻¹) into each tank to obtain the desired larval stocking density. The temperature treatments had four replicates each of the three temperatures of 15°C, 20°C and 25°C. All eggs were from the same batch (first 24 hours). The temperature experiment was run using the intensive rearing method, where there was a high density of larvae using the 'pseudo green water' technique.

Larvae ($n=5-10$) were randomly selected for a sample every 3 days at 9:00 am. The sampling started when the eggs had hatched (0 DPH), making the sampling days between temperatures different. Five to ten larvae were taken from each tank and photographed using a Nikon Digital Sight Fi 1 digital camera attachment on a stereomicroscope (Nikon SMZ1500, fitted with a HR Plan Apo (1X) WD 54 lens). The total length (TL-length of fish from tip of the lower jaw to the caudal fin) (Mihelakakis *et al.* 2001; Fielder *et al.* 2005),

absorbance of the yolk-sac and oil globule, repletion rate (presence of food in the gut), appearance of swimbladder and tail flexion were recorded using the image analysis software NIS Elements Basic Research 3.2. Both the yolk-sac axes (L - major length, H - minor length) and oil globule diameter (d) were measured in order to calculate the volume of each. Using the equations suggested by Blaxter and Hempel (1966) and Cetta and Capuzzo (1982), the yolk-sac volume (VYS) and the oil globule volume (VOG) were calculated to the nearest cubic millimeter.

To calculate the volume (VYS), both the lengths of the axes of the spheroid yolk-sac were measured and placed into equation 1:

$$VYS = 4/3\pi(L/2)(H/2)^2 \quad \text{Equation 1}$$

To find the volume of the oil globule (VOG), the diameter was measured and used in equation 2:

$$VOG = 4/3\pi(d/2)^2 \quad \text{Equation 2}$$

The Specific Growth Rate (SGR) for different periods in the larvae's early life was calculated using equation 3:

$$SGR (\%) = 100 \times \left(\frac{\ln TL_2 - \ln TL_1}{\text{time}} \right) \quad \text{Equation 3}$$

where TL_1 and TL_2 indicate the initial and final mean total length (mm), respectively (Koumoundouros *et al.* 1999; Biswas *et al.* 2005). Time refers to the number of days between TL_1 and TL_2 .

At the end of the experiment (30 DPH) the surviving larvae per tank were counted and weighed on a mass balance to the nearest 0.001 mg. The presence of a swimbladder was assessed under a microscope. When calculating survival all replicate tanks were taken into account and the total number of larvae sampled from each specific tank was subtracted

from the total number added at the start of the experiment. The mean survival percentage per temperature was then calculated.

Final survival was calculated for each tank at the end of the 30-day rearing period using equation 4:

$$\% \textit{Survival} = \left(\frac{N_f}{N_i} \right) * 100 \quad \text{Equation 4}$$

Where N_f refers to the final number of larvae present and N_i to the initial number of fertilised eggs added. With this information the mean survival percentage per treatment was calculated.

Tank setup

The 50 L rearing tanks were randomly numbered from 1 to 12. Of these twelve tanks there were four replicates for each temperature treatment. The tanks were heated individually and as such were totally independent of each other. The tanks were supplied with semi-recirculated sea water that was treated via a sand filter, bio-filter and protein skimmer. The water then entered reservoirs above the tanks. The 15°C reservoir was attached to a water-chiller that recirculated the water keeping it at the required temperature. The reservoir used to supply water to the 20°C and 25°C treatment tanks contained two heaters that were set at 20°C. Heaters were set at 20°C and 25°C and placed inside each 20°C and 25°C treatment tank to keep the water at the correct temperature.

Each tank had a centre standpipe surrounded by a 250 µm mesh barrier cylinder. The air and water supply was placed inside this cylinder as to not cause injuries, mortalities and inhibit the larvae's movement. Surface skimmers were not used in the rearing tanks as the standpipe allowed the water to overflow from the surface. The flow rate into the 20°C and 25°C experiment tanks was kept constant at approximately 150 ml min⁻¹, while in the 15°C tanks the flow rate was approximately 300 ml min⁻¹. This was done to ensure that the room temperature did not affect the water temperature in the tanks. During the first 10 DPH the flow rate in all tanks was reduced to half, to ensure the larvae were not impeded by the current and were able to feed successfully on the exogenous food sources.

Feeding and addition of microalgae

All larvae in the experiments were fed rotifers, *Brachionus plicatilis*, enriched with microalgae, *Nannochloropsis oculata*, at 9:00 hours and 15:00 hours every day from 3 DPH to 30 DPH. These additions helped to maintain the correct density of approximately 10 rotifers ml⁻¹. The rotifers were added by hand from a culture tank that had similar salinity to the experimental tanks and a water temperature of 18-20°C.

The 'pseudo green water' technique was used and *Nannochloropsis* was first added at 3 DPH to all the experiment tanks. The concentration was kept at approximately 6-12x10⁶ cells ml⁻¹ by daily additions. It was added at the same time as the rotifers. The microalgae and rotifers were added to all tanks a day earlier than recommended to allow the more developed larvae at 25°C, and possibly at 20°C, to successfully initiate first-feeding. *Artemia* sp. nauplii were fed to the larvae at 9:00 h and 15:00 h from 17 DPH until the end of the experiment. *Artemia* numbers were assessed and adjusted to 3-5 individuals ml⁻¹ using a stock culture of nauplii. These were added to the tanks by hand.

Water quality measurement

Temperature, salinity, pH and DO₂ were measured on every day of sampling to the nearest 0.1°C, 0.1‰, 0.1 pH unit and 0.1 mg L⁻¹, respectively using a water quality probe (WTW Multi 350i). Light intensity (lux) was measured using a digital light meter (Toptronic T630).

Data analysis

The data from the larvae from the first 6 DPH were analysed separately, as this was the only time in the experiment that there were enough larvae from each tank to represent the growth and development at each temperature. After this time there were not enough larvae from the 25°C rearing tanks to provide a representative sample. The larvae from the 15°C and 20°C tanks were subsequently assessed together and compared from 0 DPH to 30 DPH. All measurements are given as the mean ± standard error followed by the measured range in parentheses.

For all analyses statistical significance was accepted at $P < 0.05$. Null hypotheses were rejected at $P < 0.05$. The effect of temperature on the growth of the larvae was analysed using the statistical computing environment R (R Development Core Team, 2012). The R package 'nlme' was used to realise the fitting of linear mixed-effect models. A linear mixed-effect model was used to assess if there was any significant difference between larval growth at the three treatment temperatures. There were three treatments in the experiment being temperature (15°C, 20°C and 25°C). The effect of the tank was a nested variable inside the temperature variables and was specified as a random variable. This was done to assess if the tank had a significant effect on the variable being analysed. Length data were ln transformed to be able to assess the linear relationship over time per replicate tank from each treatment. By comparing the slopes of the linear trendlines the effect of the random variable of tank was assessed.

Regression analyses were conducted and the growth equation for total length was calculated for 20°C and 15°C treatments.

Ogives were created to assess the effect of temperature on the proportion presence of swimbladder inflation, repletion rate and notochord flexion. The day-at- (50%) presence (d_{m50}) was determined using the total number of the sampled larvae per tank per sampling day and assessing the success and failure of the morphological changes mentioned above. The proportion of the larvae at the specific DPH and the day-at-(50%) presence was estimated by fitting it to a two-parameter logistical model:

$$P(d) = 1 + \exp\left(-\frac{(d_2 - d_{m50})}{\delta d}\right)^{-1} \quad \text{Equation 5}$$

In the model $P(d)$ refers to the proportion of larvae that have undergone the morphological changes of feeding, swimbladder inflation and notochord flexion at day post hatch d and δd represents the steepness parameter of the logistic ogive. The maximum likelihood estimates for feeding, swimbladder inflation and notochord flexion were fitted by minimizing the negated binomial log-likelihood function.

Swimbladder inflation never reached a proportion of 1 for the larvae reared at 15°C and 20°C. Therefore the asymptotic presence (p_{∞}), which is normally set at 1 when all the sampled larvae have swimbladder inflation, feeding or flexion present, is estimated as an additional parameter (Winker *et al.* 2012). Thus making the d_m50 the size at which half the population attained p_{∞} .

4.3. RESULTS

During the larval rearing experiment the mean values of salinity, pH and DO₂ were similar for all groups within the same temperature and between the different temperatures. Salinity had a mean value of 33.9 ± 0.2 ‰ (min-33.2 ‰; max-34.5 ‰) ($n=132$), pH had a mean value of 6.55 ± 0.12 pH units (min-6.4 pH units; max-6.9 pH units) ($n=132$) and DO₂ had a mean value of 6.05 ± 0.25 mg.L⁻¹ (min-5.6 mg.L⁻¹; max-6.5 mg.L⁻¹) ($n=132$). The temperature within the tanks fluctuated within 1°C of the specified temperatures. The light intensity at the surface of the tanks had a mean value of 653 ± 10.9 lux (min-600 lux; max-700 lux) ($n=12$).

Larvae in the 25°C replicate tanks showed high mortalities after 6 DPH and only six fish survived until the end of the experiment at 30 DPH. Therefore, the data captured only from the three first samplings (0 DPH – 6 DPH) were used to assess the growth and development of the larvae at 25°C. The 25°C data were not used in the overall analysis to assess the effect of temperature on the growth and condition of the larvae.

Embryonic Development:

Timing of embryonic development stages

The sequence of development stages of the eggs was the same at all three temperatures, although the time from fertilisation to hatching decreased as temperature increased (Figure 4.1). At 15°C, the first signs of hatching occurred at 48 hours after fertilisation, while at 20°C and 25°C, hatching occurred at 36 hours and 30 hours, respectively. The timing of the cell divisions for the first 3 hours was similar at all three temperatures, with all eggs at the morula stage after this time. After this time there was a far greater time difference in

development stages between the three temperatures. The longest time between stages at all three temperatures was between the gastrula stage and the appearance of the embryo.

Mortalities

The highest percentage of mortalities occurred at 15°C, with 68.75% of the eggs dying.

Mortality percentage at 20°C and 25°C was 54.17% and 58.33%, respectively.

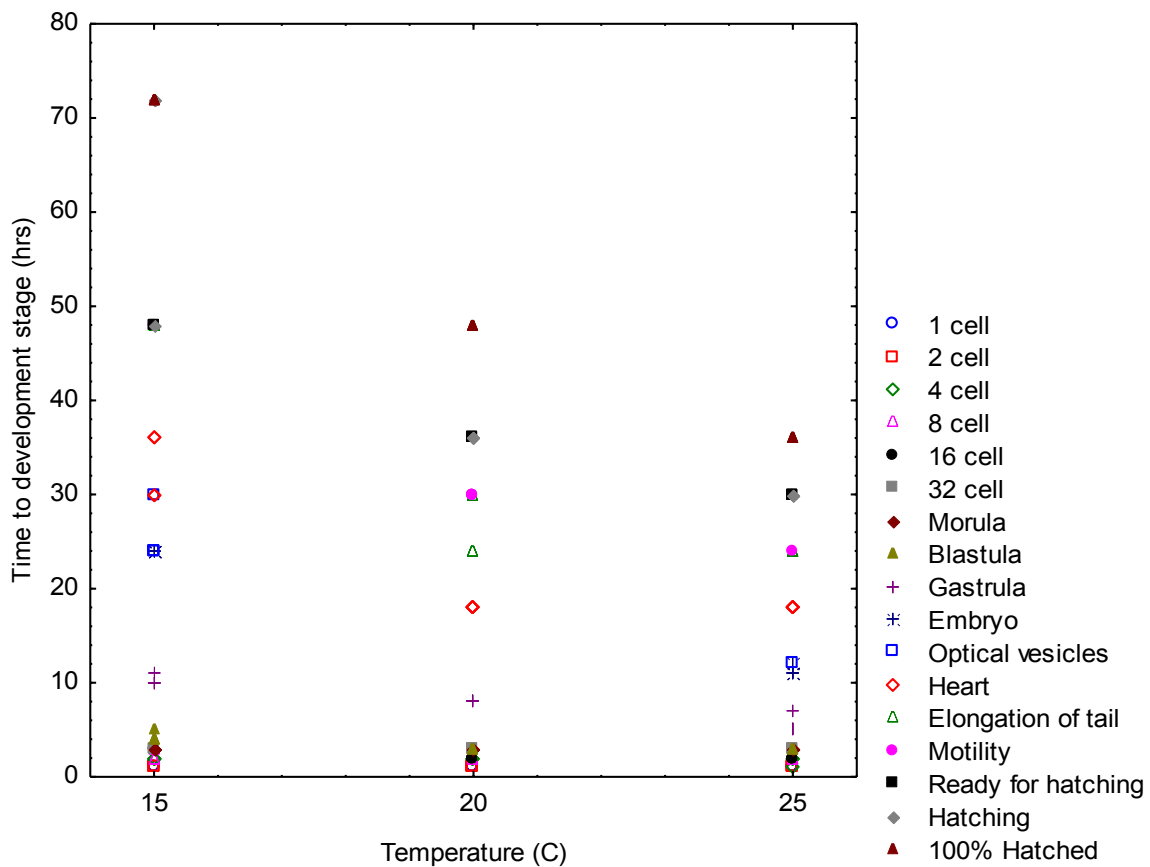


Figure 4.1: Sequence (time to development stage) of the main stages in the embryonic development of *Rhabdosargus globiceps* at three different incubation temperatures.

Larval development:***Yolk-sac and oil globule absorption***

The yolk-sac volumes of the larvae kept at the three temperatures were initially different at hatching (0 DPH). The results suggest an inversely proportional relationship between initial yolk-sac volume and temperature. At 15°C the larvae hatched with a mean yolk-sac volume of $0.172 \pm 0.01 \text{ mm}^3$ (min-0.132 mm^3 ; max-0.244 mm^3) ($n=10$), at 20°C the larvae hatched with a mean yolk-sac volume of $0.156 \pm 0.007 \text{ mm}^3$ (min-0.125 mm^3 ; max-0.191 mm^3) ($n=10$) and at 25°C the larvae hatched with a mean yolk-sac volume of $0.150 \pm 0.014 \text{ mm}^3$ (min-0.095 mm^3 ; max-0.231 mm^3) ($n=10$).

The rate at which the yolk-sac was used was directly proportional to the temperature, with the larvae at 25°C having the fastest depletion rate (Figure 4.2). The greatest depletion of the yolk-sac for all larvae occurred between 0 DPH and 1 DPH. At this time the larvae at 25°C had the largest mean yolk-sac volume loss of 0.125 mm^3 (82.9%), while the larvae at 20°C and 15°C had a mean yolk-sac volume loss of 0.107 mm^3 (68.65%) and 0.111 mm^3 (65.74%), respectively (Figure 4.2). The yolk-sac was completely depleted at 5 DPH at 15°C, 4 DPH at 20°C, and 3 DPH at 25°C.

From the time of hatching to the full absorption of the yolk-sac and oil globule (0 DPH – 6 DPH) the SGR for the larvae at 15°C, 20°C and 25°C was 3.19%, 4.13% and 4.44%, respectively. The depletion of the oil globule followed the same pattern as yolk-sac depletion with the larvae at 25°C having the fastest rate of absorption and the larvae at 15°C having the slowest (Figure 4.3). However, the greatest loss of volume at 20°C and 25°C occurred between 1 DPH and 2 DPH, whereas at 15°C the greatest loss of volume occurred between 2 DPH and 3 DPH. The oil globule was completely depleted at 6 DPH, 5 DPH and 5 DPH at 15°C, 20°C and 25°C, respectively.

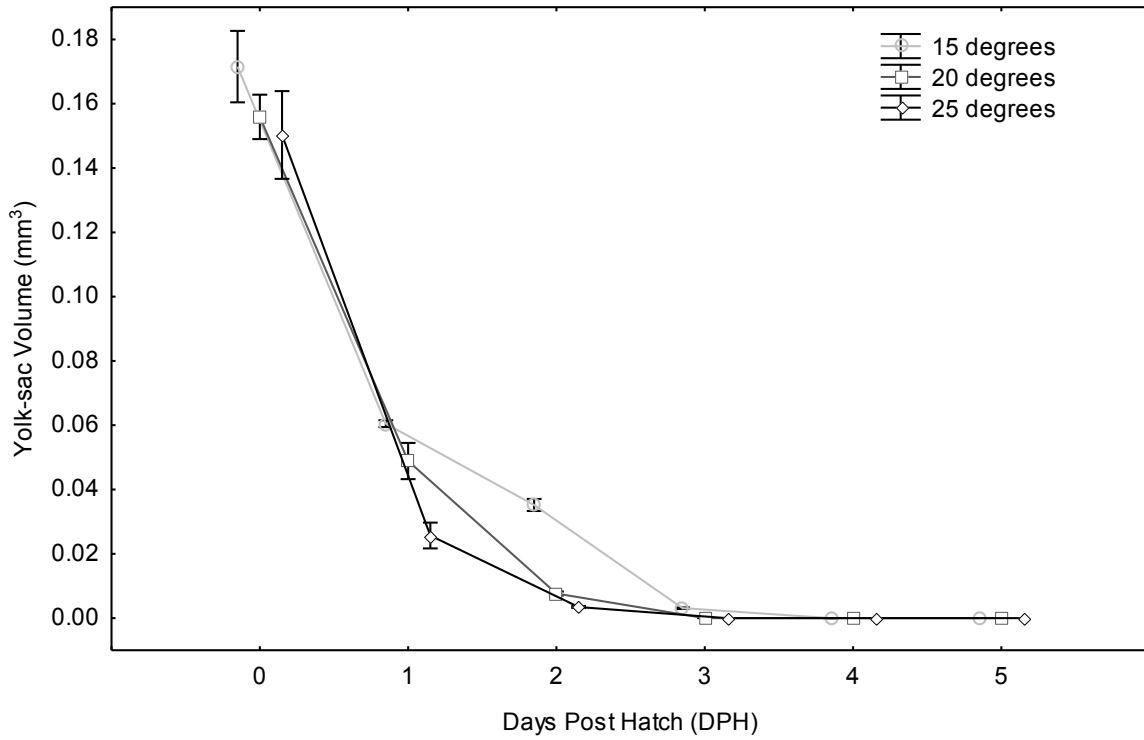


Figure 4.2: Depletion of the yolk-sac (mean \pm SE) in *Rhabdosargus globiceps* larvae over the first 5 DPH from larvae reared at 15°C, 20°C and 25°C.

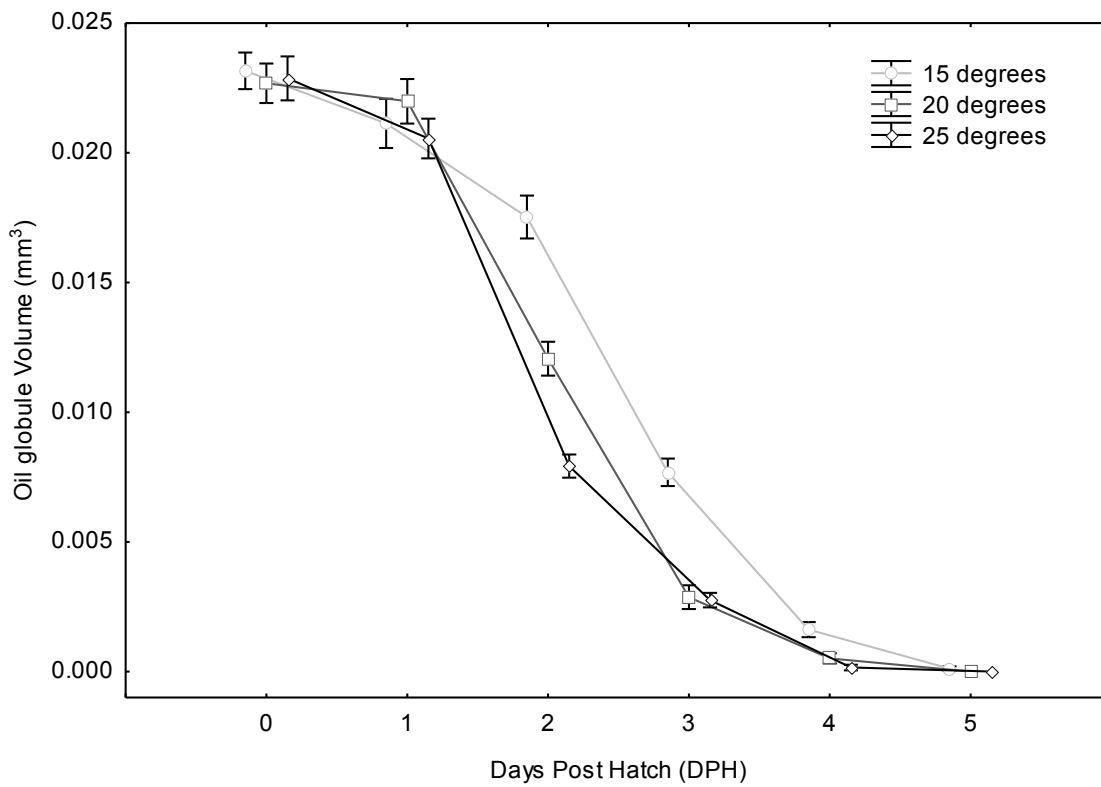


Figure 4.3: Depletion of the oil globule (mean \pm SE) in *Rhabdosargus globiceps* over the first 5 DPH from larvae reared at 15°C, 20°C and 25°C.

Abnormalities

A small proportion (7%) ($n=30$) of the sampled larvae reared at 25°C showed deformities, in regards to the position of the oil globule (Figure 4.4). The oil globule was found to not be on the same position as the larvae reared at 15°C and 20°C. These larvae were not used in the total length, yolk sac or oil globule measurements.

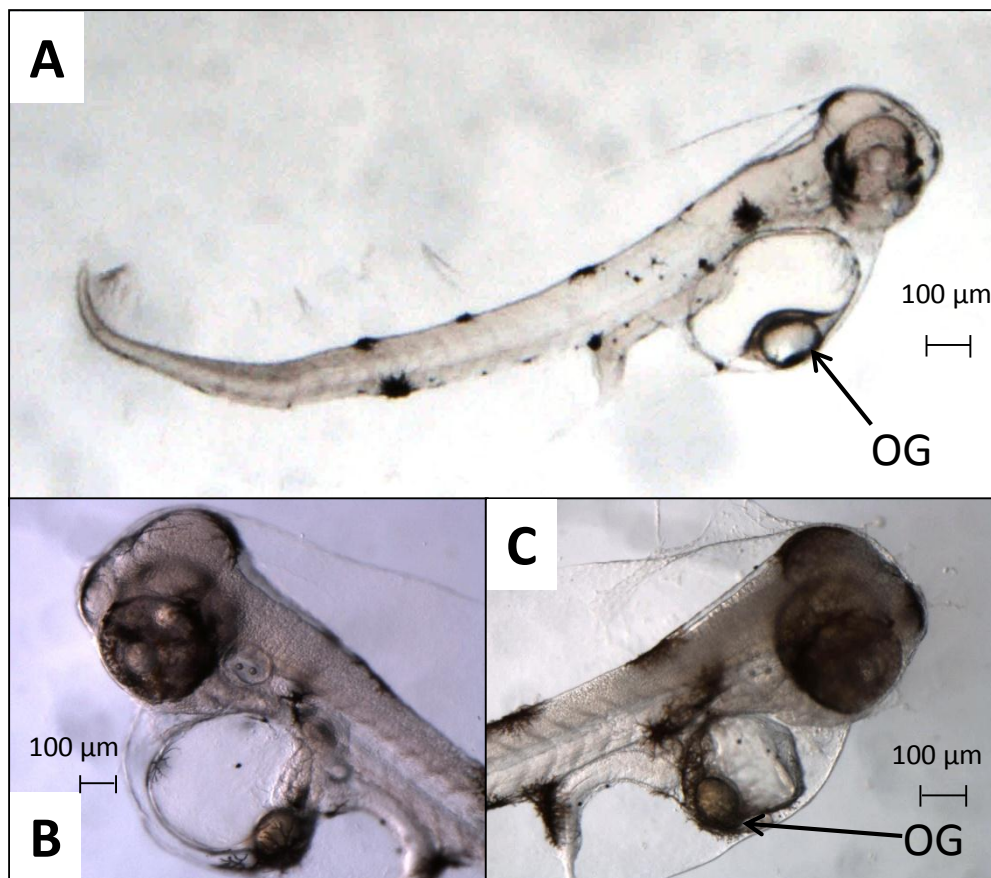


Figure 4.4: A – 1 DPH *Rhabdosargus globiceps* larvae reared at 25°C. Oil globule (OG) positioned at the bottom of the yolk-sac (abnormal position). B – 2 DPH *Rhabdosargus globiceps* larvae reared at 15°C. Oil globule positioned correctly. C – 2 DPH *Rhabdosargus globiceps* larvae reared at 20°C. Oil globule positioned correctly.

Repletion rate

Food was first apparent in the larvae reared at 25°C. Microalgae and rotifers were present in the gut of $12.5 \pm 9.5\%$ (min-0%; max-40%) ($n=40$) of the sampled larvae at 3 DPH (Figure 4.5). No larvae from the 20°C or 15°C rearing tanks had food in their guts at 3 DPH. However, at 6 DPH a mean of $71 \pm 9.9\%$ (min-57%; max-100%) ($n=24$) of the sampled larvae from the 20°C rearing tanks and $56.8 \pm 14.3\%$ (min-20%; max-90%) ($n=27$) from the 15°C rearing tanks had fed (Figure 4.5). At 6 DPH the percentage of larvae with food in their guts from the 25°C rearing tanks was $57.3 \pm 10.5\%$ (min-29%; max-80%) ($n=22$).

The percentage of feeding larvae increased over the 30 day period, with the larvae reared at 20°C and 15°C reaching 100% of larvae fed by 15 DPH. The percentage of larvae that had fed at 15°C varied between 80 and 100% from 15 DPH to 30 DPH respectively, while the percentage of fed larvae at 20°C varied between 95 and 100%.

The results from the ogive two-parameter logistic model show that the larvae reared at 15°C had a day-at-(50%) presence of feeding after 7.88 DPH ($\delta_d = 3.53 \text{ day}^{-1}$, Figure 4.5). Larvae reared at 20°C had a day-at-(50%) presence of feeding after 6.94 DPH ($\delta_d = 2.33 \text{ day}^{-1}$, Figure 4.5). The effect of temperature on the proportion of larvae that were actively feeding was significantly different between 15°C and 20°C ($\chi^2 = 7.37$, $df = 2$, $P = 0.025$).

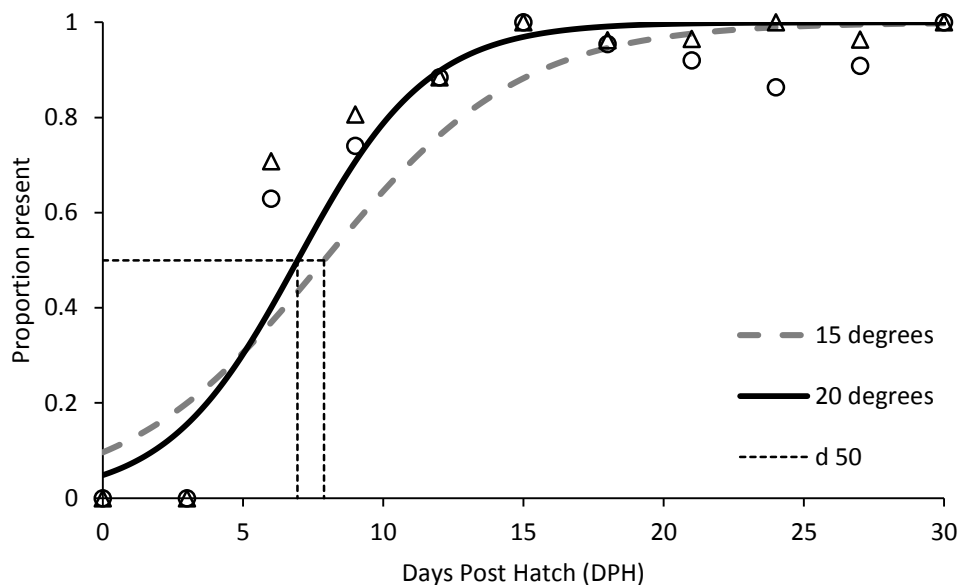


Figure 4.5: Two-parameter logistical model fitted to the observed proportion of sampled *Rhabdosargus globiceps* larvae from both 15°C and 20°C tanks that were shown to be feeding over the period of 0 DPH to 30 DPH. ($\chi^2 = 7.37$, $df = 2$, $P = 0.025$). From 15°C, $n=301$. From 20°C, $n=320$.

Swimbladder inflation

Swimbladder inflation was first apparent on 3 DPH from the larvae sampled from the 25°C tanks at a frequency of $2.5 \pm 2.5\%$ (min-0%; max-10%) ($n=30$). At 6 DPH the percentage of larvae with inflated swimbladders at 25°C and 20°C were $12.25 \pm 7.3\%$ (min-0%; max-29%) ($n=22$) and $14.25 \pm 9.5\%$ (min-0%; max-40%) ($n=24$), respectively, with no presence of swimbladders in the sampled larvae reared at 15°C (Figure 4.6). Swimbladders first appeared in the sampled larvae reared at 15°C at 9 DPH with a frequency of $36 \pm 6.4\%$ (min-17%; max-44%) ($n=27$). For the remainder of the experiment the percentage of swimbladder inflation from the sampled larvae from the 20°C tanks differed significantly to that from the 15°C tanks. The percentage of swimbladder inflation at 15°C was lower than that found at 20°C from the first appearance to 30 DPH (Figure 4.6). Neither of the groups of sampled larvae had a 100% record of successful swimbladder inflation over the rearing period.

The results from the ogive two-parameter logistic model show that the larvae reared at 15°C had a day-at-(50%) presence of swimbladder inflation after 9.19 DPH ($\delta_d = 1.26 \text{ day}^{-1}$, Figure 4.6), while larvae reared at 20°C had a day-at-(50%) presence of swimbladder inflation after

7.63 DPH ($\delta_d = 0.85 \text{ day}^{-1}$, Figure 4.6). The effect of temperature on the proportion of larvae with swimbladder inflation was found to be significantly different between 15°C and 20°C ($\chi^2 = 28.86$, $df = 3$, $P < 0.0001$).

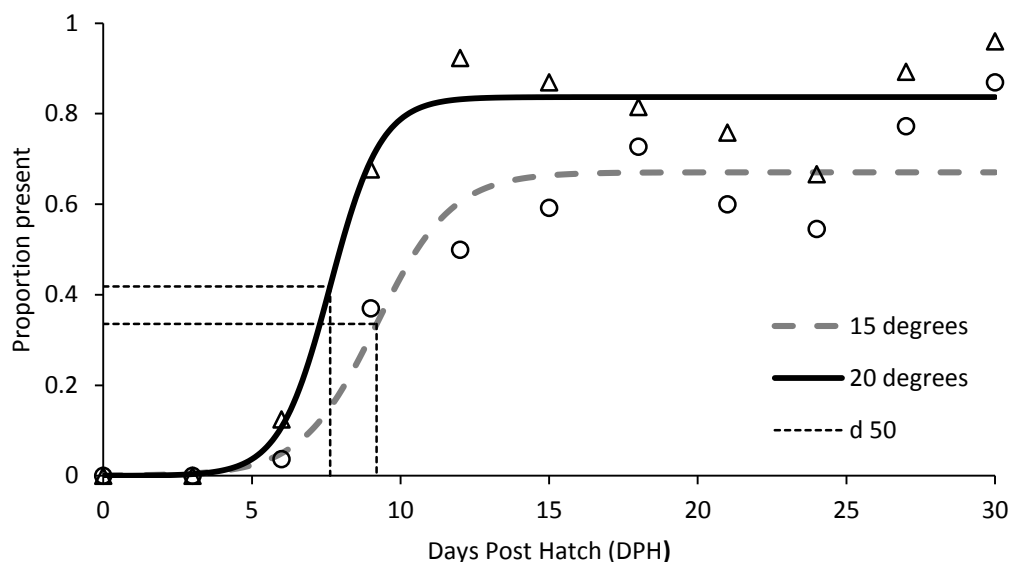


Figure 4.6: Two-parameter logistical model fitted to the observed proportion of sampled *Rhabdosargus globiceps* larvae from both 15°C and 20°C tanks that have achieved swimbladder inflation over the period of 0 DPH to 30 DPH. ($\chi^2 = 28.86$, $df = 3$, $P < 0.0001$). From 15°C, $n = 301$. From 20°C, $n = 320$.

Notochord flexion

Notochord flexion was first noticed in the sampled larvae from both 15°C and 20°C rearing tanks at 15 DPH (Figure 4.7). The larvae reared at this time at 20°C had a flexion percentage of $51.75 \pm 13.65\%$ (min-20%; max-80%) ($n=22$), while $19.25 \pm 9.23\%$ (min-0%; max-44%) ($n=27$) of the larvae reared at 15°C had undergone flexion. Notochord flexion frequency reached 100% for sampled larvae from the 20°C rearing tanks at 18 DPH. At 15°C the sampled larvae only reached 100% notochord frequency at 24 DPH.

Larvae reared at 15°C had a day-at-(50%) presence of notochord flexion on 17.54 DPH ($\delta_d = 1.52 \text{ day}^{-1}$, Figure 4.7). Larvae reared at 20°C had a day-at-(50%) presence of notochord flexion on 14.98 DPH ($\delta_d = 0.18 \text{ day}^{-1}$, Figure 4.7). The effect of temperature on the

proportion of larvae with notochord flexion was found to be significantly different between 15°C and 20°C ($\chi^2 = 33.44$, $df = 2$, $P < 0.0001$).

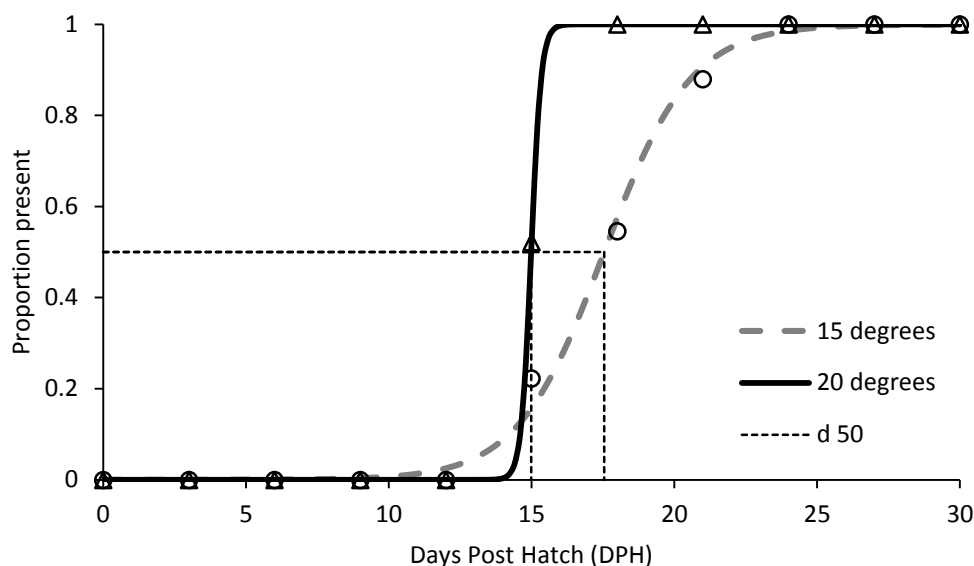


Figure 4.7: Two-parameter logistical model fitted to the observed proportion of sampled *Rhabdosargus globiceps* larvae from both 15°C and 20°C tanks that had undergone notochord flexion over period of 0 DPH to 30 DPH. ($\chi^2 = 33.44$, $df = 2$, $P < 0.0001$). From 15°C replicate tanks, $n = 301$. From 20°C replicate tanks, $n = 320$.

Growth

Temperature had a significant effect ($P < 0.05$) on the growth rate between the larvae reared at 15°C and 20°C (Figure 4.10). At hatching the sampled larvae from the 20°C and 15°C rearing temperatures had the same mean total length of 2.29 mm, with SE of ± 0.02 mm (min-2.01 mm; max-2.38 mm) and ± 0.01 mm (min-2.03 mm; max-2.37 mm) ($n=40$), respectively. The larvae reared at 25°C were slightly smaller at 2.17 ± 0.02 mm (min-1.95 mm; max-2.33 mm) ($n=40$) at hatching. From 6 DPH onwards the larvae reared at 20°C were greater in mean total length than the larvae reared at 15°C. The average SGR from 0 DPH to 6 DPH at 15°C, 20°C and 25°C was 7.45%, 9.64% and 10.36%, respectively (Table 4.1). Over the entire experiment, from 0 DPH to 30 DPH the average SGR at 15°C and 20°C was 12.46% and 14.98% respectively. At 30 DPH the larvae reared at 20°C had reached a total length of

11.87 ± 0.34 mm (min-9.21 mm; max-14.92 mm) ($n=25$), compared to the larvae reared at 15°C that had a mean total length of 9.01 ± 0.23 mm (min-5.87 mm; max-10.63 mm) ($n=23$) (Figure 4.8).

Table 4.1: Mean specific growth rate (SGR) of *Rhabdosargus globiceps* (% increase day⁻¹) at three treatment temperatures.

SGR (% increase day ⁻¹)	15°C	20°C	25°C
0 – 6 DPH	7.45	9.64	10.36
0 – 30 DPH	12.46	14.98	NA

Exponential growth equation for larvae reared at 20°C

$$y = 2.2785e^{0.0565x} \quad (R^2 = 0.967, n=320)$$

Exponential growth equation for larvae reared at 15°C

$$y = 2.2765e^{0.0466x} \quad (R^2 = 0.9497, n=301)$$

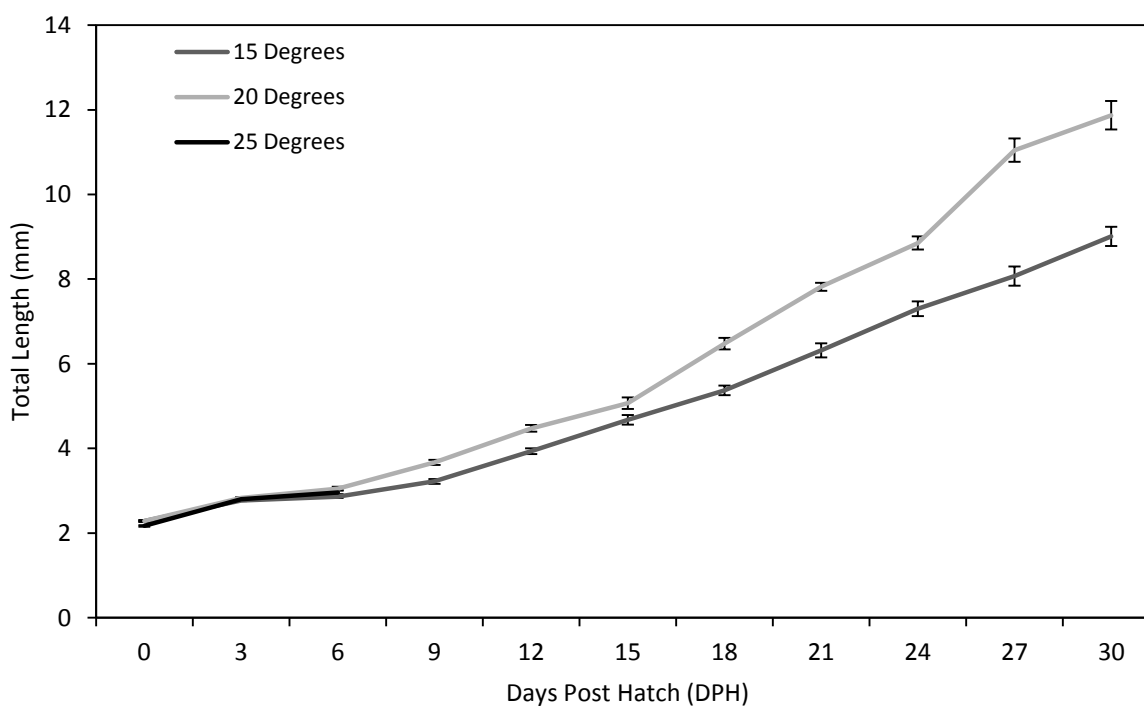


Figure 4.8: Mean total length ± SE of *Rhabdosargus globiceps* from 0 DPH to 30 DPH reared at 15°C, 20°C and 25°C.

Comparing the growth of the larvae between the replicate tanks allowed for the random effect of the tanks to be assessed. Tanks of the same temperature had very similar linear slope values (Figure 4.9). This calculated linear slope value for each tank shows that the effect of the tanks was slight. The linear slopes for the 20°C tanks are greater than the linear slopes for the 15°C tanks, indicating a faster growth rate (Figure 4.9).

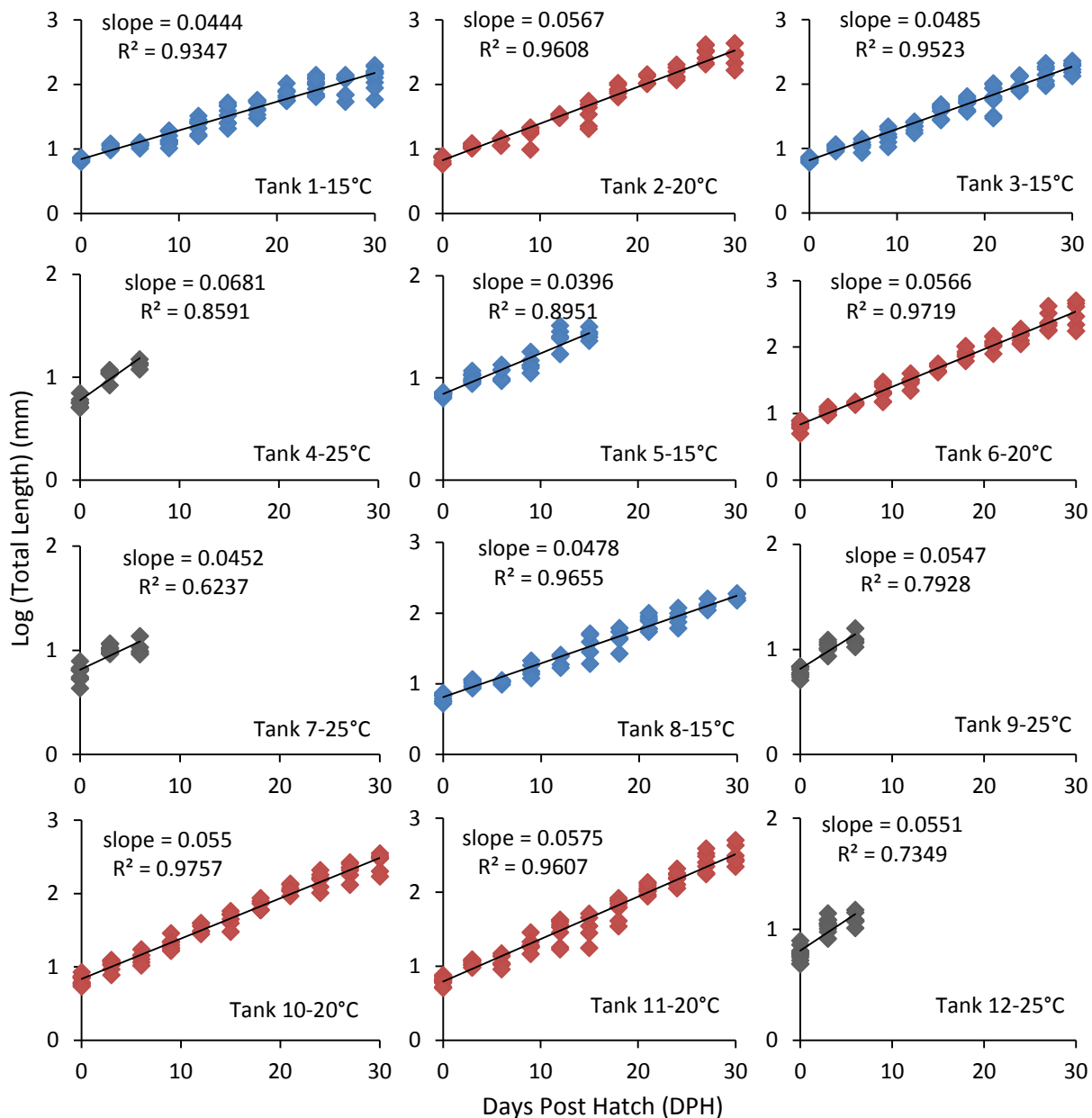


Figure 4.9: Log transformed data of total length illustrating the slope gradient per tank. Tank numbers are arranged from left to right. 15°C tanks are blue. 20°C tanks are red and 25°C tanks are grey. Repeated samples were taken from 0 DPH to 30 DPH. Insufficient larvae to sample from 6 DPH for all 25°C tanks and from 15 DPH for tank 5.

The results gained from the R-analysis clearly show that the difference in temperature between the rearing tanks had a significant effect on the growth rate of the larvae (Figure 4.10). The significant difference in growth was first recorded at 6 DPH.

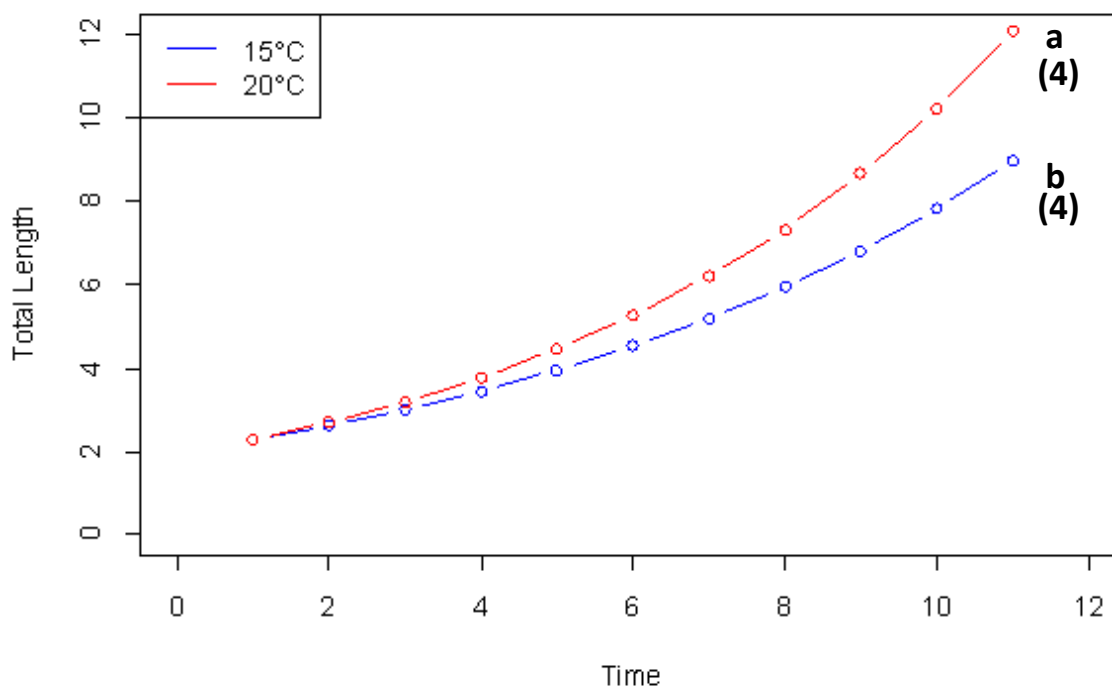


Figure 4.10: Results from the linear mixed-effects model. Larvae had the same initial start point. Larvae reared at 20°C had significantly greater growth than larvae reared at 15°C. Points with different letters are significantly different ($P < 0.05$); (n) = number of tanks sampled. The x-axis of Time refers to the sample number.

Biological performance

Temperature had a significant effect on the survival of the larvae. Only a total of 6 individuals survived from two of the 25°C tanks. The larvae reared at 15°C and 20°C had similar survival. A total of 111 individuals survived from three of the 15°C replicate tanks (Table 4.2). A total of 108 individuals survived from the four replicate tanks of the 20°C tanks (Table 4.2). These surviving larvae were used to record the final survival percentage, mean wet weight and mean percentage of swimbladder inflation per temperature. The wet weight of the larvae increased with the increase in temperature.

Table 4.2: Final percent survival, total length, wet weight, and swimbladder inflation of *Rhabdosargus globiceps* larvae grown at three different temperatures from 0 to 30 DPH.

Temperature (°C)	Survival (%)	Total length (mm)	Wet weight (mg)	Swimbladder inflation (%)
15	0.75 ± 0.47	9 ± 0.23	16.4 ± 0.8	86.96
	(0-2)(4)	(5.9-10.6)(3)	(10-23)(3)	(3)
20	0.73 ± 0.25	11.9 ± 0.34	45.2 ± 2.9	96
	(0.08-1.3)(4)	(9.2-14.9)(4)	(21-71)(4)	(4)
25	0.04 ± 0.032	16.2 ± 0.96	142.1 ± 28.6	100
	(0-0.13)(4)	(13.9-18.5)(2)	(69-241)(2)	(2)

Data are means ± standard errors. Range (min-max) is displayed in parenthesis. (n) Represents the number of replicate tanks.

4.4. DISCUSSION

This is the first study that has assessed the effect temperature has on the successful rearing of *Rhabdosargus globiceps* over the first 30 DPH. The egg and larval stages of teleosts are recognised as the most sensitive stages to environmental factors during their life cycle (Berlinsky *et al.* 2004). The rate of growth and development generally increases with temperature, until the thermal threshold is reached and either malformations or death occur (Berlinsky *et al.* 2004). The results from this research illustrate the significance of temperature during the embryonic development and the larval rearing process.

Larvae reared at 15°C and 20°C survived to 30 DPH, while the larvae reared at 25°C died post 6 DPH. The *Rhabdosargus globiceps* population in Saldanha Bay has a spawning season from September to February (Attwood *et al.* 2010), when water temperature is approximately 14.5 - 20°C (Clark *et al.* 2009). Kucharczyk *et al.* (1997) state that the suitable temperature for embryo development is closely correlated to the natural water temperature during the spawning season. The temperature range chosen to rear the larvae was therefore very similar to the ambient water temperatures present under natural spawning conditions. Optimal rearing temperature ranges also coincided with the natural spawning temperatures of other sea bream species, such as the silver sea bream, *Sparus sarba*, and *Pagrus auratus* (Fielder *et al.* 2005).

It has been noted by Battaglene and Talbot (1992) that metamorphosed *Pagrus auratus* did not show any sign of stress at temperatures up to 31.5°C, confirming that temperature has a greater effect on embryos and early larvae. This also suggests that the different stages in the fish's life may require different temperatures for optimal growth and development. Some of the effects of temperature on early larval development are time and size-at-hatching, yolk-sac and oil globule utilisation, growth, metabolic demand and feeding rate (Beitinger & Fitzpatrick 1979; Jobling 1981; Fuiman *et al.* 1998; Koumoundouros *et al.* 2001; Fielder *et al.* 2005; Radonic *et al.* 2005). Throughout the experiment the anticipated effect of accelerated development at a higher temperature was realised.

Egg quality directly affects the viability of the larvae (Radonic *et al.* 2005). The assessment of egg quality is crucial in order to explain if the low survival rate during larval rearing is a consequence of larval viability at hatching or egg quality (Giménez *et al.* 2006). The control

of egg quality for a potential aquaculture species is necessary for the development of reproduction techniques (Lahnsteiner & Patarnello 2004). Even commercially important cultured sea breams, such as *Sparus aurata*, *Pagrus pagrus* and *Dentex dentex*, produce eggs of variable quality, but because the broodstock spawn such a large number of eggs there is still a large number of larvae (Lahnsteiner & Patarnello 2004; Mylonas *et al.* 2004; Giménez *et al.* 2006). The quality of eggs and larvae is recognised as a constraint of production (Brooks *et al.* 1997).

The embryonic development of *Rhabdosargus globiceps* was affected by temperature, in terms of speed of development and the quality of larvae at hatching. As hypothesised, at a higher temperature there was a faster rate of development. The same relationship has been found in other sea bream species and other fish species (Kucharczyk *et al.* 1997; Klimogianni *et al.* 2004; Radonic *et al.* 2005). The larvae reared at 25°C displayed irregular positions of the oil globule and were smaller at hatching. Therefore, the higher temperature of 25°C produced larvae of a lower quality. The percentage of mortality was highest at 15°C, although the hatched larvae showed signs of good quality. Further work should be carried out on biochemical egg quality markers for *Rhabdosargus globiceps*.

Assessments of the hatched larvae at 0 DPH suggest that the temperature affected the embryo before hatching. The size of the larvae as well as the volume of the yolk-sac at hatching decreased with an increase in temperature. This suggests that the energy requirements of these embryos increased while inside the egg (Klimogianni *et al.* 2004), and that the growth of the embryo was impeded at higher temperature (Batty *et al.* 1993; Berlinsky *et al.* 2004). Additionally, the difference in depletion of endogenous food reserves and the fact that larvae reared at 25°C had the greatest SGR over the first 6 DPH (10.36 %) demonstrate the change in metabolic rate brought about by the high temperature.

The yolk-sac and oil globule lasted longer at 15°C than at 20°C and 25°C, thus supporting the larvae for a longer time through the transitional period and delaying the onset of active feeding. The timing of yolk-sac absorption and the efficiency with which the larvae use this energy can allow for more robust, larger larvae that can actively feed at an earlier stage (Berlinsky *et al.* 2004; Yufera & Darias 2007).

The faster use of endogenous food reserves at 25°C may have given these larvae the greatest SGR at the end of the yolk-sac stage, but they were still smaller in size than the larvae reared at 20°C. This effect of temperature has been recorded in *Dicentrarchus labrax*, where the larvae displayed high 'developmental plasticity' in their morphometry at hatching, onset of feeding and thereafter (Koumoundouros *et al.* 2001). The hatched and reared larvae in this present study showed similar occurrence of 'developmental plasticity' between the different rearing temperatures possibly due to physiological compensations between allometric growth and ontogenetic development (van Maaren & Daniels 2001).

At 25°C, the high metabolic rate coupled with abnormalities of the oil globule, that supplies needed lipid food reserves during the time of transition between endogenous and exogenous food sources, could be recognised as two main factors that led to the death of the larvae (Yufera & Darias 2007). However, at a high temperature there are many biological actions that could be impeded and affect survival, such as feeding and digestion, swimbladder inflation, notochord flexion and fin development (Fuiman *et al.* 1998). It has been recorded that high temperatures can accelerate the process of ontogeny more than the growth rate (Fuiman *et al.* 1998). This would explain the smaller size of the larvae reared at 25°C at 6 DPH and the appearance of swimbladder inflation at 3 DPH, a full sampling time earlier than the larvae reared at 20°C and 15°C. The larvae reared at 15°C and 25°C had a similar feeding percentage at 6 DPH, which may suggest that the small size of the larvae at 6 DPH may have impeded feeding. At 25°C the larvae could not function because of altered morphological ontogeny (Koumoundouros *et al.* 2001), therefore it can be said that the larvae reached their biological tolerable limit.

In terms of growth, the few larvae reared at 25°C that survived grew faster than the larvae reared at 20°C and 15°C. Larger and heavier larvae were produced at 25°C after 30 DPH. The surviving larvae were heavier than their counterparts in the 15°C and 20°C tanks and reached a wet weight of 142.1 ± 28.57 mg. This is three times the weight of the larvae from the 20°C tanks, and nine times larger than the weight of the larvae from the 15°C tanks, which reached a wet weight of 45.19 ± 2.94 mg and 16.44 ± 0.8 mg respectively. However, the success of survival of larvae reared at 20°C and 15°C illustrates the appropriate temperature window for early larval growth and development of *Rhabdosargus globiceps*.

When comparing the growth of the larvae reared at 20°C and 15°C, it could be shown that the larvae reared at 20°C performed significantly better. They were longer and heavier than the larvae reared at 15°C, with a slightly lower survival percentage, although only by 0.02%. These differences in mean TL appear as early as 6 DPH in the larval stage and as the larvae get older the gap widens. One of the factors that it could be attributed to is thyroid function, as this is recognised as a key regulator of metamorphosis (Tanaka *et al.* 1998). At a higher temperature the thyroid function is stimulated, thus accelerating development relative to growth, meaning that the size at metamorphosis is decreased with an increase in temperature (Tanaka *et al.* 1998). With larger and more morphologically developed larvae the likelihood of successful active feeding is increased. Future studies should test this hypothesis.

The elevated temperature not only increased the growth, use of endogenous food reserves and reduced the time to hatching, but it also accelerated the time to active feeding and the critical development of morphological features, such as swimbladder inflation and notochord flexion. The timing of these ontogenetic events can also be altered due to quality and quantity of food sources (Koumoundouros *et al.* 1999), but these other parameters could not have contributed significantly to the observed changes in morphology and ontogeny, as they were similar between treatments. Similarly, the effect of the genotype on larval performance should also be excluded, as the treatment populations were from the same batch of eggs of common origin (Koumoundouros *et al.* 2001). These parameters can be used to assess the overall health of the larvae.

Active feeding, swimbladder inflation and notochord flexion were recorded significantly earlier and at higher percentages in larvae reared at 20°C than at 15°C. The day-at-(50%) presence for feeding, swimbladder inflation and notochord flexion at 20°C was 6.94 DPH, 7.63 DPH and 14.98 DPH respectively, while at 15°C they occurred at 7.88 DPH, 9.19 DPH and 17.54 DPH, respectively. The earlier onset of feeding, swimbladder inflation and notochord flexion for the larvae reared at 20°C allowed growth to increase and a gap between the larvae reared at 15°C to form. With the earlier onset and higher percentage of these features in the larvae reared at 20°C, the condition of the larvae improved.

To conclude, this chapter provided evidence that varied temperatures cause 'developmental plasticity' to occur, which had a significant effect on the growth and overall development of the *Rhabdosargus globiceps* eggs and larvae. The sequence of development stages of the eggs was the same at all three temperatures, although the time taken from fertilisation to hatching decreased as temperature increased. Due to the lowest mortality percentage between the treatments, 20°C is the recommended temperature for incubation. At 25°C survival of the larvae was poor, indicating that the larvae reared at this temperature reached and exceeded their thermal tolerable limit. Larvae reared at 20°C and 15°C showed relatively good survival rates with larvae at 15°C having a slightly better survival. However, the larvae reared at 20°C had a significantly better growth rate and a higher proportion of the larvae were more successful when actively feeding and initiating swimbladder inflation, thus producing a higher quality product in a shorter time. As with many other teleosts, *Rhabdosargus globiceps* showed that at very high temperatures the morphological development was impeded, but at an appropriate high temperature, within the range of the species thermal tolerance, the morphological development was accelerated (Fuiman *et al.* 1998; van Maaren & Daniels 2001). The recommended rearing temperature, taking into account the trade-off that occurs between survival and growth, for *Rhabdosargus globiceps* would be 20°C.

CHAPTER 5

Conclusion

The biological feasibility of *Rhabdosargus globiceps* as a potential aquaculture candidate was assessed by examining the fundamental aspects of reproduction and the early life stages. This endemic sea bream is found in the family Sparidae, which consists of species that have had considerable success in the international aquaculture industry. Some of these species include *Sparus aurata*, *Pagrus pagrus* and *Pagrus major* (Shields 2001; Basurco *et al.* 2011). It has been recognised that the production of eggs and the larval rearing phase provide the greatest threat to loss of quality and survival, and therefore have been recognised as the biggest bottlenecks in production for most aquaculture species (Dhert *et al.* 1998; Crespo *et al.* 2001; Zohar & Mylonas 2001). Consequently, this research represents the first documented investigation into aspects of broodstock conditioning, egg embryonation and larval rearing for *Rhabdosargus globiceps* to 30 DPH as an initial indication of the biological feasibility of this species as a prospective aquaculture candidate species in South Africa.

Rhabdosargus globiceps showed attributes of a good aquaculture species as well as biological feasibility of production. The wild-caught broodstock adapted to captive husbandry conditions without any difficulty and appeared unstressed and healthy for the entirety of the research without any mortality. They readily accepted commercial pelleted feed despite the frequency of handling due to the research objectives and general husbandry activities. Furthermore, the fish were not aggressive and displayed a calm nature, which can also be regarded as favourable traits for domestication and aquaculture. As a gonochoristic species the broodstock could easily be sexed according to colouration (Griffiths *et al.* 2002). The broodstock were successfully spawned on two occasions using compressed photothermal cycles and the larvae were successfully reared to post 30 DPH. The larvae were heavily sampled and data concerning their growth and development over this 30 day period were recorded in detail. Though the rearing experiments of the larvae

stopped at 30 DPH, the larvae were successfully reared to post 150 DPH and showed good tolerance to captive conditions.

Conditioning cycles using photoperiod and temperature regimes have been used successfully to achieve the induction of spawning with other fish species, including those used for aquaculture (Zanuy *et al.* 1986; Mañanós *et al.* 1997; Morehead *et al.* 2000). These environmental factors were chosen to be used to manipulate reproductive maturation in *Rhabdosargus globiceps*, as fish, especially at higher latitudes, show a strong synchronicity with the seasonal changes in daylength and the timing of development and maturation (Bromage *et al.* 2001; Pankhurst & Porter 2003). In all cases of induced spawning the manipulated conditions are those that coordinate with the internal endocrine processes allowing for the eggs to be released when conditions are optimal for the survival of the larvae. For temperate fish species found at the mid to high latitudes the start of spring provides the best conditions (Bromage *et al.* 2001).

Rhabdosargus globiceps in Langebaan Lagoon, from where the broodstock were collected for this study, followed the same period of synchronicity with the environmental conditions as shown by Attwood *et al.* (2010) and began spawning in August. The use of the 3 month compressed photothermal cycle, as determined by the natural conditions recorded from Langebaan Lagoon, proved successful with the captive broodstock showing correlated increases of estradiol 17 β concentration, oocyte diameter and oocyte maturation. These factors are recognised as good indicators of reproductive maturation (Mylonas *et al.* 2010). The ability to analyse the estradiol 17 β concentration through optimized ELIZA means that the reproductive maturation of *Rhabdosargus globiceps* can now be assessed without the need of invasive biopsies thereby reducing the need to handle the fish excessively and consequently reducing the associated stress that may have a negative effect on gametogenesis (Cleary *et al.* 2002).

The application of induced spawning techniques over 6 months, used in Chapter 3, and over a 3 month cycle, used in Chapter 2, were successful with good quality and number of eggs being produced from both spawnings. On the second induced spawning the estimated total number of fertilised eggs produced over the four days was 257600. This technique of

induction of spawning can be used to produce a supply of eggs on a year-round basis (Bromage *et al.* 2001).

Adapting the procedure for larval rearing of *Sparus aurata* for the rearing of *Rhabdosargus globiceps* larvae, with the use of the 'pseudo green-water' technique was shown to produce viable larvae (Moretti *et al.* 1999; Papandroulakis & Divanach 2002). The survival of *Rhabdosargus globiceps* larvae under these conditions displays the similarity of requirements of the larval at this stage to other aquaculture sea bream species. The addition of *Nanochloropsis oculata* microalgae helped to stabilise conditions in the tanks and provide enrichment for the rotifers in circulation. This method has been shown to produce survival two to five times better than the use of the clear water method (Papandroulakis & Divanach 2002).

The development of *Rhabdosargus globiceps* larvae showed no abnormalities regarding the ontogeny. As with other sea bream species such as *Sparus aurata*, *Porgy porgy*, and *Dentex dentex* the yolk-sac and oil globule play vital roles in the initial growth and development after hatching (Sarasquete *et al.* 1993; Conides & Glamuzina 2001; Santamaría *et al.* 2004). The larvae moved onto exogenous food sources successfully, although mortalities were high at this time. However, as mentioned in Chapter 3, this is a common occurrence at this time of rearing for the majority of aquaculture sea bream species. The timing to first-feeding, swimbladder inflation and notochord flexion was recorded and can be used for future experiments where the normal development of *Rhabdosargus globiceps* larvae is examined.

The performance of *Rhabdosargus globiceps* larvae at 15°C, 20°C and 25°C displays the significant effect temperature has on development and survival. The condition of the larvae is highly dependent on successful feeding and swimbladder inflation (Chatain 1989; Parra & Yúfera 2000). The 20°C treatment produced the best results, in terms of proportion of sampled larvae that had fed and initiated swimbladder inflation. The larvae reared at this temperature in Chapter 4 also had the best growth, reaching 11.87 ± 0.34 mm (min-9.21 mm; max-14.92 mm) ($n=25$) at 30 DPH, and had a mean survival percentage of $0.73 \pm 0.25\%$ (min-0.08%; max-1.29%) ($n=4$). Comparison to other aquaculture species and potential aquaculture species shows that total length and survival percentage is similar to that achieved from similar studies. Successfully cultured sea bream such as the two populations

of *Pagrus auratus*, found in Japan and Australia reached a total length of 13 mm and 11.8 mm at 29 DPH (Battaglione & Talbot 1992). Similarly, *Porgy porgy* and *Acanthopagrus latus* reach approximately 11 mm and 9.75 mm at 30 DPH (Mihelakakis *et al.* 2001). Garratt *et al.* (1989) reared santer seabream, *Cheimarius nufar* (Valenciennes, 1830), to a survival percentage of 0.1-0.5% after 150 days, and stated that this species may hold potential to be an aquaculture candidate. *Acanthopagrus latus* had a survival percentage ranging from 0.7-27.5% after 50-56 days (Leu & Chou 1996). Larval survival of *Rhabdosargus globiceps* will be improved with further optimisation of growing and larval rearing conditions as has been shown for other sea bream species that have been used in aquaculture for a longer time show better survival percentages. For example *Sparus aurata* has been shown to have a survival percentage of $11.7 \pm 1.31\%$ under optimum conditions at 32 DPH (Tandler *et al.* 1995).

From the two larval rearing experiments a total of 300 larvae survived. For the first trial of larval rearing of this species and after these populations were intensively sampled this is a relatively high number. The 75 survivors from the first rearing experiment (Chapter 3) are now displayed at the Two Oceans Aquarium, Cape Town, and the 225 survivors from the second rearing experiment (Chapter 4) are housed at the Department of Agriculture, Forestry and Fisheries (DAFF) Aquaculture Research Facility, in Seapoint, Cape Town, South Africa.

Future research recommendations

The overall production protocol and methods, from broodstock conditioning to larval rearing techniques need to be optimized. Various factors affect the quality of the eggs and the growth and development of the larvae. Narrowing down the best conditions, feed and techniques would allow for increased production of quality eggs and larvae and the reduction of cost per individual (Caballero *et al.* 1999; Lee 2003).

The use of controlled photoperiod and temperature cycles have been shown to induce oocyte maturation (Bromage *et al.* 2001; Biswas *et al.* 2010). However, this technique can result in not all broodstock becoming reproductively mature at the same time, which can affect egg collection and larvae production (Zohar & Mylonas 2001). More research into the

reproductive dysfunctions and alternate induction methods for *Rhabdosargus globiceps* needs to be done. The reproductive dysfunction that is most common in broodstock is the failure to undergo final oocyte maturation in females, and poor sperm production in males (Mylonas *et al.* 2010). Yet, using the sustained-release GnRHa-delivery system, that is embedded in the fish in the form of an implantable pellet of cholesterol, ethylene-vinylacetate implants or biodegradable microspheres, these dysfunctions can be reduced and the production of good quality eggs can be maximised (Mylonas *et al.* 2010). This hormone method stimulates the gradual release of pituitary luteinising hormone (LH) leading to the natural advancement of the plasma steroids that bring about oocyte maturation and spermiation (Mylonas & Zohar 2001). This method coupled with environmental cues could provide the most efficient way to induce maturation, ovulation and spawning. Methods of hormone injection are mostly done multiple times, which incurs much unwanted handling stress on the fish that can lead to a reduction in spawning capacity of the broodstock and reduction in the egg quality (Schreck *et al.* 2001; Cleary *et al.* 2002).

The biochemical composition of the eggs should be assessed to study if the artificially produced eggs differ to those from the wild (Brooks *et al.* 1997; Lahnsteiner & Patarnello 2004). A good quality batch of artificially produced eggs could then be identified. Biochemical composition of the eggs correlates closely with the larval development and survival percentage (Lahnsteiner & Patarnello 2004). By understanding the levels of biochemicals in good quality eggs, it would be known when batches of eggs can be discarded due to poor quality, thus saving on production costs and effort.

Larval rearing using intensive, semi-extensive and extensive methods can produce varied results in terms of growth and survival of a particular species (Divanach & Kentouri 2000). Due to the negative effect that high stocking densities can have on survival, growth and performance under intensive rearing conditions, many commercial farms are choosing to use semi-intensive (mesocosm) techniques (Leu & Chou 1996; Papandroulakis & Divanach 2002). The use of intensive rearing techniques has seen great success with sea bream species, however, these techniques are not seen as the most appropriate way to rear possible new species of which little is known (Divanach & Kentouri 2000). The semi-intensive rearing technique have been shown to solve the biological problems associated

with larval rearing and their human, technical and economic costs (Papandroulakis *et al.* 2004b). Less competition for food and stability of conditions makes the semi-intensive rearing technique a more natural method of production and has shown better results (Divanach & Kentouri 2000). Further research must be done on the most appropriate rearing technique and conditions for *Rhabdosargus globiceps*. The use of varied rearing techniques may lead to greater diversification of the species used for aquaculture, which will advance its sustainable development.

With the small size of *Rhabdosargus globiceps* at hatching and the mortalities observed at 4 DPH to 7 DPH, it is suggested that the larvae struggle to ingest prey items during first-feeding. Alternative feeds for the larvae to initiate first-feeding successfully and grow optimally need to be trialled, such as the smaller strain of rotifer, *Brachionus rotundiformis*, Tschugunoff, 1921, with added HUFA (high unsaturated fatty acid) enrichment diets and naturally occurring prey such as copepods, which have been shown to have higher nutritional value (Planas & Cunha 1999; Stottrup 2000; Lubzens *et al.* 2001). The use of microdiets that replace rotifers as feed can substantially reduce hatchery production costs (Lee 2003). Although, total replacement of rotifers with microdiets is not recommended, as this reduces growth and the knowledge of the suitability of appropriate microdiets for early fish larvae is limited (Fernandez-Diaz & Yúfera 1997; Planas & Cunha 1999). There is also a reason for research into the use of automated rearing systems that provide a higher level of intensification with simplified production methods and techniques, which ensure predictable outputs (Dehasque *et al.* 1997). The technologies used in larval rearing of sea bream still have inherent problems, which need to be addressed in order to develop them to be more sustainable and reliable. With these improvements in the rearing techniques, production would improve and the causes of inhibited growth and low survival of individuals could be identified (Planas & Cunha 1999).

Chapter 4 assessed the effect of temperature on the growth and development of the eggs and larvae. Only three temperatures were used for the assessment, making optimisation and the narrowing down of the most effective temperature still possible, although these temperatures provide good insight into the larvae's temperature threshold. Temperature is viewed as the most important environmental factor during larval rearing (Jobling 1981; van

der Kraak & Pankhurst, 1996), yet there are more environmental factors that can maximise survival and growth of the larvae.

Combinations of photoperiod, salinity and temperature produce different results. Osmoregulation in the larvae is controlled by the interactive effects of temperature and salinity, due to this they should be examined together to help determine the best conditions for optimal performance and tolerance (Fielder *et al.* 2005). When considering the correct photoperiod, one should use a longer light regime. The natural photoperiod of 15 hours light was used for all larval rearing experiments in this study. The 24 hour light regime has been used in *Sparus aurata* and *Pagrus auratus* larval rearing as this increases the time that the larvae can actively feed, thus increasing their growth rate (Boeuf & Le Bail 1999). However, the optimal photoperiod may differ between species and performance and development may change. For example, under a 24 hour light regime *Pagrus auratus* had a better feeding rate and growth rate, but there was a lower swimbladder inflation rate than larvae reared at 18 hours of light (Fielder *et al.* 2002). This species of sea bream performed better at 18 hours light 6 hours dark.

To justify the culture of *Rhabdosargus globiceps* on a commercial level there needs to be a market survey that describes the demand for, not only cultured fish, but more specifically, cultured *Rhabdosargus globiceps*. It is a well-known eating fish with good tasting flesh (Van der Elst 1988). However, there needs to be a demand for the product to create profit. At the end of this research it was found that *Rhabdosargus globiceps* has now become a species of high interest for aquaculture in South Africa and further research will be carried out under the Department of Agriculture, Forestry and Fisheries. For an industry in its infancy, the fish based aquaculture projects need a greater selection of species. With a small range of coastline that is not affected by wave and current action, the selection of the species to be used in these areas is fundamental in making aquaculture projects viable.

Overall, the biological capacity of *Rhabdosargus globiceps* to become an aquaculture candidate showed very positive results. Established induction and larval rearing techniques have thus been shown to be able to be successfully used on endemic species. These positive results combined with the species' similarity to successful aquaculture sea bream species

suggest that culturing of this species is biologically feasible. However, these techniques need to be refined and broodstock management and larval rearing optimized so that this process of production is regarded as a totally viable option for aquaculture in South Africa.

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