Characterization of the Early Development and Quality of Red Snapper (*Lutjanus campechanus*) Eggs and Larvae in Aquaculture Conditions

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CHARACTERIZATION OF THE EARLY DEVELOPMENT AND QUALITY OF RED SNAPPER (*LUTJANUS CAMPECHANUS*) EGGS AND LARVAE IN AQUACULTURE CONDITIONS

by

Agnès Bardon Albaret

Abstract of a Dissertation Submitted to the Graduate School of The University of Southern Mississippi in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

December 2014
Abstract

Characterization of the early development and quality of red snapper (*Lutjanus campechanus*) eggs and larvae in aquaculture conditions

by Agnès Bardon Albaret

December 2014

The quality of red snapper *Lutjanus campechanus* eggs is highly variable and unpredictable in aquaculture, leading to high mortality during early larval rearing. In this work, the viability of red snapper eggs was investigated from fertilization until larvae expired due to exhaustion of vitellin reserves. The studied spawns were obtained via strip spawning wild-caught (n=17) and captive (n=7) females following hormonal induction. The fertilization rate, the hatch rate, and the duration of survival of unfed larvae post hatch were weakly correlated to each other, revealing occurrence of distinct and independent components of egg quality.

Spawns from captive females were characterized by a longer latency interval between hormonal induction and ovulation, lower fecundity, and lower hatch rates, as compared to those from wild females. Among the wild brood fish, a positive correlation was observed between the age of the female and the hatch rate.

The proximate composition, fatty acid, and amino acid profiles of the ova did not differ significantly between spawns from captive and wild females.
RNA-sequencing analysis revealed that the standardized measure of gene expression differed significantly between wild and captive groups for 1,349 mRNA transcripts. Variation in hatch rate was significantly related to changes in 1,304 transcripts abundance. Other egg quality variables were only associated with variation in abundance of smaller subsets of transcripts (392-696), suggesting that maternal mRNAs had a more pronounced effect on the embryonic development. More than 40% of the transcripts related to hatch rate were also associated with female age, suggesting that the better hatching success of eggs from older females is related in part to maternal mRNAs content.

During challenge experiments conducted to determine oxygen and ammonia requirements during incubation and early larval rearing, significant mortalities were observed when dissolved oxygen levels were lower than 3 mg L\(^{-1}\) and unionized ammonia levels greater than 0.2 mg L\(^{-1}\).

Continued exploration of the relationships between egg quality parameters and endogenous characteristics of ova or developing embryos is warranted.
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The smallest unit of the analysis is the relationship. No entity has specific identity in itself alone. Therefore, nothing "is" everything “becomes.” ~ Scott Gilbert, developmental biologist.
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CHAPTER I

INTRODUCTION

Marine Aquaculture

During the past half century, rapid increase in fishing efforts worldwide led to full or over-exploitation of 80% of wild fish stocks which often result in spectacular collapses, particularly in small pelagic fish stocks (Kleisner et al. 2013). Despite shifts of fishing effort towards alternative species to compensate for the loss of harvest in overfished stocks, global capture by fisheries have remained stable at about 90 million tons during the last three decades. In the same time, the development of fish culture technologies allowed for the expansion of food fish production via farming at an average rate of 8.8% per year, outpacing population growth and thereby increasing fish production per capita (FAO, 2012). Freshwater fish account for 70% of current aquaculture production, and marine aquaculture is currently mostly oriented towards the culture of mollusks. Despite the current low contribution of marine fishes to global aquaculture production, there is a major interest in developing the culture of these species because they support large markets, and several wild stocks are overfished which leads to stagnation or decrease of fisheries harvests. The development of aquaculture production for those species is limited by technical issues, in particular difficulties to achieve some of the essential life history phases in culture conditions. Specifically, the lack of control of reproduction and larval development are identified as primary bottlenecks for almost all species candidates in marine aquaculture (Mylonas et al. 2010).
The selection of species for marine aquaculture has been driven mostly by their commercial value. In many cases, restrictions applied to fisheries due to overfishing have resulted in price increases and/or reduced availability of fisheries products, thereby stimulating interest in developing aquaculture of target species. In addition, in several countries including the United States, aquaculture for stock enhancement and/or restoration has generated a lot of interest in recent years (Camp et al. 2013). Aquaculture for stock enhancement aims to increase the availability of a given species through the release of cultured juveniles when natural recruitment is limited (Lorenzen et al. 2010). Restoration aquaculture is also of interest when local wild stocks are severely depleted or extinct (Blaylock et al. 2000). This demand-driven approach leads to direct efforts towards promoting local production of indigenous species in accordance with the regional demand of consumers (Telehea and Fontaine 2011).

The red snapper (*Lutjanus campechanus*) is one of the most economically important marine fish species in the southeastern United States, especially in the Gulf of Mexico (GOM), where it is targeted by both recreational and commercial fisheries. Red snappers are found on offshore continental shelf habitats of the western Atlantic Ocean. Adults are commonly associated with benthic structures such as oil rigs or natural reefs, typically at depths between 10 and 130 m (Moran 1988), while juveniles are found in shallower waters. Red snapper are large predatory, opportunistic bottom feeders that forage on a variety of benthic crustaceans and small fishes (Wells et al. 2008). They are long-lived with a maximum reported age of 57 years (Allman et al. 2005).
The reproductive season of red snapper extends from May to September in the GOM (Collins et al. 1996; Woods et al. 2003). Males from the South Atlantic Region of the United States can reach sexual maturity at age 1 (200 mm TL) and are all mature at 3 years old (378 mm TL). Females in the same region can reach sexual maturity at 2 years old (287 mm TL) and are all mature at age 4 or 435 mm TL (White and Palmer 2004). In the northern GOM, the smallest mature female was 267 mm and two years old, and 90% of the females sampled were mature at age 3 (Woods et al. 2003). Differences among regions within the GOM were noted for age and size at the first maturity of females, with females maturing younger and at a smaller size offshore of Alabama than of Louisiana (Jackson et al. 2007; Woods et al. 2003). Red snappers are characterized by their high annual fecundity (46 to 60 million eggs per female per year), a single female being able to spawn more than three million eggs in a single spawning event (Brown-Peterson et al. 2008; Collins et al. 2001). They are batch spawners, spawning every 3 to 4 days during the reproductive season (Brown-Peterson et al. 2008; Woods et al. 2003). Relative and absolute fecundity vary with age, size of the fish, and also differ among regions (Brown-Peterson et al. 2008). Spawning occurs mid-afternoon in the wild (Jackson et al. 2006) and in captivity (Papanikos et al. 2008), but spawning behaviors have not been described yet for this species. Similar to most lutjanid species, the red snapper spawn pelagic, spherical eggs, approximately 0.8 mm in diameter and bearing a single oil globule (Rabalais et al. 1980; Woodard 2003). The duration of incubation in snappers ranges generally from 16 to 30 hours, depending on the species (Emata et al. 1994; Leu et al. 2003) and the temperature (Peña et al.
2014). Even though most lujanid larvae hatch with an elliptic, nearly round yolk (Hamamoto et al. 1992), *L. campechanus* larvae carry a particularly elongated yolk sac, whose tip extends beyond the snout. The extreme anterior placement of the oil globule might play a role in buoyancy for the pelagic larvae (Williams et al. 2004). Red snapper larvae are pelagic for a period of up to one month before settling on open benthic habitats of the continental shelf at depths between 18 and 55 m (Gallaway et al. 2009; Geary et al. 2007).

Red snapper abundance decreased by an estimated 90% between the 1970s and the 1990s (Goodyear and Phares 1990), owing to overexploitation by commercial and recreational fisheries, high juvenile mortality due to the shrimp-trawl bycatch, and habitat change (Christman 1997; Gallaway et al. 1998). While management efforts led to a perceived recovery of the stock in the late 2000’s, the 2010 Deepwater Horizon oil spill into the GOM coincided with the timing of the red snapper maturation and spawning season and may have impacted populations of the northern GOM. In Alabama, post settlement densities of age-0 red snapper in fall 2010 were similar to those observed in previous years (Szedlmayer and Mudrak 2014), but the abundance of juvenile red snapper observed during SEAMAP surveys in 2010 and 2011 was the lowest on record since 1994 in the eastern GOM (SEDAR 31 2013). The stock is currently federally managed, and while it is considered in recovery in the GOM, fisheries’ harvests are still severely restricted, with a federal recreational fishing season of six weeks in 2012 (SEDAR 31 2013) further reduced to 34 days in 2013 and to only nine days for 2014 (NOAA/NMFS 2014). Despite this reduced fishing effort, more than 8,631 metric tons valued at approximately $58.05 million were
reported for recreational and commercial landings in 2012, highlighting the popularity of this fish in the Gulf (NOAA/NMFS 2012).

Because of its high value both as a food and game fish and its recent overfished status, the red snapper is a primary candidate for the developing marine aquaculture industry in the United States. The potential benefits of red snapper aquaculture include a commercial supply of cultured red snapper as an alternative to wild caught fish and direct contributions to efforts to rebuild wild stocks through the production and release of fingerlings in stock enhancement programs (Blaylock et al. 2000; Brennan et al. 2007).

Initial attempts to culture red snapper date back to the 1970s when Arnold et al. (1978) reported spontaneous spawning of red snapper held in tanks. A few years later, Minton et al. (1983) developed a method to hormonally induce gamete maturation of adult red snapper caught in the wild, and artificially fertilizing eggs obtained by manual stripping. Continued hatchery production work has largely relied on the latter approach, in part because of the difficulties associated with obtaining fertilized volitional spawns of breeders held in tanks (Watanabe et al. 2005). More recently, Papanikos et al. (2008) obtained encouraging results with spontaneous spawning of captive red snapper maintained under natural and accelerated photothermal regimes, highlighting the possibility to produce eggs from captive broodstock under controlled conditions. However, a large proportion of eggs spawned by captive red snapper were unfertilized, and spawning activity varied widely among spawning tanks (Papanikos et al. 2003). In addition, the quality of eggs obtained during spawning of captive or wild females in the hatchery is highly variable and unpredictable,
and leads to high mortality during early life stages (Papanikos et al. 2003; Phelps et al. 2009).

Problem Identification

The problem targeted in this work is the high variability and unpredictability of the quality and viability of eggs obtained during spawning of red snapper females. Variability occurs when broodstock are held captive for the entire maturation period and also when they are caught in the wild immediately prior to spawning induction. The unpredictability of egg quality and associated mortality during early developmental stages prevents the identification of suitable husbandry protocols for the culture of early life stages. Better control of the quality and viability of red snapper eggs is therefore a matter of utmost importance. High priorities for research in the field include (1) to acquire a better understanding of factors influencing egg quality in order to design husbandry methods that will maximize the viability of fry and (2) to develop accurate predictors of the quality and viability of fry in order to enable selection of high quality progeny for red snapper larval trials.

Egg Quality Concepts

In the fish farming industry, the quality of an egg has been defined as its potential to produce viable fry (Kjørsvik et al. 1990). This definition implies that high quality eggs are successfully fertilized and subsequently develop into an autonomous individual, while low quality eggs do not complete early development and perish. An essential component of this concept is the identification of a sequence of developmental events undergone by the embryo and larva and is attributed to egg characteristics. Once this sequence of events is
identified, success rates during the selected developmental events can be used to characterize egg quality. Brooks et al. (1997) proposed that successful embryonic development, characterized by the hatch rate, be the ultimate measure of egg quality. Similarly, Bobe and Labbé (2010) defined egg quality as the ability of the egg to be fertilized and to subsequently develop into a normal embryo. On another hand, Bromage et al. (1994) defined high quality eggs as those exhibiting low levels of mortality at fertilization, hatch and up to first feeding; they would also be expected to produce the healthiest and fastest growing fry. This second definition is more inclusive and assesses fry viability when larvae initiate exogenous feeding, thereby capturing long-term effects of egg quality. In this dissertation, egg quality is defined as the potential of an egg to produce viable fry, as measured by high fertilization rates, and high survival to hatching and until first feeding.

This definition does not include potential effects past first feeding. While these are certainly important, the viability of fry during the early stages that follow first feeding can be difficult to measure without disturbing red snapper larvae and inducing mortality. In addition, once larvae begin exogenous feeding, typically a couple of days post hatch, egg quality effects on survival and growth quickly become confounded with a variety of environmental and husbandry factors that are not always well controlled, especially with emerging species like the red snapper where culture protocols are still in development. Characterization of egg quality through the acquisition of exogenous feeding capability can be performed more reliably, which is critical to enable comparison among studies and experimental groups as needed to study factors of variation of egg quality.
Following ovulation, the egg of marine fishes is fertilized and will undergo embryonic development to hatch into a larva. Larvae will then use endogenous reserves (yolk or vitellus) until they successfully transition to exogenous diets. Survival of the egg can be appraised at different times of its development. Mortality events at specific developmental stages (fertilization, hatch, and before, at, and after initiation of exogenous feeding) are often not correlated to each other and could reflect failures of multiple developmental mechanisms (Migaud et al. 2013). Insights can be gained on the origin and mechanisms of these failures through studying the characteristics of eggs in relation with the time and/or developmental stage where failure occurs (Bonnet et al. 2007). The definition of a developmental scale (e.g., a sequence of developmental events on a time scale under standard conditions) in relation to mortality estimates can potentially reveal which phenomena are involved in the developmental failure and enhance assessments of egg quality (Bobe and Labbé 2010).

A mature fish ovum is an oocyte arrested in metaphase II of meiosis and released from its surrounding follicular cells in the ovary. This unfertilized egg contains its own DNA, RNAs, and enzymes and is constituted of proteins, amino acids, lipids (fatty acids, very low density lipoproteins and vitellogenins), carbohydrates, vitamins (ascorbic acid, vitamins A and E), and growth factors with specific functions important for the proper development of the embryo (Lubzens et al. 2010). All of these characteristics of the egg contribute to egg quality and will be subsequently referred to as endogenous characteristics.

The modulating effects of multiple factors such as broodstock nutrition, age, stress, and genetic background determine the endogenous characteristics
of the final oocyte, and by extension, its developmental competency (Li and Leatherland 2012). The nutritional components required for embryonic development and early larval growth are incorporated into the oocytes during oogenesis (Brooks et al. 1997; Kjørsvik et al. 1990). These nutrients are obtained principally through mobilization of reserves from muscle and liver tissues of the mother (Adams 1999). Therefore, the nutritional status of female broodstock largely determines the success of its progeny to reach first feeding (Bobé and Labbé 2010). The importance of amino and fatty acid content for gamete quality has been demonstrated in multiple fish species (Rønnestad and Fyhn 1993; Finn et al. 1995). In particular, a deficiency in highly unsaturated fatty acids (HUFA) of the n-3 series in broodstock diets has been shown to negatively affect the reproductive outcome of several species (Rainuzzo et al. 1997). For example, eggs from wild and captive broodstock of Atlantic cod Gadus morhua have been found to differ in composition (Lanes et al. 2012), possibly reflecting inadequate diets of captive broodstock.

Genetic effects also play a significant role into embryo viability (Von Siebenthal et al. 2009). One major role of genetic factors directly relevant to egg quality is that of the maternal RNAs incorporated into the egg during oogenesis, which control the initial stages of embryonic development (Lindeman and Pelegri 2010; Pelegri 2003). The importance of these maternal factors was established in recent studies of the correlation between the expression of specific maternal genes and egg viability at hatch (Mommens et al. 2010).

Besides the endogenous factors discussed above, egg viability can also be influenced by the parameters of the environment experienced by the egg after
ovulation and release into the external environment (further referred to as exogenous factors). The physicochemical composition of the water (temperature, salinity, and oxygen) can induce a stress on the embryos and influence the quality of fry. Egg quality could be high at fertilization but decrease in quality and viability if they encounter such stressors. Notably, hypoxia was proven to induce vertebral malformation during fish embryonic development (Sawada et al. 2006; Shang and Wu 2004), and elevated ammonia concentrations were shown to reduce the survival rate of newly hatched red drum larvae (*Sciaenops ocellatus*, Holt and Arnold 1983). The relative roles of endogenous and exogenous factors on egg viability and fry fitness remain unclear, and several factors are necessary to tentatively predict the potential of fish larvae to survive (Brooks et al. 1997). Prediction of egg quality is an important issue for practical hatchery production. To be useful, parameters to be used for the determination of egg quality should be determined early during spawning or embryo development and should be simple enough to measure in hatchery conditions so that they can be used in a timely manner to make decisions to use spawns for larval trials (Kjørsvik et al. 1990).

Studies of red snapper spawning including assessments of egg quality in aquaculture conditions were recently summarized by Phelps et al. (2009). Fry mortality is particularly elevated during the first 36 hours post hatch (hph), where it is estimated to average 60% (Phelps et al. 2009). Peaks of mortality are reported during embryonic development before gastrulation (Papanikos et al. 2003; Woodard 2003) and then, at different times post hatch (e.g., 10-20 hph; GCRL-USM unpublished data). Additional mortality is observed when the larvae
transition from endogenous to exogenous feeding (Williams et al. 2004). The kinetics of these mortality episodes have not been clearly documented in red snapper because survival was only recorded at one arbitrary pre-set time point (usually 36 hph). In particular, it is not clear if mortality peaks happen at specific developmental stages. In order to interpret the mechanisms and causes of developmental failures in low quality eggs, identify key developmental stages affected by egg characteristics, and determine egg quality criteria for red snapper, the first research goal is to establish a developmental scale within the incubation and early larval phase, referencing mortality events using this developmental scale.

The value of selected parameters measured at or shortly after spawning for the purpose of predicting egg quality in red snapper was examined in a preliminary study by Bourque and Phelps (2007). Even though a positive relationship was found between fecundity and larval survival at 36 hph, post-ovulation characteristics such as buoyancy, fertilization success, and egg and oil globule sizes were not related to larval viability. Some parameters measured before spawning (date of spawn or latency time of ovulation) were included in the study and showed a relationship with egg quality when grouped in a combination of variables. Other parameters (such as female condition coefficient K, and oocyte stage before hormonal stimulation) should be examined in order to improve prediction of egg quality at early developmental stage, and to assist in the selection of spawns used to initiate larval trials.

The role of endogenous and exogenous factors on egg quality is still poorly documented in red snapper. Nutrient content and the quality and quantity
of maternal RNAs are primary candidate determinants of egg quality. Major
differences in both nutritional factors and maternal RNAs may be induced by the
rearing environment during oogenesis, when brood fish are held captive and
thus, comparisons of these profiles between eggs of captive and wild females
seems warranted to elucidate disruptions of oogenesis leading to low quality
eggs in culture conditions. Diets supplemented with HUFA of the n-3 series did
not improve natural spawning performances of captive red snapper broodstock,
although effects on the fatty acid composition of the fertilized eggs were not
determined (Papanikos et al, 2008). Leucine, valine, lysine and alanine, serine,
and asparagine were the most abundant essential and non-essential free amino
acids respectively contained in newly fertilized red snapper eggs obtained after
hormonal induction and artificial fertilization. However, these eggs exhibited more
than 60% embryonic mortality. The role of these amino acids on egg viability as
well as their optimum level are still unclear (Hastey et al. 2010).

Information on the effects of exogenous factors on egg quality is also
relatively limited in red snapper. Incubation at high density facilitates the harvest
of newly hatched larvae from incubators for stocking into larval rearing tanks.
However, high density during incubation can lead to the rapid deterioration of the
water quality, in particular reduced levels of dissolved oxygen or elevated levels
of ammonia. When stocked at densities higher than 1 egg mL$^{-1}$ during incubation,
red snapper embryos exhibited lower larval survival at 36 hph (Bourque and
Phelps 2007), but specific factors involved in these mortality events were not
identified. The tolerance of red snapper eggs to environmental stressors such as
the increase of ammonia levels or hypoxia resulting from high stocking density
during incubation in intensive recirculating systems therefore remains to be formally evaluated.

Research Hypotheses, Objectives, and Outline

This project addresses four primary hypotheses:

Hypotheses

H₁ – Low egg quality leads to mortality and developmental failures at critical stages of the early development of embryos and larvae.

H₂ – Red snapper eggs from different batches and different females vary in quality due to different endogenous characteristics (primary candidates include nutrient and maternal mRNA content).

H₃ – Eggs from captive and wild females are expected to differ due to distinct environmental conditions during oogenesis – the quality of eggs from captive females is hypothesized to be reduced in comparison to that of eggs from wild females due to inadequate nutrition, stress of confinement, and endocrine disruptions of oogenesis induced by the captive environment.

H₄ – Egg quality can be altered post fertilization if environmental conditions are unsuitable during incubation. Major external factors unstudied to date in red snapper are related to stocking density, i.e., concentration of toxic ammonia and hypoxia.

These hypotheses led to the formulation of the following research objectives:
Objectives

1. Establish a baseline concept of egg and larval quality by characterizing the early development of eggs and larvae and the kinetics of mortality of embryos and larvae with reference to a developmental scale. This includes assessing the correlation between rates of developmental failure at different embryonic and larval stages (i.e., Is there one versus multiple egg quality parameters?).

2. Develop predictors of egg quality that can be measured at early developmental stages to assist in decision of using spawns for larval trials.

3. Study the role of endogenous parameters affecting the egg and larval quality (nutrients and mRNA content of ova) and their variation due to maternal phenotypic traits, with an emphasis on the difference between wild and captive broodstock.

4. Evaluate the effect of exogenous factors related to incubation at high density in culture conditions (elevated ammonia, hypoxia) on egg quality. Specific objectives include (1) assessing the range of tolerance of red snapper eggs (i.e., the range that does not induce direct mortality during or immediately after exposure) and (2) evaluating the effects of sub-lethal conditions on subsequent viability of larvae post hatch.

Outline

The dissertation is divided into five chapters that address the above objectives. Chapter II focuses on the definition of the baseline concept of egg and larvae quality of red snapper with the description of the larval survival and ontogenetic development. In Chapter III, the value of various candidate female
and spawn parameters for the purpose of predicting egg quality is evaluated. In Chapters IV and V, the egg nutrient content and the maternal RNA present in the ova (endogenous factors) are studied in relation to egg quality and, finally, in Chapter VI, the effects of environmental (exogenous) factors on egg and larval quality are examined.
CHAPTER II
CHARACTERIZATION OF THE DEVELOPMENT AND MORTALITY
OF RED SNAPPER EMBRYOS AND EARLY LARVAE AND
DEFINITION OF EGG QUALITY PARAMETERS

Introduction

The variability and unpredictability of egg quality is one of the major factors limiting the development of aquaculture (Brooks et al. 1997; Migaud et al. 2013). Poor egg quality results in high embryonic and larval mortality (Bromage et al. 1994; Kjørsvik et al. 1990), leading to increased costs of hatchery production due to the time and resources spent on egg batches with low survival potential. Good quality eggs have been defined as those exhibiting low mortality through fertilization, hatching, and first feeding (Bromage et al. 1994). While the ultimate assessment of developmental success and egg viability is integrated in measurements of survival and fitness at or after first feeding, a comprehensive assessment of egg quality may include the evaluation of survival rates at multiple time points during embryonic and early larval life (Bobé and Labbé 2010).

The first major developmental step where egg quality can be assessed post ovulation is fertilization. Successful fertilization is usually determined by observing the first embryonic cleavage (Bromage et al. 1992; Kjørsvik et al. 2003). While success at fertilization is a component of the overall egg quality, empirical data indicate that the fertilization rate is not always correlated with subsequent developmental success (Kjørsvik et al. 1990). In Atlantic halibut Hippoglossus hippoglossus, fertilization and hatching rates were positively correlated ($r = 0.57$, Babiak et al. 2008), but they were not in Persian sturgeon
Acipenser persicus ($r = 0.22$, Yousefian et al. 2010). Thus, the fertilization rate can be high, but in some cases, embryos undergo mortality during the incubation period (from fertilization to hatch) resulting in poor hatch rates. Indeed, the initiation of zygotic transcription that occurs during the mid-blastula transition (MBT) in zebrafish Danio rerio has been documented to be a critical period for embryo survival and an important developmental stage for the evaluation of the quality of eggs (Abrams and Mullins 2009; Kimmel et al. 1995; Lubzens et al. 2010). The lack of correlation between fertilization and hatch rates may thus reflect that different mechanisms and egg quality parameters are involved in fertilization success compared to successful completion of embryonic development.

Following hatch, mortality has been reported to peak at the onset of exogenous feeding in several studies (see Yúfera and Darias 2007 for review). A critical parameter that appears correlated with success at first feeding is the duration of the transitional feeding period. The transitional feeding period in larvae is the phase during which feeding ability has been developed and feeding can commence, but some vitellin reserves are still present to support the energetic demands of prey capture (Kamler 2002). The source of nutrients and the energy necessary to continue the larval development change from the yolk reserves to the ingested food (Balon 1986). To achieve this transition, the vitellus needs to support the larva long enough to initiate exogenous feeding. When the transition is successful, exogenous food provides the supply of nutrients required for subsequent maintenance and growth. Alternatively, if vitellus reserves are insufficient or inadequate, the larva does not have the necessary resources to
transition to exogenous feeding and dies. In consequence, evaluation of egg quality also requires determining the potential for the larva to initiate exogenous feeding. A positive correlation between mortality during the transitional feeding phase and the hatch rate has been reported in some studies (e.g., in the Pacific red snapper *Lutjanus peru*, $r = 0.73$, Moguel-Hernández et al. 2013), but the relationship was less pronounced in others (e.g., $r < 0.50$ in a study of the common dentex *Dentex dentex* (Giménez et al. 2006). These moderate correlations suggest that developmental failures at these different stages may involve different mechanisms and that viability through first feeding needs to be evaluated as a separate egg quality trait. Finally, while specific early mortality episodes have been well documented in many aquaculture species (fertilization, hatch, and first feeding), it is important to note that developmental failure and mortality can also occur at other less typical developmental stages. For example, high mortality events occurred in induced spawn studies of Atlantic croaker *Micropogonias undulatus* and red snapper a few hours after hatch but long before first feeding and when larvae still had abundant vitellin reserves (Bardon-Albaret and Saillant, unpublished results). The causes of these mortalities and the exact developmental phases affected are still unclear.

These observations indicate clearly that egg quality must be studied at multiple developmental stages. For a better interpretation of potential causes and mechanisms, it also seems essential to record mortality events with reference to a developmental scale. A necessary pre-requisite to studies of egg quality is therefore to describe the early development and its kinetics, so mortality events can be referenced to facilitate interpretation of potential causes and mechanisms
involved in these developmental failures. This allows also comparing kinetics of mortality of embryos and larvae in various spawns and experimental groups, showing the different outcomes needed to investigate factors influencing egg viability at different stages and/or define predictors of egg quality.

When reared in captivity, most red snapper female broodstock initiate oogenesis (Bardon-Albaret et al. in press). However, oocyte maturation is infrequent, spontaneous spawns in tanks are scarce, and typically unfertilized (Phelps et al. 2009 for review). Although volitional tank spawns lead to a better quality of the fry (Papanikos et al. 2003), their rarity, unpredictability, and low fertility lead to the use of artificial propagation techniques (hormonal induction and \textit{in vitro} fertilization) to obtain spawns for aquaculture production. Treatment with human chorionic gonadotropin (hCG) has been shown to be effective for the induction of gamete maturation and release in red snapper (Minton et al. 1983). However, the quality of eggs obtained by this method is highly variable.

High mortality has been observed during the first 60 hours of post fertilization (hpf) of red snapper larval culture, where it is estimated to average 60% (Phelps et al. 2009). Peaks of mortality were reported during embryonic development (Papanikos et al. 2003) and during the first day of life while vitellin reserves were still abundant (e.g., 36-48 hpf; GCRL-USM unpublished data). During studies of induced spawns of red snapper, relatively high frequencies of deformed embryos were reported at the end of the cleavage period, before the mid blastula transition (Woodard 2003), suggesting that a large fraction of embryonic mortality occurs at these early stages. Mortality of larvae also occurred later, within 10 to 12 hours of hatch (Bardon-Albaret et al. unpublished
At this stage, the larvae rely only on endogenous feeding provided by maternal resources allocated in the ova during the oogenesis process, and larvae are not yet ready to initiate exogenous feeding. Additional mortality was observed during the transitional feeding phase, from mouth opening occurring at 3 days post fertilization (dpf) to the first few days of exogenous feeding (Williams et al. 2004). The kinetics of these mortality episodes have not been clearly documented in most studies of red snapper early development because survival was only recorded at one arbitrary pre-set time point (usually 36 hph, or 60 hpf, Phelps et al. 2009). In particular, it is not clear if major mortality peaks happen at one or multiple specific developmental stages and if these developmental failures are correlated to each other. Preliminary results on the larval development of red snapper produced in aquaculture focused only on the digestive system (Chiluiza 2003) using outdated hatchery protocols.

In this chapter, the development of red snapper embryos and early larvae was described based on Chiluiza’s (2003) study and updated to current culture conditions to facilitate the referencing of mortality events and developmental failures. Then, the kinetics of mortality were examined in spawns from captive and wild broodstock to investigate the viability of eggs at various developmental stages and compare it between spawns from females held in captivity and spawns from females captured in the wild immediately before spawning. In order to define egg quality parameters for further study in subsequent chapters, the correlation of survival at various developmental stages was studied to evaluate if multiple developmental failures do occur at multiple stages independently,
possibly reflecting different causes and mechanisms and whether one or multiple egg quality variables need to be defined and studied.

Materials and Methods

Because of the rarity and low fertility of volitional spawns of red snapper females, the studies of the kinetics of development and mortality events and egg quality in this work were all performed using spawns obtained by hormonally inducing females red snapper with mature oocytes, and strip spawning of eggs when ovulation was observed followed by *in-vitro* artificial fertilization. Two protocols were used to collect spawns from females presented in these spawning experiments. A first group referred to as captive females were caught in the wild and held in captivity for at least one year prior to spawning experiments. Hence, the captive holding period encompassed the entire gametogenesis. The second group, referred to as the wild females, were caught in the natural environment the day of induction, transported to the laboratory, then immediately selected and induced following routine hatchery procedures at the Thad Cochran Marine Aquaculture Center of The University of the Southern Mississippi, Gulf Coast Research Laboratory (TCMAC-USM-GCRL). The handling of females from the two groups for hormonal induction, spawning, and evaluation of spawns was identical to allow comparison of the results. All spawning trials on wild and captive broodstock were performed between 2010 and 2013, during the natural spawning season reported for wild red snapper in the northeastern GOM (Collins et al. 1996).
**Broodstock Acquisition**

Red snapper broodstock used in spawning trials were collected between May 2010 and September 2013, 20 to 25 miles south of the Alabama coast. Fish were caught by hook and line during charter boat trips, at depths between 15 to 25 meters. Fish target size ranged between 1.4 and 4.3 kg. Fish in this size range were likely to have reached sexual maturity and could better tolerate handling than larger fish. The sex of each fish was determined at capture by the observation of sperm release in males following gentle abdominal pressure or by visual examination of the aspect of the genital papilla for the female: the opening of the oviduct appearing as an open slit across the papilla. Fish were held in a round live well on the boat and then transported in oxygenated tanks with thermal insulation to the facilities at the TCMAC-USM-GCRL. The temperature and salinity in the transport container were adjusted to match the conditions in the wild habitat at the time of capture.

**Prophylaxis Procedures and Acclimation of Captive Fish**

Upon arrival at the hatchery, fish destined to be kept as captive broodstock received prophylactic treatments. They were transferred into a static bath of freshwater for 5 minutes to eliminate most of the external parasites, followed by a static bath of an anthelmintic drug (Praziquantel at 5 mg L\(^{-1}\)) during 24 h to expel parasitic worms from their gastrointestinal tract. Fish were then allocated to quarantine tanks containing 0.2 mg L\(^{-1}\) of copper to exterminate any external parasitic organisms left. This concentration of copper was maintained during the entire period of the quarantine by adding copper sulfate solution when the concentration decreased below a minimum threshold of 0.17 mg L\(^{-1}\). Fish
were held in quarantine for at least 3 weeks, where they were acclimated to hatchery feeding practices before being allocated to their destination tanks pending gill arches were free of parasites.

Each brood fish was implanted with a passive integrated transponder (PIT) tag to allow for individual identification. They were maintained in four 10-m³ tanks with a sex ratio of 1:1 per tank. Each tank was connected to individual recirculating seawater filtration system. The artificial photothermal period simulated natural seasonal variations previously recorded in offshore Mississippi waters (NOAA National Data Buoy Center, Station 42067). Red snapper brood fish were fed a fresh diet composed of fish, shrimp, and squid at a 2:1:1 ratio respectively, three times per week to satiation. This diet is similar to the diet of red snapper in the wild (Szedlmayer and Lee 2004) and was determined superior to the supplemented diets evaluated by Papanikos et al. (2008) regarding reproductive performance. During the gametogenesis and spawning periods, the diet was partially substituted twice a week with a supplement that consisted of lecithin (0.2%) and a vitamin premix (2%) prepared according to Moon and Gatlin (1991), mixed with fish-meal (3%) and fish-oil (3%), and combined in a gelatin based preparation that was distributed at 1% BW.

Fish selection, Hormonal Induction, and Spawning

Selection of captive fish. Examination of gamete maturation status began when the temperature in the tanks reached 26°C, corresponding to conditions at the beginning of the natural spawning season of wild red snapper. Fish were individually weighed and measured. The males were selected for spawning experiments when they released sperm following application of a gentle pressure
on both sides of the abdomen toward the papilla. The oocyte maturity stage of females was determined via observation of a sample of oocytes obtained by an intra-ovarian biopsy. Biopsies were performed using a Frydman® memory form polyethylene catheter (1.1-mm inner diameter, with a rounded end, and integrated metal guide wire, CDD laboratory) inserted through the genital papilla into the middle portion of one of the ovaries. A small sample of oocytes was gently drawn by suction while the cannula was slowly withdrawn. Oocytes were scattered in a petri dish and covered with Serra’s solution (or EFA, ethanol:formalin:acetic acid, 6:3:1 by volume) to clear the cytoplasm and determine the oocyte maturation stage. Females with fully-grown oocytes (diameter>300 µm, with cytoplasm entirely clear with EFA) were selected, and pictures of the biopsy were taken for subsequent staging using criteria described in the next chapter.

Selection of wild fish. Mature wild caught red snapper were captured and directly transported to the hatchery facility as described above. Fish were selected for spawning experiments based on the criteria described above for captive fish (oocyte stage and presence of sperm). They were weighed, measured, and then hormonally induced. Hormonal induction occurred within 8 hours of fish collection.

Hormonal induction. Sixty-seven wild and 20 captive females were selected during eight trials, all using the same protocol. For each spawning trial, 4 to 5 wild caught males were also induced and used for fertilizing the ova from selected females. Gamete maturation was induced with a single intramuscular injection of human Chorionic Gonadotropin (hCG, VWR Scientific Products Inc.,
Suwanee, GA) at a dose of 1,100 IU kg\(^{-1}\) of body weight for females and 550 IU kg\(^{-1}\) for males (Minton et al. 1983). Following injection, fish were transferred into 400-L aquaria (4 to 5 fish per aquarium) where they could be easily observed during the induction period. Aquaria were connected to a recirculating seawater filtration system maintained at 27±1°C and 30±2 psu. Fish were individually marked by a specific cut on their caudal fins to ease visual identification of individual females within an aquarium during the monitoring of ovulation. In our experimental conditions, ovulation occurred between 24 to 35 hours post injection for 52% of the induced females.

*Monitoring of ovulation and strip spawning.* Females were checked for ovulation beginning 24 hours post induction. As soon as ovulation was detected by the release of clear, hexagonal-shaped ova (ovulated eggs) following a gentle pressure on the flanks of the female, the fish was anesthetized by adding 100 mg L\(^{-1}\) of Tricaine Methane Sulfonate (MS-222) to the water in the holding tank. A male was simultaneously sedated in a separate bath with the same dose of MS-222. Ova were stripped from the female into a graduated bowl containing 500-mL of seawater. Sperm was simultaneously stripped from the male into the bowl and immediately mixed with the eggs. Sperm was added in large excess (>1 mL per spawn) to ensure that the milt was not a limiting factor for fertilization. Two to five minutes were allowed for fertilization following addition of the sperm. After fertilization, a 3-mL sample was taken from the homogenized mixture of eggs in seawater, and the total number of eggs in the sample was determined under a dissecting microscope. The total number of eggs of the spawn (fecundity parameter) was estimated by extrapolation of the count to the volume containing
the spawn. The eggs were then stocked in an incubator at 1 egg mL\(^{-1}\) and homogenized via gentle aeration until the determination of the fertilization rate at 1 hpf. The fertilization rate was determined by counting the proportion of eggs having completed the second embryonic division (n\(\geq\)100 eggs). Hatching rates were estimated at 36 hpf by counting the numbers of hatched larvae and unhatched eggs in two replicate samples of approximately 100 eggs each, kept in separate 1-L glass beakers in static conditions (26±1°C, 30±1 psu). The low density of eggs in the beakers ensured oxygen was largely in excess, and degradation of water quality due to unhatched embryos was minimal (data presented in Chapter VI).

*Monitoring Post Spawning*

*Development of red snapper embryos.* Immediately following the determination of the fertilization rate, random samples of fertilized eggs were transferred to 30 1-L beakers (100 eggs per beaker estimated volumetrically) and maintained in static conditions (25±1°C, 30±1 psu) throughout the embryonic development period. The hatch rate of embryos incubated in the above static conditions was similar to the hatch rate estimated in recirculated incubation systems. In a preliminary trial (trial 1), the spawn from one female was monitored every two to four hours. The second trial (trial 2) was performed using an equal mixture of eggs from four spawns with similar fertilization rates (81 to 95%) and fertilized within a 2 h time window. Observations were performed every hour from 1 to 30 hpf. The eggs from a subsample of a single designated beaker for each hour were observed under a dissecting microscope at 400x magnification. Pictures were taken using a digital camera (Jenoptik) mounted on a microscope
Developmental stages were diagnosed with reference to Woodard (2003) and Kimmel et al. (1995).

Development of red snapper larvae. Larvae were observed in beakers and collected at 1, 3, and 5 dpf from larval culture tanks to describe organogenesis. On each sampling day, larvae were concentrated onto a 50 µm mesh, anaesthetized with an excess of MS-222 (10 mg L$^{-1}$), and fixed in Bouin’s solution (composed of picric acid, formalin and glacial acetic acid) for 24 h. Following the fixation period, larvae were rinsed overnight with freshwater and dehydrated through immersion in alcohol baths of increasing concentration from 60% to 100% to complete dehydration prior to embedding in paraffin. Larvae were stained with Fast Green FCF during the dehydration process to help orienting and positioning them in the molds during embedding. Serial longitudinal sections 3 to 5-µm thick were obtained using a rotary microtome. Embedded tissue sections were mounted on labeled microscope slides in a warm water bath and allowed to dry for at least 2 hours. Paraffin was removed from these sections with xylene substitute (Sigma-Aldrich), then stained in Hematoxylin 2 and counterstained in Eosin Y (Richard-Allan Scientific). Tissues were covered for observations with coverslips affixed with a mounting medium (Richard Allan Scientific). Pictures were taken at 400 x magnification with a Nikon DMX 1200C digital camera mounted on a Nikon Eclipse 50i microscope and captured with ACT-1C version 1.01 camera control software. Then, contrasts of microphotographs were enhanced with GIMP 2.8.

Kinetics of mortality post fertilization. The survival of eggs and starved larvae from spawns of captive and wild broodstock was monitored over time.
Random samples of eggs from each studied spawn were stocked with approximately 100 eggs L\(^{-1}\) in 14 1-L beakers filled with sea water (30±1 psu), maintained in static conditions at 26±1°C, and treated identically throughout the experiment. Two of the beakers were randomly selected every 24 hours beginning at 36 hpf. The number of live and dead larvae and unhatched eggs were counted in the two replicate beakers within a 2 hour window and recorded. Because of the potential stress inflicted on the larvae by the process of counting, replicate beakers were counted once and then discarded. Subsequent survival counts were performed on other duplicate beakers from the 14 beakers stocked for each spawn and treated identically. Sets of duplicate beakers were counted every 24 h until 100% mortality was reached (maximum 7 dpf at 26°C and 30 psu in all cases).

*Statistical Analyses*

All percentage data were subject to angular (arcsine square root) transformation to improve normality. Survival post hatch was standardized to the hatch rate to distinguish mortality post hatch from embryonic mortalities. The percentage of surviving larvae was compared among days post fertilization in a one way ANOVA in Statistical Analysis Software (SAS®) version 9.3 (SAS Institute, NC) using PROC GLM (time was treated as a fixed factor), followed by a post-hoc Fisher’s Least Significant Difference (LSD) test. For each spawn, the day immediately preceding the first significant decrease in larval survival was determined and will be referred hereafter as the survival duration. The survival duration was kept for further analyses in the subsequent chapters as a measure of egg quality for the spawn.
Student’s t-test was performed using PROC TTEST to compare end point spawning parameters (fertilization, hatching rates and survival duration) measured on the spawns from wild and captive females with the female origin (wild vs captive) treated as a fixed factor.

Pearson’s coefficients of correlation ($r$) between fertilization, hatch rate, and survival duration were estimated using PROC CORR. Differences for all analyses were considered significant at $P$-values of 0.05 or less. All numerical data are reported as the means ± standard deviations (SDs), unless otherwise specified.

Results

*Development of Red Snapper Embryos*

Red snapper ova (unfertilized eggs) were buoyant, transparent, and enclosed a yolk with soft peach color. They contained a large quantity of yolk unevenly allocated within their cytoplasm (telolecithal ova). Usually, one single and unpigmented oil globule was present, and a thin extracellular coat, the chorion, surrounded the ova.

In both trials of embryogenesis observation, the first cleavage began approximately 45 minutes post-fertilization producing two zygotic cells (blastomeres) of equal size (two-cell stage, Table 3). The cleavage of the egg was incomplete (meroblastic) and arose only in a part of the egg at the animal pole (discoidal), forming a blastodisc. Fifteen minutes after the first cleavage, the second division appeared along an axis perpendicular to that of the first division (four-cell stage, 1 hpf, Figure 1 A).
Figure 1. Photographs of embryonic development of red snapper *L. campechanus*. (A) 4-cell stage, 1 hpf, (B) 30% epiboly, blastula stage, 6 hpf, (C) 75% epiboly, gastrulation stage, 10 hpf, (D) 4-8 somites, neurulation stage, 14 hpf, (E) pigmentation and organogenesis, 20 hpf, and (F) ready to hatch, 26 hpf. Scale bar represents 500 µm.
The two subsequent divisions (8 and 16-cell stages) followed a highly synchronous, reproducible, and symmetrical pattern of meridional and equatorial cleavages. After the fourth division (32 and 64-cell stage, around 2 hpf), cleavage became more asymmetrical, and the cells were not always arranged regularly.

The blastula period began at the 128-cell stages (Kimmel et al. 1995). Cells were arranged in multiple layers, and at 3 hpf they started to form a ball-like cluster of cells on top of the yolk, the blastoderm. The cells of the blastoderm continued to divide and peripheral cells started to extend over the yolk towards the vegetal pole. This first cell movement observed, i.e., epiboly, marked the beginning of the gastrulation period. During gastrulation, the blastoderm cells continuously moved down around the yolk by epiboly and covered a third of the egg at 6 hpf (30% epiboly, Figure 1 B). At 50% epiboly (7 to 9 hpf), the epiboly process slowed down, and a thickened ring around the yolk at the blastoderm margin (the germ ring) was visible. Ten to 12 hpf (Figure 1 C), the margin of the blastoderm advanced to cover 75% of the yolk, while the cells eventually narrowed along the dorsal midline forming the notochord, the precursor of the embryonic axis.

Pigmentation was detected between 18 and 20 hpf when a few dendritic melanophores spread over the body. Melanophores increased in size and number as the embryos were closer to hatch. Increase in body depth was visible at 22 hpf, particularly at the head region (Figure 1 E). Spasmodic contractions of the tail in the most advanced embryos started between 22 and 24 hpf and increased in frequency until the first hatch was observed at 24 hpf. Hatching
period extended for about 6 h, with most larvae hatching around 26 hpf (Figure 1 F), leading to a total embryonic development time of 24 to 30 h.

*Development of Red Snapper Larvae*

Newly hatched larvae observed at 36 hpf (1 dpf, Figure 2 A) were translucent and static in the upper phase of the water column. Their elongated yolk sac extended to the ventral part of the body, in an ovoid shape underneath the head, and included a single oil globule located at the anterior tip. The mouth was closed, the digestive tract poorly developed, and the eyes not pigmented. The digestive tract was rudimentary and consisted of a straight undifferentiated tube lying dorsally to the yolk sac, with the posterior portion curved toward the anus (Figure 2 B). No digestive organ was present as reported in most marine fish larvae at hatching. On the dorsal part of the anus at the curvature of the digestive tract, two pronephric ducts (future kidneys) fused together and the posterior junction joined the digestive tract. Between hatching and 3 dpf, the buccal and gill cavities developed in the anterior part of the gut.

Intense organogenesis gave rise to the mouth opening synchronously with the establishment of the eye pigmentation at 3 dpf (84 hpf, Figure 2 C). Pectoral buds were visible, and the larvae maintained a more horizontal position in the water column. Consequently, healthy larvae were more dispersed and present in the upper part of the water column, while weaker larvae were inactive at the bottom of the beakers. The endogenous reserves (yolk and oil globule) had been rapidly consumed such that no yolk was present, and approximately 75% or less of the oil globule remained at that stage which corresponded to the transitional feeding period where larvae need to initiate exogenous feeding.
Figure 2. Microphotographs of early larvae development of red snapper *L. campechanus*. External view (Figure 2 A, C, and E) and sagittal histological sections (H-E, 100x, Figure 2 B, D, and F) at different developmental stages A and B: hatching, 36 hpf, C and D: mouth opening, 3 dpf, E and F: 5 dpf. A, anus; DT, digestive tract; E, eye; GA, gill arch; Ggl, gas gland; H, heart; HG, hindgut; L, liver; MG, midgut; OG, oil globule; P, pancreatic tissue; PN, pronephric tubules; SB, swimbladder; UB, urinary bladder; and YS, yolk sac.
Histological analysis revealed that the incipient gut had evolved into foregut, midgut, and hindgut (Figure 2 D). Intestinal constrictions determined the midgut (future pseudo-stomach), denoted as a swelling between the pyloric sphincter and the intestinal valve, respectively. The gill arches began their formation. Liver, pancreatic tissue, pronephric tubules, and swimbladder appeared as small differentiated cell clusters at this age.

Morphological changes observed at 5 dpf were due to organogenesis taking place to adapt to the exogenous feeding, with very little growth in body size (Figure 2 E). The differentiated mouth structures enabled suction feeding, and the eyes were fully pigmented and functional. The development of the pectoral fins allowed short periods of active swimming, and the swim bladder started to inflate due to the developing gas gland, which improved stabilization of the larvae position in the water column and allowed active swimming.

The walls of the digestive tract started to thicken, and the size of the liver, pancreatic tissue, and gut folds enlarged (Figure 2 F). Pronephric tubules increased in size and number. The heart connected the hepatic vein to the gills and consisted of three chambers (1) the sinus venosus and atrium still linked on the dorsal part, (2) the ventricle, and (3) the bulbus arteriosus on the ventral part.

Kinetics of Mortality during Early Larval Development

Table 1 summarizes the results obtained from the spawns of 31 red snapper females (23 wild and 8 captive) monitored for survival until complete mortality was observed (up to 7 dpf). The fertilization rate of the 31 studied spawns averaged 83.7 ± 19.5% and ranged from 28.6 to 100.0%. About 90% of the spawns had fertilization rates higher than 71%; the four other spawns ranged
between 29.6 to 56.9% fertilization (Figure 3 A). Fertilization rates were slightly lower for the spawns from captive fish compared to the spawns from the wild fish (76.8 ± 21.6% and 86.2 ± 18.6% on average, respectively), but the difference among the 31 spawns was not significant (P=0.1570). The mean hatching rate for all 31 studied spawns was 64.6 ± 31.2% (Table 1). The distribution of hatch rates had greater variance (0 to 95%, Standard Deviation SD=31.2%) than that of fertilization rates (SD=19.5%, Figure 3 B).

Table 1

*Date, female identification number, mean fertilization rates with 95% confidence interval, mean hatch rates ± SD, and survival duration* (in dpf), of 31 spawns from captive and wild red snapper.*

<table>
<thead>
<tr>
<th>Date (2011-2013)</th>
<th>Female</th>
<th>Fertilization ± SD</th>
<th>Hatch ± SD</th>
<th>Survival duration *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Captive fish</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17-Aug 2011</td>
<td>1</td>
<td>89.72 ± 1.87</td>
<td>63.88 ±</td>
<td>1</td>
</tr>
<tr>
<td>12-Jul 2012</td>
<td>2</td>
<td>90.74 ± 2.10</td>
<td>73.10 ±</td>
<td>1</td>
</tr>
<tr>
<td>12-Jul 2012</td>
<td>3</td>
<td>28.57 ± 3.96</td>
<td>0.00 ± 0.00</td>
<td>0</td>
</tr>
<tr>
<td>12-Jul 2012</td>
<td>4</td>
<td>71.29 ± 4.03</td>
<td>31.39 ±</td>
<td>6</td>
</tr>
<tr>
<td>25-Jul 2012</td>
<td>5</td>
<td>71.34 ± 5.13</td>
<td>36.03 ±</td>
<td>4</td>
</tr>
<tr>
<td>25-Jul 2012</td>
<td>6</td>
<td>96.67 ± 0.49</td>
<td>82.31 ±</td>
<td>4</td>
</tr>
<tr>
<td>25-Jul 2012</td>
<td>7</td>
<td>87.50 ± 2.35</td>
<td>56.54 ±</td>
<td>4</td>
</tr>
<tr>
<td>25-Jul 2012</td>
<td>8</td>
<td>78.33 ± 3.64</td>
<td>39.06 ±</td>
<td>4</td>
</tr>
<tr>
<td>Captive fish</td>
<td>mean</td>
<td>76.77 ± 21.58</td>
<td>47.89 ± 26.55</td>
<td>3.0</td>
</tr>
<tr>
<td>n=8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild fish</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17-Aug 2011</td>
<td>1</td>
<td>89.84 ± 2.02</td>
<td>88.56 ±</td>
<td>4</td>
</tr>
<tr>
<td>23-May 2012</td>
<td>2</td>
<td>87.18 ± 2.37</td>
<td>93.58 ±</td>
<td>5</td>
</tr>
<tr>
<td>23-May 2012</td>
<td>3</td>
<td>92.68 ± 1.70</td>
<td>85.05 ±</td>
<td>5</td>
</tr>
<tr>
<td>23-May 2012</td>
<td>4</td>
<td>87.59 ± 2.49</td>
<td>64.40 ±</td>
<td>3</td>
</tr>
<tr>
<td>23-May 2012</td>
<td>5</td>
<td>94.57 ± 1.37</td>
<td>74.88 ±</td>
<td>1</td>
</tr>
<tr>
<td>23-May 2012</td>
<td>6</td>
<td>91.30 ± 1.83</td>
<td>77.75 ±</td>
<td>5</td>
</tr>
<tr>
<td>3-Jul 2012</td>
<td>7</td>
<td>99.67 ± 0.11</td>
<td>95.09 ±</td>
<td>5</td>
</tr>
<tr>
<td>3-Jul 2012</td>
<td>8</td>
<td>99.34 ± 0.22</td>
<td>92.07 ±</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 1 (continued).

<table>
<thead>
<tr>
<th>Date</th>
<th>Trial</th>
<th>Hatch Rate Mean</th>
<th>Hatch Rate SD</th>
<th>Fertilization Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Jul 2012</td>
<td>9</td>
<td>33.89 ± 13.54</td>
<td>7.64 ± 0.83</td>
<td>0</td>
</tr>
<tr>
<td>3-Jul 2012</td>
<td>10</td>
<td>96.99 ± 1.09</td>
<td>92.45 ±</td>
<td>5</td>
</tr>
<tr>
<td>3-Jul 2012</td>
<td>11</td>
<td>96.62 ± 0.78</td>
<td>92.56 ±</td>
<td>4</td>
</tr>
<tr>
<td>3-Jul 2012</td>
<td>12</td>
<td>100.00 ± 0.00</td>
<td>86.53 ±</td>
<td>2</td>
</tr>
<tr>
<td>3-Jul 2012</td>
<td>13</td>
<td>79.72 ± 5.56</td>
<td>85.74 ±</td>
<td>5</td>
</tr>
<tr>
<td>26-Jul 2012</td>
<td>14</td>
<td>89.06 ± 2.16</td>
<td>91.65 ±</td>
<td>5</td>
</tr>
<tr>
<td>26-Jul 2012</td>
<td>15</td>
<td>89.23 ± 2.15</td>
<td>68.08 ±</td>
<td>4</td>
</tr>
<tr>
<td>26-Jul 2012</td>
<td>16</td>
<td>95.28 ± 0.99</td>
<td>26.95 ±</td>
<td>6</td>
</tr>
<tr>
<td>12-Sep 2012</td>
<td>17</td>
<td>94.62 ± 1.77</td>
<td>72.74 ±</td>
<td>1</td>
</tr>
<tr>
<td>12-Sep 2012</td>
<td>18</td>
<td>91.79 ± 2.06</td>
<td>94.81 ±</td>
<td>1</td>
</tr>
<tr>
<td>12-Sep 2012</td>
<td>19</td>
<td>54.86 ± 6.42</td>
<td>0.00 ± 0.00</td>
<td>0</td>
</tr>
<tr>
<td>12-Sep 2012</td>
<td>20</td>
<td>35.80 ± 5.73</td>
<td>0.00 ± 0.00</td>
<td>0</td>
</tr>
<tr>
<td>24-May 2013</td>
<td>21</td>
<td>93.62 ± 1.39</td>
<td>59.17 ±</td>
<td>5</td>
</tr>
<tr>
<td>24-May 2013</td>
<td>22</td>
<td>98.45 ± 0.34</td>
<td>78.10 ±</td>
<td>5</td>
</tr>
<tr>
<td>24-May 2013</td>
<td>23</td>
<td>89.74 ± 1.95</td>
<td>92.05 ±</td>
<td>5</td>
</tr>
</tbody>
</table>

| Wild fish | mean | 86.17 | 70.43 | 3.6 |
| n=23      | SD   | 18.64 | 31.04 | 2.0 |
| Overall   | mean | 83.74 | 64.59 | 3.4 |
| n=31      | SD   | 19.52 | 31.18 | 2.0 |

* The method used to determine this parameter is described in the statistical analysis section of the materials and methods.

The four spawns with low fertilization success (<70%) displayed hatch rates lower than 10% (mean 2.5%). The hatch rates of spawns from captive females were significantly lower than those from wild females (47.8 ± 26.6% versus 70.4 ± 31.0%, *P*=0.0198). For the 23 spawns from wild females, the distribution of hatch rates followed a bimodal pattern, with four spawns displaying hatch rates lower than 30%, while the remaining 19 spawns had hatch rates greater than 60%. The embryonic mortality rate, calculated as (1 – hatch rate) divided by fertilization rate for each spawn averaged 29% in spawns from captive females versus 16% in spawns issued from wild females, but the difference
between the two groups was not significant \((P=0.1081)\). Four out of eight spawns from captive females had hatch rates lower than 40%. Hatch rates for the four other spawns from captive females ranged between 56.5% and 82.3% (Table 1, Figure 3 B).

When all 31 spawns were included in the analysis, the Pearson’s correlation \(r\) between the hatch rate (standardized hatch rate = divided by fertilization rate) and the fertilization rate was 0.73 \((P<0.0001, \text{ Table 2})\). However, the four outliers with fertilization <70% and low hatch rates accounted for most of this relationship. Once these four specific spawns were removed from the analysis, the correlation \(r\) between fertilization and standardized hatch rates of the 27 remaining spawns was only 0.24 \((P=0.2336)\), indicating a low correlation between the fertilization and hatch rates when fertilization was higher than 70%.

The survival duration for the 31 spawns averaged 3.8 ± 2.0 dpf, ranged from 0 to 6 dpf, and did not differ significantly between wild and captive spawns \((P=0.496)\). The frequency distribution of survival duration had one main mode at 4 to 5 dpf, reflecting the survival duration of larvae dying from starvation following the exhaustion of vitellin reserves (Figure 3 C). The four spawns with fertilization rates lower than 70% had a hatch rate that was significantly lower than the fertilization rate due to elevated embryonic mortalities. Therefore, the duration of survival for these four spawns was 0 dpf according to the methodology used to determine the duration of survival parameter (i.e., day before the significant decrease in survival). Seven other spawns had a survival duration between 1 and 3 dpf, meaning that the significant decrease in survival rate happened before or within 24 hours following mouth opening.
The survival duration was moderately correlated to fertilization \((r=0.59, P=0.0005)\) and hatch \((r=0.55, P=0.0015)\) rates, and these correlation coefficients did not differ significantly from zero when the four outlier spawns discussed above (fertilization rate <70\%) were removed from the analysis (Table 2, \(n=27\)).

The lack of a strong relationship between fertilization and hatch rates and survival duration indicates that some spawns can have a good fertilization, or a good hatch rate but poor survival duration and vice versa.

**Table 2**

*Pearson’s correlations \(r\) between fertilization rate, mean standardized hatch rates, and survival duration for spawns obtained from captive and wild red snapper (see text for details). Correlations were calculated using all available data \((n=31)\) and with spawns with fertilization >70\% \((n=27)\). Significance level of correlations was * \(P<0.05\), ** \(P<0.01\), or *** \(P<0.001\)*

<table>
<thead>
<tr>
<th></th>
<th>Fertilization</th>
<th>Standardized hatch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=31)</td>
<td>(n=27)</td>
</tr>
<tr>
<td>Standardized hatch</td>
<td>0.73***</td>
<td>0.24</td>
</tr>
<tr>
<td>Survival duration</td>
<td>0.59***</td>
<td>-0.13</td>
</tr>
</tbody>
</table>
Discussion

The first objective of this work was to describe the sequence of developmental events undergone by red snapper embryos and early larvae and their timing during the period when these processes are under the influence of maternal factors. Monitoring began at the collection of ova and ended when 100% mortality of starved larvae was observed. Successful larvae survived several days once they had acquired the capacity to ingest and digest exogenous food and were expected to have high probability of success.
transitioning to an autonomous life. This monitoring protocol encompassed the entire period when high quality fry feed on their endogenous reserves and therefore captured the majority of direct maternal effects on early development. The viability of embryos and larvae was assessed through estimation of fertilization rates, hatch rates, and survival rates of hatched larvae every 24 hours until complete mortality. These data served as the main criteria for further analyses of egg quality conducted in the subsequent chapters. The observation of variable rates of embryonic mortality between captive and wild spawns is one of the major findings of this study and will require further investigations to detect the particular embryonic stages where mortality occur for a better understanding of the causes and mechanisms involved in developmental failures prior to hatch. In order to provide baseline data for such studies, the monitoring of the early development conducted in this study included kinetics of embryonic development where developmental stages were recorded at regular intervals between fertilization and hatch.

*Development of Red Snapper Embryos*

The embryonic development of *L. campechanus* was followed 27 h until partial hatch in trial 1 and lasted 24 to 30 hours at 25°C from fertilization to complete hatch in the second trial. The kinetics observed in this work were generally consistent with previous descriptions by Rabalais et al. (1980) and Woodard (2003, results of all 4 experiments summarized in Table 3).
Table 3

*Time and developmental stages of red snapper embryos.*

<table>
<thead>
<tr>
<th>hpf</th>
<th>This study, trial 1 1 Female, 25°C</th>
<th>This study, trial 2 4 Females, 25°C</th>
<th>Rabalais et al. 1980 (23-25°C)</th>
<th>Woodard, 2003 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4 to 8-cell</td>
<td>&gt; 64-cell</td>
<td>2-cell</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>64-cell</td>
<td></td>
<td>&gt; 16-cell</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Late blastula</td>
<td>512 to 1k-cell</td>
<td>Yolk Syncytial Layer</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>30% epiboly</td>
<td>Dome</td>
<td>Start of epiboly</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>75% epiboly - Neurulation</td>
<td>60% epiboly - Neurulation</td>
<td>Neurulation</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>10 somites</td>
<td>Neurulation to 4-6 somites</td>
<td>Cranial regionalization</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>20 somites + pigments</td>
<td>&gt; 14 somites</td>
<td>&gt; 15 somites</td>
<td>Pigments</td>
</tr>
<tr>
<td>22</td>
<td>Movements</td>
<td>&gt; Pigments</td>
<td>&gt; 18 somites + Pigments</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>first hatch</td>
<td>first hatch</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>first hatch</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>30% hatch</td>
<td></td>
<td>100% hatch</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>100% hatch</td>
<td></td>
<td>First hatch</td>
<td></td>
</tr>
</tbody>
</table>

The initial cell cleavage was observed within one hour post fertilization in all studies. The embryo rapidly developed in a blastula and initiated gastrulation at 6 to 8 hpf in both trials. When epiboly reached 30% of the distance between the animal and vegetal poles, the embryo attained the mid-blastula transition (MBT, Aanes et al. 2011; Abrams and Mullins, 2009; Dosch et al. 2004; Tadros and Lipshitz 2009). At the MBT, a subset of maternal mRNAs and protein are expected to be eliminated, and the transcription activity of the zygotic genome is activated (Harvey et al. 2013; Vesterlund et al. 2011). Consequently, development prior to the completion of the MBT is under the control of maternal RNAs and eggs with deficient maternal RNA content likely display developmental
failures initiated prior to completion of this phase (Lindeman and Pelegri 2010).
This is reflected for example in studies conducted in other species where asymmetry in the early divisions of embryos was found to be correlated with low hatch rate of embryos (Shields et al. 1997).

Between 12 and 16 hpf, segmentation of the body in different somites began followed by organogenesis, including head and tail differentiation, eye formation, and brain regionalization. At 18 hpf, the growing red snapper embryo became pigmented and developed neural tube, somites, notochord, and head structures, i.e., the common characters to all vertebrates (Ballard 1981). This stage, called the pharyngula period (or phylotypic stage) is considered the most morphologically and transcriptomically conserved period among vertebrate embryos (Comte et al. 2010; Irie and Kuratani 2011). While the developmental period that follows the MBT includes complex differentiation of the embryos, a hypothesis that could be formally tested in future studies is that this period, largely controlled by transcription of the zygotic genome, is less impacted by maternal effects and variation of egg quality. Assessment of the viability of spawns prior to and after the MBT would be useful in future monitoring of egg quality in order to formally test this hypothesis. The next major event was the hatch of embryos that was observed between 24 and 30 hpf.

Although these experiments and the studies of Woodard (2003) and Rabalais et al. (1980) were all conducted at the same temperature, the timing of embryonic stages differed among experiments. Rabalais et al. (1980) reported an advanced stage of cleavage (early blastula) at 1 hpf. In contrast; Woodard (2003) described the first cell division of the embryonic development only at 1 hpf. In the
present study, the first division occurred 45 min after fertilization with most of the embryos found at the 4 to 8-cell stage one hour after fertilization. Although the three studies showed differences in developmental rates as early as the first divisions, these differences did not amplify over embryogenesis, and the observed ranges of hatching times only differed moderately (Table 3). Variation among embryos in the rate of early embryonic development was studied in the zebrafish by Schmidt and Starck (2004). Differences among embryos were particularly apparent during the early organogenesis, but the magnitude of differences in developmental rates among embryos was reduced in the later stage. The interconnectivity of embryonic elements established around the pharyngula period required a high degree of coordination, resulting in stronger internal developmental constraints, and in more homogeneity across individuals in the timing of the later phases of embryonic development. This hypothesis is consistent with the moderate range of time to hatch among-spawns at a given temperature which would be due to individual variations in developmental rates occurring primarily during the initial embryonic stages.

The potential causes of differences in developmental rates among embryos and spawns (including between the two experiments conducted in this work) are multiple. Embryos could differ in their genetic potential for developmental kinetic. This may include differences in genetic material transmitted by the mother in the form of maternal messenger RNAs controlling early development (Pelegri 2003). The influence of maternal mRNAs on developmental success has been demonstrated in rainbow trout *Onchorynchus mykiss* (Bonnet et al. 2007) and striped bass *Morone saxatilis* (Chapman et al.
2014) and will be discussed in Chapter V. Maternal mRNAs could determine in part the development rates of embryos, a hypothesis that could be examined during analyses of the transcriptome (and developmental rate) of single embryos (Jong et al. 2010).

Differences in early developmental rates may also be related to egg viability as suggested by the association between asymmetrical early divisions and failure to hatch (Ajduk and Zernicka-Goetz 2013). Another potential source of difference among spawns and/or studies could be inconsistent recording of developmental stages. The developmental stage reached by embryos was not assessed at the same time post-fertilization in all studies, which may have resulted in the false inference of large differences in developmental rates among spawns due to overestimation of the time to reach a given stage in some experiments. Mis-identification of some developmental stages is another potential source of error that could be involved in these differences.

Finally, although all studies were conducted at the same reported temperature, minor environmental differences including subtle differences in temperature may have occurred. For example, Woodard (2003) found that hatching occurred at 30 hpf at 25°C and only 21.75 hpf at 26°C, a difference in hatching time of 8.25 h (27%) for a change in temperature of only 1°C. Thus, temperatures may have varied among the studies by ±0.3°C, which could lead to hours of differences in terms of hatching time. Despite the caveats discussed above, differences in developmental rates among embryos are strongly suggested by the variation in hatch time in this study and in studies of other species (Korwin-Kossakowski 2012; Pauly and Pullin 1988; Schmidt and Starck 2004). Within a spawn,
variation in hatching time was approximately 3 to 6 h (this study, and data from Rabalais et al. 1980), suggesting that differences of this range among spawns are possible. The practical implications of these findings are related to the design and interpretation of studies involving the determination of embryo survival prior to hatch. The differences among studies and among embryos within a spawn render the a priori prediction of the time to completion of a developmental phase hazardous. In consequence, experiments may need to involve sampling embryos at a pre-set time-interval, measuring mortality rates to determine the developmental stage in relevant samples (i.e., immediately preceding and following mortality of a detectable fraction of embryos). Indirect measures of embryonic mortality such as cell viability coloration (PrestoBlue®) could be used to rapidly assess the viability of embryos in serial samples for that purpose if the dye molecule is small enough to penetrate the chorion (<500 Da, Coward et al. 2002).

**Development of Red Snapper Larvae**

The early larval development of *L. campechanus* observed in this study was similar to previous reports for this species and other lujanids (Allen 1985; Chiluiza 2003; Leu et al. 2003; Williams et al. 2004). Newly hatched larvae had a rudimentary digestive tract with no major digestive organs, like most marine fish larvae at hatching. During the endogenous feeding period, the larvae experienced rapid morphological changes preparing for the transition to exogenous feeding.

In the conditions of our experiment, red snapper mouth was opened at 3 dpf, the digestive organs were developing (liver and pancreatic tissues), and
the digestive tract was differentiated into foregut, midgut, and hingut indicating that the larvae could initiate feeding. The endogenous reserves remaining at that time were limited to approximately 75% of the initial oil globule. For several other lutjanids incubated at temperatures between 23 and 28°C, the yolk sac was also fully absorbed on the day of the mouth opening (Boza-Abarca et al. 2008; Hamamoto et al. 1992; Leu et al. 2003; Lim et al. 1985; Suzuki and Hioki 1979; Watanabe et al. 1998; Zavala-Leal et al. 2013). The rate of depletion of yolk reserves and the transitional window period are determined in part by abiotic conditions, in particular rearing temperature (Kamler 2008). In *L. campechanus* reared at 28°C, yolk reserves were completely exhausted on the day of mouth opening (Williams et al. 2004), suggesting that the transitional period was almost non-existent at that temperature, meaning that larvae had little energy reserve remaining by the crucial 24 hours of first feeding. Thermal effects on endogenous reserves depletion have been described in many teleost species, with death due to exhaustion of vitellin reserves and starvation occurring earlier at higher temperatures (Fukuhara 1990; Kamler 2008).

A practical consideration based on these observations is that in aquaculture conditions, lowering the incubation temperature to 26°C could improve the success rate of larvae through first feeding by increasing the time window of mixed feedings (endogenous and exogenous). These results also have implications for the assessment of egg quality and viability. Assuming a rearing temperature of 26°C applied in this study, larvae surviving a longer time following opening of the mouth (3 dpf) are expected to experience a longer
transitional period and be given a chance to successfully initiate exogenous feeding.

The observation of high survival rates of unfed larvae in spawns after 4 dpf or more suggests that sufficient vitellin reserves were still present at 3 dpf to support the initiation of feeding activity and the transition to exogenous feeding, thus providing an ultimate measure of fry quality. This egg quality concept was therefore evaluated in this work by classifying spawns in two categories. Spawns were inferred as high quality when no significant decrease in survival was detected until 4 dpf or later, and low quality was inferred when survival decreased significantly at or before 3 dpf. The rationale behind this classification is that in all studied spawns, a significant decrease in survival was followed by complete mortality within 24 hours, thus, the surviving larvae after that day had likely reached a point of non-return (PNR), beyond which larvae are deprived nutritionally and unable to initiate feeding even if prey is available (Blaxter 1988).

**Kinetics of Mortality during Early Larval Development**

The second objective of this chapter was to examine the distribution of mortality of embryos and early larvae through time and the correlation of mortality rates at different development steps to define relevant criteria to be used in assessment of egg quality. The first assessment of developmental success was the fertilization rate. In this study, spawns with high fertilization rates were preferentially selected, so interpretation of the observed variability of fertilization rate is limited.
A primary factor impacting the fertility of eggs and potentially its subsequent embryonic development is the timing of ova collection post ovulation. Delay in ova collection leads to post-ovulatory ageing, or over-ripening, which decreases egg quality very rapidly after ovulation in most warm water fish species (Bobe and Labbé 2010). Ova generally survive in vivo for a few hours after ovulation at the most, depending on the species and their natural temperature (Bromage et al. 1994; Legendre et al. 1996; Linhart and Billard 1995). In red snapper, the viability of ova typically drops within one hour after ovulation (Phelps et al. 2009). The accurate timing of red snapper ovulation is therefore paramount for successful artificial fertilization. In the conditions of our experiments where sperm was used in large excess, fertilization rates lower than 70% likely signaled post ovulatory ageing; therefore, they were not retained (except for the four spawns with low fertility collected initially). Spawns with such low fertilization rates should also be avoided for production purposes as high embryonic mortality is expected to occur in overripe spawns. The results of the four spawns with low fertilization rates support this hypothesis as their hatch rate was very low. Other factors such as oocyte stage at induction, time in spawning season, and female condition could impact fertilization rates (Adams 1999; Żarski et al. 2011a) and will be examined in the Chapter III.

Developmental success from fertilization to hatching was assessed by estimation of hatch rates. Hatch rates were standardized to the fertilization rate to evaluate the relationship between these two parameters. A first notable result is that spawns obtained from captive red snapper females had significantly lower hatch rates than those of wild females, indicating higher rates of embryonic
mortality. The superior quality of eggs from wild fish over captive fish has been related to environmental influences (Brooks et al. 1997). Captive red snapper females typically reach less advanced pre-maturation stages (Bardon-Albaret et al. in press) and rarely spawn spontaneously in tanks (Papanikos et al. 2003). The ovulation induced in our experiment was therefore the first ovulation of the spawning season for these females. In contrast, wild females are typically collected during their active spawning season when they can spawn every 4 days (Brown-Peterson et al. 2008). The lower viability of produced embryos from captive females may be related to the lower competency of oocytes at induction due to the lack of reproductive activity of captive females. Vitellin reserves could also be of lower quality in captive fish due to deficiencies or an inadequate balance of certain nutrients in the food as was demonstrated in several species (Lanes et al. 2012; Morehead et al. 2001; Pickova and Brännäs 2006), and this hypothesis will be explored in Chapter IV.

The correlation between hatch rate and fertilization rate was moderate, and once the four putatively overripe spawns discussed above were removed from the dataset, the correlation was very low and non-significant, indicating independence of the two parameters and potentially different mechanisms impacting these two egg quality measures. Several hypotheses discussed above can be formulated about the cause and mechanisms involved in embryonic mortalities (e.g., failure initiated prior to the mid-blastula transition due to deficient maternal mRNAs). Monitoring of mortality rates at different stages of embryonic development facilitates the interpretation of experiments.
In seven spawns, hatch rates were high, but a significant mortality peak was observed before 3 dpf, rapidly followed by complete mortality. Despite high fertilization and hatching rates (>70%), survival was less than 30% at mouth opening for these spawns. These mortalities were not related to a difference in the absorption rate of the endogenous reserves which were still available when larval mortalities were occurring. Additional data about spawns displaying this pattern of mortality need to be obtained to diagnose the causes and mechanisms of these developmental failures.

The last main mortality event occurred at the time of yolk resorption. As discussed above, energy reserves support the larva for a limited period which varies among species, egg and larval size, and temperature (Miller et al. 1988). In red snapper, the short mixing period of endogenous and exogenous feeding often results in a high mortality at first feeding in aquaculture (Williams et al. 2004) and requires optimization of first feeding protocols to promote successful transition of a large proportion of larvae. When vitellin reserves are exhausted, starved larvae rapidly reach the point of non-return (PNR), beyond which exogenous feeding cannot be initiated and mortality is inevitable (Blaxter 1988). The PNR and starvation mortality are reached sooner in species with small larvae than in species with large larvae (Miller et al. 1988; Robert et al. 2013) and typically occur as early as 24 hours after the exhaustion of vitellin reserves for tropical marine fish larvae (Fukuhara 1990). In red snapper, the mortality peak around 4 and 5 dpf is considered to reflect the exhaustion of endogenous resources. High survival rates at this later stage of development identify the quality of endogenous reserves contained in the yolk sac and the intrinsic
survival potential of the larvae (Giménez et al. 2006). This potential was measured in two ways. First by the duration of survival (number of days before a significant decrease in survival rate was recorded) and then using the quality parameter defined above, where 4 dpf was set as an inclusive threshold to define high quality eggs. Correlation analysis indicated that the survival duration was independent from the fertilization and hatch rates once the four putative overripe spawns were removed from the dataset, suggesting that different causes and mechanisms were involved in these parameters.

The main conclusion of the study of survival kinetics is therefore that in red snapper, the three egg quality parameters fertilization, standardized hatch rate, and survival duration were at best weakly correlated to each other. Some spawns were in the higher range for hatch rate but had short survival and vice versa. The lack of correlations between these variables indicates that different and independent developmental failures are involved in the three types of mortality events. For that reason, all egg quality variables (fertilization, hatch, survival duration, and quality) will be analyzed in subsequent chapters seeking to define predictors of egg quality (Chapter III) and characterize the role of endogenous factors on egg viability (Chapters IV and V).
CHAPTER III
EVALUATION OF FEMALE AND SPAWN CHARACTERISTICS AS PREDICTORS OF RED SNAPPER EGG AND LARVAL QUALITY

Introduction

The need for efficient methods to predict egg quality for aquaculture has motivated a vast amount of research over the past few decades (Bobè and Labbé 2010; Brooks et al. 1997; Kjørsvik et al. 1990 for reviews). Efforts to assess reproductive performance, in particular to anticipate the viability of fry, are limited by the lack of standardization of methods and by the paucity of criteria that have been shown to be reliable and effective in marine fishes (Thorsen et al. 2003). The previous chapter established that routinely measured end-point parameters of egg quality (fertility and hatching rates) were not significantly correlated to one another or to the potential of larvae to transition from the maternally controlled endogenous feeding phase to exogenous feeding. The simple measurement of fertilization and/or hatching rates is thus insufficient to predict the viability of larvae at first feeding. Other egg quality criteria need to be identified to allow predicting success at all the steps of the early larval development that are under maternal influence.

A criterion is suitable for the purpose of predicting egg quality when (1) it can be measured before spawning or rapidly post spawning to allow selecting spawns prior to initiation of larval rearing trials, (2) it is highly correlated with one or all of the egg quality parameters determining viability through first feeding (fertilization, hatch rate, duration of the survival over the transitional feeding period), and/or (3) the measurement method is rapid, accurate, and compatible
with assessment before or during strip spawning. In addition, one single parameter may not be sufficient to predict viability to first feeding, integrating success at fertilization, hatch and through the transitional feeding period as discussed above. In that case, multiple characters may need to be included to achieve reliable prediction, and efforts need to be made to identify a minimum number of measurements that will allow robust assessment of egg and larval viability as a whole.

Several variables have been suggested as indicators of progeny success in aquaculture. Before fertilization, the egg size is one of the simplest and most widely used parameter to estimate a priori egg quality (Thorsen et al. 2003). Larvae hatched from larger eggs would be expected to have more reserves, permitting a longer transitional period and giving them higher survival potential (Kamler 2005). However, this parameter is not necessarily a good predictor of fertilization and/or hatching success (Llanos-Rivera and Castro 2004). Indeed, egg sizes varies significantly among clutches of batch spawners species, with a decrease in egg sizes over time as the spawning season progresses (Chambers and Legget 1996) that impedes the identification of appropriate values to predict egg quality. Furthermore, the spawn of red snapper, like many other highly fecund marine fishes, are characterized by small eggs and larvae (Grimes 1987), making it difficult to accurately distinguish differences in egg size and detect a relationship between this parameter and larvae viability (Bourque and Phelps 2007).

When females are hormonally induced for ovulation, the timing of induction and the duration of the latency period between hormonal induction and
fertilization of ova can impact significantly the reproductive output and the quality of a spawn. For example, egg quality was improved, and latency time was more homogenous when Senegalese sole *Solea senegalensis* females were induced in the early morning compared to those induced at mid-day or late evening (Rasines et al. 2013). Besides, if eggs are not collected immediately after ovulation, their quality decreases rapidly due to over-ripening. Degradation has been hypothesized to occur within an hour post fertilization in red snapper (Phelps et al. 2009) and is expected to lead to low fertilization and hatch rates (Legendre et al. 1996; Linhart et al. 2001). While detection of ovulation is relatively subjective and susceptible to experimental error, the duration of latency in association with other pre-spawning parameters allows for the accounting of delayed egg collection when it happens and improving the accuracy of assessments of spawn quality.

Another parameter that has been proposed as a predictor of egg quality is the distribution, transparency, and homogeneity of lipid droplets in unfertilized ova. This trait was positively correlated with embryonic viability in Eurasian perch *Perca fluviatilis* (Żarski et al. 2011a), different species of sea bream (Lahnsteiner and Patarnello 2005), and salmonids (Lahnsteiner et al. 2008; Mansour et al. 2007). However, while present in the autumn spawning period, this correlation was not detected in rainbow trout during spring spawning events (Ciereszko et al. 2009), and oil droplet diameter was not correlated to larval survival at first feeding in common snook *Centropomus undecimalis* (Neidig et al. 2000), suggesting that the value of lipid droplets as a predictor of egg quality minimally requires careful evaluation before being used in a new species.
Properties of the ovarian fluid released with gametes during external fertilization of fishes are expected to play an important role in the fertilization success (Rosengrave et al. 2009), particularly by enhancing conspecific sperm motility duration and swimming trajectory (Gasparini and Evans 2013; Yeates et al. 2013). The pH of the ovarian fluid in Caspian brown trout *Salmo trutta caspius* decreased significantly with increasing occurrences of broken unfertilized ova in a spawn (Naghdi-Tabrizi et al. 2011). The pH of the ovarian fluid of turbot *Scophthalmus maximus* (Fauvel et al. 1993) and channel catfish *Ictalurus punctatus* (Chatakondi and Torrans 2012) were correlated with fertilization and hatch rates, respectively, suggesting that ovarian fluid pH can be used as a predictor of these measures of egg quality.

Previous studies on induced spawns of red snapper revealed no clear relationship between egg quality and post ovulation characteristics such as egg diameter, lipid droplet size and distribution, or fertilization rate (Bourque and Phelps 2007). Therefore, the objective of this work was to expand the evaluation of candidate predictors of egg quality to include a broader panel of parameters measured at early stages of the spawning process in order to identify a set of measurements that could be used to assist in the selection of good quality spawns for larval trials.

**Materials and Methods**

Procedures for broodstock acquisition, fish selection for spawning trials, hormonal induction, and strip spawning are detailed in Chapter II. Candidate criteria were assessed using data collected during seven spawning trials. Trials were performed during the 2011 and 2012 spawning seasons and are
summarized in Table 1 (Chapter II). Only spawns with a minimum fertilization rate of 70% were kept for analysis in order to minimize the influence of post-ovulatory aging of oocytes on the results. Based on this criterion, a total of 24 spawns were selected for the study. Seventeen females referred to as wild were caught offshore of the Alabama coastline, brought to the laboratory, and induced with gonadotropin within eight hours of their capture. The seven remaining females referred to as captive females were maintained in captivity during at least one year prior to hormonal induction under conditions described in the previous chapter.

*Spawning Procedures and Data Acquisition*

Multiple parameters were recorded for each spawn and evaluated as predictors of egg and larval quality. The number of days elapsed between the beginning of the spawning season (considered to be 1st May in the northern GOM; Collins et al. 1996) and the date of the spawn was included as a parameter in the analysis. Female weight (Wt, in g) and total length (TL, in cm) were measured before hormonal induction. Fulton’s condition coefficient K was then calculated for each individual according to the formula $K = 100 \frac{Wt}{TL^3}$.

Ovarian biopsy samples were obtained from each female by use of a Frydman® memory form polyethylene catheter, and oocytes were cleared in Serra’s solution (EFA, described in Chapter II) to determine the most advanced maturation stage reached by oocytes in selected females at the time of induction. Pre ovulatory oocytes were classified into five stages reflecting consecutive changes of the aspect of the vitellus in maturing oocytes described by Żarski et al. (2011b):

Stage I: uniform yolk, no oil droplet visible; Stage II: small and poorly visible oil
droplet filling the entire cytoplasm of the oocytes; Stage III: oil droplets well defined, peripheral hyalinization; Stage IV: ring of large forming droplets centered around the germinal vesicle; and Stage V: less than 5 large oil droplets coalesced with diameter about 1/4 of the germinal vesicle diameter.

The latency period, defined as the time interval between hormonal stimulation and the time when ovulation was detected, was recorded for each contributing female. The pH of the unfertilized ova was measured immediately when ovulation was detected from a subsample of the spawn obtained by manual stripping in a dry 10-ml beaker. The pH was measured using a pH pen-meter (YSI®, pH-100 with piercing electrode). The remaining ova were stripped from the female into a graduated bowl containing 500 mL of seawater and immediately fertilized by mixing sperm striped from one or two males using methods described in Chapter II. Fecundity was measured as the total number of eggs released by the female and was estimated volumetrically right after fertilization by counting the number of eggs in a subsample of the spawn. Fecundity was standardized to the female weight to obtain the relative fecundity (Rfecundity). After spawning, contributing females were euthanized using a lethal dose of MS-222 (>400 mg L⁻¹). Otoliths were removed to determine the age by otolith increment analysis according to protocols described in VanderKooy and Guindon-Tisdel (2003). The liver was weighed and standardized to body weight to calculate the hepatosomatic index (HSI=100 (liver Wt x total body Wt⁻¹)). The fertilization rate, hatch rate, and survival of larvae recorded every 24 hours post hatch were assessed as described in Chapter II. The quality of each spawn was characterized based on the fertilization rate, the standardized hatching rate, the
duration of survival post hatch, and the quality parameter (described in the
previous chapter, high quality, or score of 1 when survival duration exceeded
4 dpf versus low quality, or score of 0 when mortality occurred before 4 dpf).

Statistical Analyses

Statistical analyses were performed using SAS 9.3® (SAS Institute, NC).
The distributions of each parameter in captive and wild females were
summarized as mean ± standard deviation (SD). The assumptions of normality
and homoscedasticity were tested using Shapiro-Wilk test in PROC
UNIVARIATE for normality and a Folded F test using the ratio of the two
variances in PROC TTEST for homoscedasticity. To improve normality of
distributions and reduce heterogeneity of variances before further statistical
analysis, variables with poor normality ($P<0.01$) were transformed. The survival
duration (in dpf) was standardized to its maximum value (6 dpf). Data expressed
in percentages (fertilization, standardized hatch rates, and standardized survival
duration) were subject to angular (arcsine square root) transformation, and the
relative fecundity was log transformed. An allometric transformation was used for
HSI. The weights of the liver and the female body were log transformed before
HSI calculation, and residuals of the resulting linear regression between the two
variables (resHSI) were used to meet the assumption of the lack of correlation
between response variables for multiple linear regressions (Anderson 2001).

An unpaired Student’s T-test implemented in PROC TTEST was used to
compare mean values of the parameters between spawns from captive and wild
females (Captivity parameter) and between spawns classified as high versus low
quality (Quality parameter), respectively (both categorical parameters). Pearson’s
correlation coefficients $r$ were calculated in PROC CORR and were determined between variables along with the probability $P$ that $r=0$. Correlations were considered significant when $P$-value was less than 0.05. Stepwise multiple linear regressions were used in PROC REG to build models predicting the continuous end-point variables (relative fecundity, fertilization, standardized hatch rate, and standardized survival duration) with an optimal subset of the variables. Because of the low number of samples available from captive females ($n=7$), multiple regressions were not attempted on this dataset. Models were built using the data from wild females ($n=17$) only due to the significant differences observed between spawns from wild and captive females for several parameters. Models were computed using a stepwise algorithm allowing variable entry and retention at the default significance level $P=0.15$. A multiple logistic regression in PROC LOGISTIC was used to build a model predicting each of the two categorical parameters (Captivity and Quality). Models were optimized using a stepwise selection approach as above. Significance levels for variable entry and retention in the model were set at a significance level of 0.1 to 0.3.

Results

Summary statistics (mean ± SD, range) for each parameter, along with the results of the Student’s T-test comparing mean values between the 17 spawns from wild females, and the 7 spawns from captive females are presented in Table 4.
Table 4

**Summary statistics (mean ± SD, range) for each parameter in spawns from wild and captive females; results of the Student’s T-test comparing these two groups (T- and P-values), with significant P-values in bold font (degrees of freedom df=22, except for Age and HSI df=21 due to one missing value).**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild (n=17)</th>
<th>Captive (n=7)</th>
<th>T-values</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date (days)</td>
<td>67.0 ± 37.0 (23 – 135)</td>
<td>85.6 ± 12.0 (73 – 109)</td>
<td>-1.28</td>
<td>0.2124</td>
</tr>
<tr>
<td>Age</td>
<td>5.9 ± 0.93 (4 – 7)</td>
<td>4.1 ± 1.2 (3 – 6)</td>
<td>3.89</td>
<td>0.0009</td>
</tr>
<tr>
<td>Wt (kg)</td>
<td>2.50 ± 0.67 (1.55 – 4.32)</td>
<td>2.58 ± 0.83 (1.43 – 3.70)</td>
<td>-0.25</td>
<td>0.8017</td>
</tr>
<tr>
<td>TL (cm)</td>
<td>56.5 ± 4.4 (49.5 – 65)</td>
<td>53.9 ± 5.1 (47.5 – 60.5)</td>
<td>1.28</td>
<td>0.2130</td>
</tr>
<tr>
<td>K</td>
<td>1.36 ± 0.12 (1.12 – 1.57)</td>
<td>1.60 ± 0.21 (1.34 – 1.94)</td>
<td>-3.74</td>
<td>0.0011</td>
</tr>
<tr>
<td>Oocyte stage</td>
<td>3.5 ± 1.5 (1 – 5)</td>
<td>2.6 ± 1.4 (1 – 5)</td>
<td>1.39</td>
<td>0.1789</td>
</tr>
<tr>
<td>Latency Time (h)</td>
<td>28.89 ± 1.06 (27.0 – 31.0)</td>
<td>32.86 ± 1.18 (32.0 – 34.5)</td>
<td>-8.09</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ova pH</td>
<td>7.44 ± 0.51 (6.56 – 8.35)</td>
<td>7.17 ± 0.54 (6.15 – 7.82)</td>
<td>1.18</td>
<td>0.2515</td>
</tr>
<tr>
<td>HSI</td>
<td>7.86 ± 1.81 (5.66 – 11.53)</td>
<td>9.48 ± 4.75 (5.04 – 17.70)</td>
<td>-0.88</td>
<td>0.4111</td>
</tr>
<tr>
<td>Rfecundity (egg.kg⁻¹)</td>
<td>100,891 ± 48,878 (23,502 – 202,024)</td>
<td>30,653 ± 21,028 (4,729 – 65,840)</td>
<td>4.56</td>
<td>0.0002</td>
</tr>
<tr>
<td>Fertility (%)</td>
<td>92.9 ± 5.3 (80 – 100)</td>
<td>83.9 ± 10.3 (71 – 97)</td>
<td>2.61</td>
<td>0.0161</td>
</tr>
<tr>
<td>Hatch (%)</td>
<td>81.3 ± 17.0 (27 – 95)</td>
<td>54.6 ± 19.7 (31 – 82)</td>
<td>3.10</td>
<td>0.0053</td>
</tr>
<tr>
<td>Survival duration (dpf)</td>
<td>3.9 ± 1.6 (1 – 6)</td>
<td>3.4 ± 1.8 (1 – 6)</td>
<td>0.54</td>
<td>0.5977</td>
</tr>
<tr>
<td>Quality (0/1)</td>
<td>0.71 ± 0.47</td>
<td>0.71 ± 0.49</td>
<td>-0.04</td>
<td>0.9698</td>
</tr>
</tbody>
</table>

The body weight and total length of females used in this study ranged between 1.43 and 4.32 kg and 47.5 to 65.0 cm, respectively. No significant difference in size or weight was detected between wild and captive females. However, Fulton’s condition coefficient K was significantly larger for captive fish (1.60 versus 1.36, \( P=0.0011 \), Table 4).

Fish responded to hormonal stimulation on average 30 hours after induction at 27°C, but wild females responded significantly earlier than captive
fish ($P<0.0001$, Table 4). Mean response time for wild fish was 28.9 hours (range 27 to 31 h) while the ovulation of captive fish was detected on average at 32.9 hours (range 32 to 34.5 hours). Relative fecundity also differed significantly between the spawns of wild and captive fish ($P=0.0002$). Even though wild females produced up to 200,000 eggs Kg\(^{-1}\) (mean 100,891 eggs Kg\(^{-1}\)), captive females only produced 30,000 eggs Kg\(^{-1}\) on average with a maximum of 65,000 eggs Kg\(^{-1}\). Despite large variability between individuals, the wild fish produced on average three times more eggs than captive fish.

Otolith increment analysis revealed that the age of female used in this experiment ranged between three and seven years old (5 years-old on average). Wild females were significantly older (5.9 years old on average) than captive ones (4.1 years-old on average, $P=0.0009$).

Because captive females differed from wild females for several pre-spawning parameters, tests of correlation between variables were conducted separately for the 17 spawns from wild fish and the 7 captive fish. Pearson’s correlations between variables from the two groups are presented in Table 5.
Table 5

Pearson’s correlations between parameters measured on wild (below diagonal) and captive (above diagonal) females and their spawns. Significant values are in bold font. Symbols denote the level of significance of correlations: * P<0.05, ** P<0.01, or *** P<0.001.

<table>
<thead>
<tr>
<th></th>
<th>Date</th>
<th>Age</th>
<th>Wt</th>
<th>TL</th>
<th>K</th>
<th>Stage</th>
<th>Latency</th>
<th>Ova pH</th>
<th>HSI</th>
<th>R fecundity</th>
<th>Fertility</th>
<th>Hatch</th>
<th>Survival duration</th>
<th>Quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date</td>
<td>1</td>
<td>0.42</td>
<td>0.02</td>
<td>0.36</td>
<td>-0.48</td>
<td>0.6</td>
<td>-0.46</td>
<td>-0.28</td>
<td>-0.54</td>
<td><strong>0.79</strong></td>
<td>0.26</td>
<td>0.16</td>
<td>-0.48</td>
<td>-0.31</td>
</tr>
<tr>
<td>Age</td>
<td>0.13</td>
<td>1</td>
<td>-0.22</td>
<td>-0.22</td>
<td>0.1</td>
<td>-0.15</td>
<td>-0.27</td>
<td>0.15</td>
<td>0.09</td>
<td>0.08</td>
<td>0.75</td>
<td>0.64</td>
<td>-0.38</td>
<td>-0.2</td>
</tr>
<tr>
<td>Wt</td>
<td>-0.59*</td>
<td>0.15</td>
<td>1</td>
<td><strong>0.91</strong>*</td>
<td>0.58</td>
<td>0.27</td>
<td>-0.01</td>
<td><strong>-0.81</strong>*</td>
<td>0.21</td>
<td>-0.15</td>
<td>-0.03</td>
<td>0.17</td>
<td>-0.68</td>
<td>-0.47</td>
</tr>
<tr>
<td>TL</td>
<td><strong>-0.50</strong>*</td>
<td>0.20</td>
<td><strong>0.92</strong>*</td>
<td>1</td>
<td>0.21</td>
<td>0.57</td>
<td>-0.09</td>
<td><strong>-0.87</strong>*</td>
<td>-0.14</td>
<td>0.2</td>
<td>0.14</td>
<td>0.02</td>
<td>-0.69</td>
<td>-0.52</td>
</tr>
<tr>
<td>K</td>
<td><strong>-0.49</strong>*</td>
<td>-0.01</td>
<td>0.46</td>
<td>0.12</td>
<td>1</td>
<td>-0.48</td>
<td>-0.04</td>
<td>-0.2</td>
<td><strong>0.78</strong>*</td>
<td>-0.67</td>
<td>0.38</td>
<td>0.5</td>
<td>-0.4</td>
<td>-0.12</td>
</tr>
<tr>
<td>Stage</td>
<td>0.34</td>
<td>0.02</td>
<td>-0.24</td>
<td>-0.26</td>
<td>-0.08</td>
<td>1</td>
<td>-0.4</td>
<td>-0.73</td>
<td>-0.73</td>
<td><strong>0.84</strong>*</td>
<td>-0.37</td>
<td>-0.32</td>
<td>-0.08</td>
<td>0.03</td>
</tr>
<tr>
<td>Latency</td>
<td><strong>0.73</strong>*</td>
<td>0.03</td>
<td>-0.27</td>
<td>-0.08</td>
<td>-0.08</td>
<td>0.26</td>
<td>1</td>
<td>0.23</td>
<td>0.34</td>
<td>-0.63</td>
<td>-0.11</td>
<td>0.01</td>
<td>0.02</td>
<td>-0.52</td>
</tr>
<tr>
<td>Ova pH</td>
<td><strong>0.55</strong>*</td>
<td>0.11</td>
<td>0.05</td>
<td>0.14</td>
<td>-0.31</td>
<td>-0.08</td>
<td>0.64***</td>
<td>1</td>
<td>0.16</td>
<td>-0.32</td>
<td>0.11</td>
<td>-0.07</td>
<td>0.49</td>
<td>0.26</td>
</tr>
<tr>
<td>HSI</td>
<td><strong>-0.51</strong>*</td>
<td>-0.26</td>
<td>-0.08</td>
<td>-0.12</td>
<td>0.14</td>
<td>-0.16</td>
<td><strong>-0.62</strong>*</td>
<td>-0.32</td>
<td>1</td>
<td><strong>-0.83</strong>*</td>
<td>0.6</td>
<td>0.69</td>
<td>-0.28</td>
<td>-0.27</td>
</tr>
<tr>
<td>R fecundity</td>
<td>-0.42</td>
<td>0.06</td>
<td>0.33</td>
<td>0.44</td>
<td>-0.16</td>
<td>0.27</td>
<td>-0.11</td>
<td>-0.38</td>
<td>0.06</td>
<td>1</td>
<td>-0.19</td>
<td>-0.29</td>
<td>0.06</td>
<td>0.24</td>
</tr>
<tr>
<td>Fertility</td>
<td>0.05</td>
<td>0.10</td>
<td>0.1</td>
<td>0.16</td>
<td>-0.05</td>
<td>0.15</td>
<td>0.2</td>
<td>-0.06</td>
<td>-0.05</td>
<td>0.13</td>
<td>1</td>
<td>0.13</td>
<td><strong>0.96</strong>*</td>
<td>-0.56</td>
</tr>
<tr>
<td>Hatch</td>
<td>0.06</td>
<td><strong>0.58</strong>*</td>
<td>-0.09</td>
<td>-0.10</td>
<td>-0.21</td>
<td>-0.01</td>
<td>-0.26</td>
<td>-0.27</td>
<td>0.11</td>
<td>0.21</td>
<td>-0.23</td>
<td>1</td>
<td>0.49</td>
<td>0.26</td>
</tr>
<tr>
<td>Survival duration</td>
<td>-0.34</td>
<td>0.04</td>
<td>-0.19</td>
<td>0.64</td>
<td>-0.09</td>
<td>-0.08</td>
<td>0.08</td>
<td>-0.03</td>
<td>0.21</td>
<td>0.39</td>
<td>-0.08</td>
<td>-0.13</td>
<td>1</td>
<td><strong>0.86</strong>*</td>
</tr>
<tr>
<td>Quality</td>
<td>-0.16</td>
<td>0.28</td>
<td>0.27</td>
<td>0.14</td>
<td>-0.17</td>
<td>-0.15</td>
<td>0.1</td>
<td>0.02</td>
<td>0.16</td>
<td>0.3</td>
<td>-0.14</td>
<td>0.19</td>
<td><strong>0.86</strong>*</td>
<td>1</td>
</tr>
</tbody>
</table>
For both captive and wild fish, significantly high positive correlations were observed between weight and length ($r>0.9$) as well as between survival duration and quality ($r>0.8$). However, none of the phenotypic traits recorded prior to spawning were significantly correlated to the end point parameters (fertilization rate, standardized hatch rate, survival duration, and quality), except for a positive correlation between the age of wild females and the hatch rate of their spawn ($r=0.58$, $P=0.018$), indicating that a better hatch rate was generally obtained with older fish in this group.

Captive and wild females showed different patterns of correlations. In the wild female group, the date of spawn was significantly negatively correlated to weight, length, the HSI, and Fulton’s condition coefficient ($r=-0.49$ to $-0.59$, $0.01<P<0.05$), indicating a decrease of these parameters as the spawning season progressed. Spawning date was positively correlated with the pH of ova ($r=0.55$) and the latency time ($r=0.73$), indicating an increase of the ova pH and the time to respond to hormonal induction over the course of the spawning season. The latency time was also significantly negatively correlated with Fulton’s condition coefficient ($r=-0.61$) and HSI ($r=-0.62$), indicating a longer response time for fish with lower condition. Finally, a significant positive correlation was found between latency time and ova pH ($r=0.64$). None of these correlations were significant in the captive fish group ($n=7$).

For the captive females group, fertilization and hatch rates were strongly correlated ($r=0.96$). Although this was not the case for wild females, the relative fecundity was positively correlated to the spawning date for captive females.
Significant positive correlations were also detected between relative fecundity and oocyte stage assessed at the time of induction \((r=0.84)\) and between HSI and Fulton’s condition factor \((r=0.78)\). Negative correlations were found between relative fecundity of captive female and their HSI \((r=-0.83)\), and between the ova pH and female weight \((r=-0.81)\) and length \((r=-0.87)\). The latter correlation indicates that the pH of the ova from larger captive females was lower than for smaller fish. The different correlation patterns between wild and captive fish probably reflect in part the reduced power of inference considering the small sample size of the captive female group \((n=7)\).

Stepwise multiple linear regression analysis was used to predict egg quality parameters defined in the previous chapter (fertility, hatch, and survival duration) as well as relative fecundity of wild females based on the parameters recorded on females and their ova at the time of spawning. The optimal regression model for the prediction of the fecundity employed one single trait; the oocyte stage assessed prior to hormonal induction. However, the model explained only a small part of the variance \((r^2=0.18)\) and was not significant \((P=0.0992)\). The stepwise model retained for standardized hatch rate included one single variable, the age of the female parent, and explained 34\% of the variance in hatch rate. These two models are described below. Coefficients are given as point estimates with their standard errors.

\[
\text{Fecundity} = 0.05 \text{ oocyte stage} + 4.80 \quad P=0.0992 \quad r^2=0.18
\]

\[
\text{Hatch rate} = 0.17 \text{ female age} + 0.29 \quad P=0.0177 \quad r^2=0.34
\]
No model could be optimized for the fertilization rate and survival duration, reflecting the lack of correlation of these two egg quality variables to any of the phenotypic traits measured in the study.

The quality parameter defined in the previous chapter grouped spawns in two categories. When no significant decrease in survival was detected before 4 dpf, spawns were classified as high quality, and when a significant mortality was observed before or at 3 dpf, spawns were classified as low quality. The mean values of all pre-spawning parameters did not differ significantly between high and low quality groups. The best model predicting the quality categories included the age of the female only when significance levels for entrance and retention of variables were set at 0.3.

Discussion

The objective of this chapter was to evaluate the value of several phenotypic traits characterizing pre-spawning females and their maturation status for the purpose of predicting the four measures of egg quality described in Chapter II. One of the hypotheses evaluated in this work was that wild and captive females differ for several phenotypic characters due to the very different environment they experienced during gamete maturation, leading to different responses and endogenous characteristics of eggs. A first important result of this study is that wild and captive females indeed appeared to differ for some of the measures of egg quality and several characters that may influence egg characteristics.
Spawns of captive females showed significantly lower hatching rates and fecundity than those of wild females. The lower reproductive performances of captive females are consistent with reproductive dysfunctions reported in this and previous studies of captive maturation of red snapper broodstock (Phelps et al. 2009). Disruptions of gamete maturation and spawning in aquaculture have been linked to lack of production and release of gonadotropin by the pituitary (Mylonas et al. 2010; Zohar and Mylonas 2001) and are hypothesized to account for the lack of spawning activity of captive red snapper (Bardon-Albaret et al. in press). In the present study, no volitional spawning occurred throughout the reproductive season, a result consistent with previous observations of captive red snapper broodstock (Papanikos et al. 2003).

Earlier phases of oocyte maturation also appeared inhibited in the captive group. The majority of wild red snapper females in the size range selected in our experiments displayed fully grown oocytes and therefore reached the spawning capable phase (Brown-Peterson et al. 2011; Woods et al. 2003). In contrast, over the course of this study, only 19 out of the 106 (18%) captive females examined were found with fully grown oocytes suitable for hormonal induction, while partial or complete lack of vitellogenesis was observed in the others. In addition, even though those 19 females had larger oocytes, their maturation stage at the time of hormonal induction tended to be lower than that of wild females (2.6±1.4 versus 3.5±1.5 in wild females), although the difference was not significant due to the large variance within the two groups. Oocytes before hormonal induction were at least in Stage IV for 58% of wild females (28.6% in captive females), while 57%
of captive fish were found with oocytes only at Stage II (23.5% for wild females). Females bearing oocytes at Stage IV have a high probability of responding to hormonal induction while the success rate is much lower when females are induced at Stage II. Stage II oocytes are less advanced in the maturation process and less likely to complete maturation in response to a single injection of gonadotropin. These results thus indicate that only a small fraction of the captive stock was engaged in the gonadotropin-dependent phase of oogenesis (Grier et al. 2009; Lubzens et al. 2010). They also suggest that the mechanisms and pathways involved in the maturation process preceding ovulation were not fully established in most of captive fish (Gohin et al. 2010; Lessman 2009).

Relative fecundity was three times lower on average for spawns from captive fish, indicating that only a small number of oocytes were responsive to the hormonal treatment and could complete the final steps of maturation and ovulation. The latency time was on average about 4 hours longer in captive females (32.9 hours post induction versus 28.9 hours for wild females), suggesting that even responsive oocytes were less maturationally competent than those in wild females (Gohin et al. 2010; Zuccotti et al. 2011). These differences resulted in lower fertilization and hatch rates in the captive group, likely reflecting disruptions of gametogenesis in these females.

Another potential factor impacting the performance of captive females is their age. The selected wild females of this study were significantly older than females kept in captivity. These differences were not intended in the study, as age could only be determined after spawning experiments via otolith increment
analysis. Females were therefore selected based on their size (and oocyte pre-
maturation status). Captive females likely grew faster than their wild conspecifics
during the year prior to spawning because feeding rates were higher in the
hatchery where food supply was not limiting and also because they did not invest
energy in gametogenesis during the reproductive season as discussed above. In
consequence, captive females may have reached the required size for induction
at a younger age than wild females leading to the almost 2 years difference in
average age between the two groups. The age at first maturity for female red
snapper in the Northern Gulf of Mexico was reported to be 2 to 4 years old
(Collins et al. 1996; Woods et al. 2003). All the wild females induced for
spawning were 4 years old or older and therefore likely initiated significant
spawning activity prior to our spawning trials. On the other hand, 57% of captive
females were 3 to 4 years old and had been caught in the wild a year prior to
spawning trials (i.e., when they were 2 to 3 years old). In that situation, some of
the captive females may have not reached sexual maturity in the wild prior to
being brought to the laboratory and, given the lack of spontaneous spawning in
rearing tanks and large disruptions discussed above, may not have completed a
full cycle of oocyte maturation and/or spawning at all prior to the hormonal
induction trials. Thus, for some of the younger fish used in trials, it may have
been their first spawning event, which would be expected to result in lower
fecundity and egg quality (Berkeley et al. 2004; Jeuthe et al. 2013; Targońska et
al. 2012; White and Palmer 2004). The small number of captive females prevents
a rigorous assessment of this hypothesis, but age and lack of reproductive
activity prior to selection for hormonal induction are certainly factors contributing
to differing results between captive and wild females. Assuming efforts to achieve
maturation and spawning of red snapper in tanks are successful, this hypothesis
could be evaluated by comparing the quality of eggs from females having
completed multiple spawning events to that in spawns obtained from first
spawning fish.

No difference in weight and length were observed between captive and
wild females, consistent with our selection of fish based on size for induction
trials. However, captive fish had a higher Fulton’s condition factor. This
parameter is interpreted as an indicator of the general fitness of the fish (Bolger
and Connolly 1989). Heavier fish for a given length (i.e., higher K) are generally
thought to be in better nutritional status. In this study, higher K likely reflects
greater food availability, and also the lack of investment in spawning prior to the
trials as discussed above. The greater condition of captive females may have
influenced nutrient availability to constitute embryos endogenous reserves during
vitellogenesis but this would not necessarily translate in higher viability of fry if
the captive diet was not adapted (i.e., if it was deficient for some specific
essential nutrients). Unsuitable broodstock diets have indeed been suggested to
lead to poor fry quality in several marine fishes (Izquierdo et al. 2001;
Zambonino-Infante and Cahu 2010), and to date, optimal diets for red snapper
broodstock have not been identified (Papanikos et al. 2008).

Because of the large differences observed between captive and wild
females including differing nutritional history and probably endocrine disruptions
as well as the potential importance of the latter factors during oogenesis, mechanisms determining egg characteristics were hypothesized to be altered in the captive groups. For this reason, the wild and captive females were treated separately during investigations on correlations between variables and prediction of egg quality parameters. The only significant correlation between egg quality and female phenotype in the wild group was that between the hatching rate and the female age. In teleost species, older females tend to produce higher quality eggs than younger ones (Targońska et al. 2012) with significant effects of the age of the female on egg size and larval growth and survival (Berkeley et al. 2004; Chambers and Legget 1996; Jeuthe et al. 2013).

Wild fish tended to be smaller at the end of the spawning season reflecting in part the reduced availability of larger fish in late summer on collecting sites, possibly due in part to fishing mortality during the red snapper fishing season (typically scheduled early in summer). This result may also reflect in part the higher responsiveness of smaller fish to hormonal induction at later stages of the spawning season. Smaller fish may have been experiencing their first active reproductive cycle and could have been still unresponsive if caught and induced at the beginning of the season (May). However, these fish could have subsequently engaged in reproductive activity during the peak of the season and become responsive to the hormonal treatment if captured and induced in August or September.

The condition of wild fish, measured by K and the HSI, also decreased during the course of the spawning season. This trend was significant and
possibly reflected the reproductive investment of fish leading to the exhaustion of reserves over the course of spawning (Galloway and Munkittrick 2006; Llanos-Rivera and Castro 2004). In this experiment, an increase of the latency time following hormonal induction was observed as the reproductive season progressed, possibly reflecting the lower condition and exhaustion of fish delaying their physiological response or a slower response to induction due to the slightly lower temperature in the hatchery induction system (kept at 26-27°C throughout the spawning season) as compared to the temperature on offshore capture sites during the second part of the maturation season (typically close to 30°C). The survival rate of red snapper larvae from spawns produced at the beginning of the spawning season was higher than that of larvae obtained when broodstock were collected in June and July in the study of Bourque and Phelps (2007). Such a correlation was not detected in the present work, possibly due to the limited number of available spawns from wild females (n=17), leading to reduced power of the dataset.

Interestingly, the correlations between traits that were significant for wild females were generally not significant in the captive group. While this result is likely due in part to the low power of inference for testing the significance of correlations in the captive group due to a reduced number of females, it may also reflect altered physiology in captive females. Research is needed to determine the causes and mechanisms of disruptions of gametogenesis in captive females and to re-evaluate the relationships between variables once maturation and spawning are achieved in captivity. Even then, differing nutrition and
environmental conditions may still require determination of separate criteria and prediction models for the quality of eggs from wild and captive females.

Female age was the only variable contributing significantly to the prediction of the hatch rate during multiple regression analysis. This result suggests that older females produced eggs with a higher viability to hatch. As discussed above, this factor was also identified in other studies as an important determinant of egg viability to hatch (Brooks et al. 1997; Jerez et al. 2012; Jeuthe et al. 2013; Kjørsvik 1994). The model only explained 34% of the total variance in hatch rate, suggesting that additional variables such as the endogenous factors evaluated in the next two chapters will be needed in order to achieve reliable prediction of this egg quality trait.

The relative fecundity was best predicted by oocyte stage at hormonal induction, although the prediction was still poor with only 18% of the variance explained. In this study, the maximum stage reached by oocytes present in a biopsy sample of induced females was recorded. A simple way to improve this assessment would be to estimate the proportion of oocytes at different maturation stages and use this variable as an indicator of relative fecundity. Another factor influencing relative fecundity and that could not easily be determined during biopsy sampling is the size of the ovaries. This parameter could be assessed using ultrasound (Novelo and Tiersch 2012) and combined with the assessment of the proportion of potentially responsive oocytes in a biopsy to improve the prediction of relative fecundity.
No prediction model could be optimized for the other egg quality measures (fertilization rate, survival duration, and quality parameter). This result is consistent with the lack of significance of correlations between these quality parameters and most of the variables evaluated. While the power of our datasets is limited due to the low number of spawns available, the present findings suggest that correlations between the pre-spawning parameters evaluated in this study and spawn quality is moderate. Additional data would be useful to assess more accurately the relationship between variables, and may lead to improved predictive models. Asymmetric divisions have even been proposed as an early criterion for the detection of low quality eggs in some species. For example, blastomere morphology and structure at the early embryonic cleavage stage were correlated with hatch rates of Atlantic cod (Avery et al. 2009; Kjørsvik 1994), Atlantic halibut (Shields et al. 1997), and Pacific hapuku Polyprion oxygeneios (Kohn and Symonds 2012). Preliminary data of the symmetry of the red snapper first embryonic divisions were not related to later survival, but these results need more research to be confirmed. This parameter can be measured relatively rapidly and could be used early to assist in the selection of spawns with high viability potential if correlated to survival in red snapper.

This study strongly suggests that spawn quality traits (relative fecundity, fertilization, hatch, and survival duration) cannot be explained by simple phenotypic variables measured on females and their gonads prior to hormonal induction. This finding is consistent with studies in other species that concluded that egg quality is very difficult to predict with confidence (Bobe and Labbé 2010;
Brooks et al. 1997; Kjørsvik et al. 1990). Other parameters not captured by these simple phenotypic measurements need to be assessed to effectively predict egg quality. Endogenous variables such as nutrient content have been shown critical to determine fry viability (Cahu et al. 2003; Zambonino-Infante and Cahu 2010) and will be examined in Chapter IV. Maternal RNA content is also a strong candidate factor impacting egg quality, in particular early embryonic mortality (Bonnet et al. 2007; Reading et al. 2012) and the association between transcriptome profiles and egg quality will be examined in Chapter V.
CHAPTER IV
ENDOGENOUS FACTORS RELATED TO RED SNAPPER EGG QUALITY:
PART A – CHARACTERIZATION OF THE NUTRIENT CONTENTS
OF OVA AND MATERNAL RESERVE TISSUES IN RELATION
TO EGG AND LARVAL QUALITY

Introduction

The development of larviculture protocols for emerging marine species candidates for aquaculture requires a reliable source of viable pre-feeding larvae. Achieving a consistent and predictable supply of high quality eggs is therefore a primary focus for marine hatcheries. High quality eggs ensure that early larvae survive until they transition to exogenous feeds can be completed. Until then, embryos and newly hatched larvae are supported by maternal reserves accumulated in the vitellus during oogenesis (Bobe and Labbé 2010). These reserves will provide energy and materials needed by the embryo and early larva for growth and metabolism (organogenesis, movement, maintenance, and the initiation of exogenous feeding), until nutrients from exogenous feeds become available (Kamler 2008; Rainuzzo et al. 1997).

The quality and quantity of nutrients present in the vitellus are therefore essential components of egg quality and viability (Bobe and Labbé 2010; Brooks et al. 1997). These nutrients are mobilized from the female parent tissues and incorporated in oocytes during oogenesis (Lubzens et al. 2010). Thus, the nutritional status of a female influences its fecundity by determining the quantity of reserves that can be transferred to the oocytes. The composition of the
female’s reserve tissues also impacts the balance of essential nutrients incorporated into oocytes which is a critical determinant of the quality of the vitellus (Migaud et al. 2013). Furthermore, the nutrient concentration of broodstock diets were shown to directly affect the biochemical composition of eggs (Fernández-Palacios et al. 2011; Fuiman and Faulk 2013), leading to significant variations in egg quality (Almansa et al. 1999). Understanding the nutritional requirements of maturing broodstocks and larval fish and designing adequate diets to meet those requirements is therefore a primary goal for marine finfish hatchery production (Bromage et al. 1994; Migaud et al. 2013). A first step in understanding these nutritional requirements is to determine the relationship between egg nutrient content and the viability of larvae to first feeding so that further research can be conducted to identify husbandry protocols and broodstock diets that will ensure production of eggs with suitable nutrient content.

Most of the research on broodstock and larval nutrition to date has focused on the role of lipids and, in particular, essential fatty acids (Migaud et al. 2013). The lipid reserves of fish eggs are used in large part as substrates for embryonic and larval energetic metabolisms but are also critical structural components in cell membranes (Sargent et al. 1999). The importance of highly unsaturated fatty acids (HUFA), in particular docosahexaenoic acid (DHA, 22:6n–3) and eicosapentaenoic acid (EPA, 20:5n–3), and their balance have been documented extensively in several marine fishes, where deficient broodstock diets for those fatty acids led to reduced fecundity, fertility, and hatching rates (Rainuzzo et al. 1997 for review). Requirements in HUFAs of the
n-6 series such as arachidonic acid (ARA, 20:4n−6) received less attention until recently, but the importance of ARA for egg quality was documented in cod *Gadus morhua* (Salze et al. 2005) and warm-water species including Mangrove red snapper *Lutjanus argentimaculatus* (Ogata et al. 2004), African moony *Monodactylus sabae* (Ohs et al. 2013), and red drum (Fuiman and Ojanguren 2011). Larvae of marine species appear unable to synthesize the long chain fatty acids (e.g., EPA, DHA and ARA) from shorter chain C18 precursors (Sargent et al. 1999) making the supply of high quantities of long HUFAs in broodstock marine diets critical. In addition, ratios of n-3/n-6 HUFAs or EPA/ARA in the diet were shown to influence eicosanoid production, in particular prostaglandins (Stacey and Goetz 1982). Prostaglandins are hormone-like lipids that have been shown to stimulate the secretion of gonadotropin and the ovulation process (Stacey 2003) and to significantly impact fish egg quality (Henrotte et al. 2010). These results highlight the potential importance of the levels and relative contents of DHA, EPA, and ARA in maturation diets of marine tropical species such as the red snapper and the need to monitor the content of eggs for various classes of fatty acids in order to understand requirements.

Fewer studies have focused on larval requirements for proteins and amino acids. The amino acid content of the yolk of marine teleost eggs is usually high and shows little variation among species (Rønnestad and Fyhn 1993). The pool of amino acids established during the final steps of oocyte maturation is involved in initiating the osmotic influx of water that leads to ovulation (Coward et al. 2002). Subsequently, free amino acids are the raw material needed for protein
synthesis during somatic growth and ontogenetic development of the embryo and pre-feeding larva (Kamler 2008). Free amino acids also serve as an energy source until the beginning of exogenous feeding (Finn et al. 1995; Rønnestad et al. 1999). While amino acids are typically classified as non-essential or essential depending on whether an organism possesses the necessary pathways for their synthesis, their dual function both as energy source and material for anabolism and growth makes the supply of both types of amino acids critical to early development (Finn and Fyhn 2010). Studies in several warm-water species showed that the amino acid content of the yolk and the relative amounts of individual amino acids contributed significantly to egg viability, and these variables were included in suites of biochemical parameters used to predict the quality of egg batches (sea bream Sparus aurata, Lahnsteiner and Patarnello 2004; and common dentex, Samaee et al. 2010). Supplementation studies also suggest that specific amino acids may be beneficial to first feeding larvae (e.g., Taurine, Salze et al. 2011). Altogether, these results suggest that the amino acid concentration of eggs and the relative content of individual amino acids may both contribute to egg quality.

Red snapper spawn small eggs with limited vitellin reserves that are consumed within a very short period after mouth opening (typically less than two days, Williams et al. 2004). Information on the nutritional requirements of broodstock, embryos and larvae is still very limited in this species. The egg contents of Valine and Isoleucine were hypothesized to be of significant importance for embryonic development based on the observation of a rapid
decrease of the concentration of these two amino acids between fertilization and hatch (Hastey et al. 2010). Enrichment of broodstock diets with fish oils providing high content in HUFAs (DHA and ARA) had moderate effects on the fatty acid content and profiles of eggs in comparison to a standard diet composed of fish, shrimp, and squid, and no clear conclusion on the effects of fatty acid contents and profiles on egg quality could be drawn (Papanikos et al. 2008). Finally, survival duration of starved larvae post hatch varies largely, ranging from 0 to 7 days as described in Chapter II, suggesting that the amount of embryonic reserves may vary among spawns although this relationship has not been characterized yet.

Current hatchery procedures at the Thad Cochran Marine Aquaculture Center involve feeding broodstock with a mixture of frozen fish, shrimp, and squid supplemented with a vitamin pre-mix (Moon and Gatlin 1991) and fish oils during the spawning season. While this supplemented diet was successfully used in other marine species to enhance gametogenesis and improve fecundity and egg quality, its suitability for red snapper broodstock has not yet been established. Also, the nutritional status of females and the effectiveness of the incorporation of nutrients in oocytes may vary between captive and wild fish due to differing feeding rates, diet digestibility, and/or to endocrine disruptions and stresses induced by the captive environment as discussed in previous chapters.

In this chapter, the proximate composition and the fatty acid and amino acid profiles of red snapper ova (unfertilized eggs) were characterized. The nutrient contents in spawns from wild and captive females were compared, and
the correlation between nutrient profiles and the four egg quality parameters defined in Chapter II was tested. The effects of female pre-spawning parameters on ova nutrient content was also examined, in particular the condition of females prior to spawning was further characterized by assessing nutrient contents in reserve tissues (muscle and liver).

Materials and Methods

Proximate composition and amino acid and fatty acid profiles were analyzed in samples of unfertilized eggs from the 24 spawns studied in the two previous chapters. Seventeen of the spawns were from wild females caught offshore of the Alabama coastline during four trips performed at the beginning (five spawns in May), during the peak (nine spawns in July) or at the end (three spawns in August and September) of the 2011 and 2012 natural spawning seasons (data detailed in Chapter III). Their diet and condition was unknown but was expected to reflect the variability among red snapper females spawning offshore of the Alabama coastline during the course of the natural spawning season. Females were brought to the laboratory and induced for oocyte maturation within 8 hours of capture using protocols described in Chapter II. The seven remaining females were maintained in captivity at the Gulf Coast Research Laboratory for at least one year prior to spawning. Captive rearing conditions are described in detail in Chapter II. Briefly, the photoperiod and temperature reflected variation in the natural environment of the north central Gulf of Mexico, and the diet consisted of frozen cigar minnows, shrimp, and squid 50, 25, 25%, respectively fed at 3-4% body weight (BW) three times a week.
Twice a week, the diet was partially substituted with a supplement that consisted of lecithin (0.2%) and a vitamin premix (2%) prepared according to Moon and Gatlin (1991), mixed with fish-meal (3%) and fish-oil (3%) and combined in a gelatin based diet that was distributed at 1% BW.

Females were selected for spawning trials as described in Chapter II and induced for spawning using a single injection of 1,100 IU hCG.kg\(^{-1}\) BW. When ovulation was detected, the female was anesthetized, and ova were collected by manual stripping for fertilization. A subsample of the ova was obtained at that time, immediately snap-frozen in liquid nitrogen, and subsequently kept at -80°C until analysis. After spawning, the contributing females were euthanized, and small pieces (1 cm\(^3\)) of liver and muscle tissues were sampled. Each tissue was individually snap frozen in liquid nitrogen and stored at -80°C until analysis.

The ova of each female were fertilized with the sperm of one or two males and the fertilization, and hatch rates were recorded as described in Chapter II. The survival of larvae post hatch was monitored, and the survival duration and quality parameter were estimated as also described in Chapter II.

A suite of parameters described in Chapter II was recorded for each female and its spawn for analysis in conjunction with nutrient content data and the four egg quality parameters (fertilization, standardized hatch rate, survival duration, and quality parameter). The suite of characters included the female size (weight and length), the Fulton’s condition coefficient K, the hepato-somatic index (HSI), the age, the oocyte stage at the time of hormonal induction, the spawning date, the relative fecundity, the latency interval, and the ova pH. Samples of ova,
muscle, and liver were shipped to the fish nutrition laboratory at Texas A & M University (TAMU) for analysis of proximate composition and amino and fatty acid profiles.

**Proximate Composition Analyses**

Samples of ova and maternal muscle and liver tissues were assayed for moisture and crude lipids and proteins. Measurements of moisture and crude proteins by the Dumas method were performed following protocols described in the AOAC (2005). Lipids were extracted with a chloroform: methanol protocol and crude lipid content was measured using the protocol described by Folch et al. (1957).

**Amino Acid Analyses**

Amino acid profiles were generated for ova, and maternal liver and muscle tissues. Analysis was performed using an Ultra Performance Lipid Chromatography technique (UPLC). Samples were prepared as described by Buentello and Gatlin (2001) and Buentello et al. (2011). Briefly, 500 mg frozen sample was homogenized with a tissue generator probe (Virtis Co., Inc., New York) in 3 mL perchloric acid (1.5M), vortexed, and centrifuged (3,000 X g for 5 min) until phase separation into supernatant and pellet. The supernatant fraction was neutralized with 2M potassium carbonate (1:1 by volume). These samples were lipid-extracted with diethyl ether (1:3 by volume, then vortexed) followed by aspiration of the ether layer. Defatted samples were filtered through 0.2 μm polycarbonate syringe filters followed by precolumn derivatization with o-phthaldialdehyde for free-pool amino acid (FAA) analysis. Likewise, the resulting
pellet (protein bound amino acids, PAA) was hydrolyzed by placing in 6N hydrochloric acid at 110°C for 24 hours, vortexed, and dried under nitrogen. The dry residue was dissolved in HPLC-water, deproteinized, and neutralized. The FAA and PAA fractions were pooled and analyzed for amino acids using an Ultra Performance Lipid Chromatography technique (UPLC-Acquity system, WatersTM, Milford, MA) as described by Pohlenz et al. (2012). Identification and quantification of amino acids were accomplished using external standards (Sigma Chemical Co., St. Louis, Missouri).

Fatty Acid Analyses
The fatty acid contents in the total lipid fraction of ova were determined by using gas chromatography (GC) after transmethylation (Craig et al. 1995, 1999). The total lipid (2 mg) was dissolved in 1.0 mL of n-hexane, and 0.2 mL of methanolic 2M NaOH solution was added. After this, the mixed solution was shaken for 10 sec., and it was allowed to stand for 1 min at 50°C, and 0.2 mL of methanolic 2M HCl solution was then added and shaken for 10 sec. The n-hexane layer was collected and concentrated. Tricosanoic acid methyl ester (Sigma Chemical Corp.) was used as an internal standard. The total lipid (10 mg) was loaded on the silica cartridge, and the NL fraction was eluted with 20 mL of chloroform-methanol (49:1 by volume), while the PL fraction was eluted with 20 mL of methanol. GC analyses were performed with a Varian 3400 system equipped with an Ultra Alloy® capillary column, a flame ionization detector (FID), and a split injector. Nitrogen gas was used as the carrier gas. The column oven temperature increased from 180°C to 280°C at a rate of 4 °C.min⁻¹ and was then
maintained at 280°C for 10 min. The injector and FID were set at 260°C and 290°C, respectively. Analysis results were expressed in mg/g wet weight.

**Statistical Analyses**

Statistical analyses were performed using SAS 9.3® (SAS Institute, NC). Proportion data were subjected to angular transformation (arcsine square root), and the normality of distributions was tested in PROC UNIVARIATE using a Shapiro Wilk test. The proximate composition of ova, muscle, and liver tissue samples from captive females, and their spawns were compared to those from wild females and spawns using an unpaired Student’s T-test in PROC TTEST. Comparisons between the two groups were conducted for both raw contents (expressed in mg.g⁻¹ wet weight), and relative contents expressed in percentage of the total amino or fatty acid content of the tissue. Pearson’s correlation coefficients between proximate composition data (crude proteins, crude lipids, moisture, and amino acid and fatty acid contents) and the parameters used to characterize females and spawns in the previous two chapters were computed in procedure PROC CORR and their significance tested. The parameters included are the female age, total length, body weight, condition coefficient K, and oocyte stage before induction, the date of spawn, the latency time between induction and ovulation, the pH of ova at ovulation, the residues of HSI after a log/log transformation, the relative fecundity, the fertility, the hatch rate, and the survival duration of the spawn.

Stepwise multiple linear regressions were performed in PROC REG to build models predicting the continuous end-point parameters of the spawns.
(relative fecundity, fertilization rates, standardized hatch rates, and the survival duration) based on the proximate composition and the amino acid and fatty acid content of ova. As in Chapter III, models were built using only data from wild females (n=17) considering the differences for multiple parameters detected between wild and captive females and their spawns. Models were computed using a stepwise algorithm allowing variable entry at the default significance level $P=0.15$. The retention criterion was decreased to a significance level of $P=0.10$ to ensure the collinearity between variables retained in the model was kept at acceptable levels (Variance Inflation Factor VIF <10, and Condition Index CI <30; Belsley et al. 1980). The quality score defined in Chapter II (quality score is 1 when no significant mortality is detected until 4 dpf, and 0 otherwise) was compared between captive and wild females and spawns with an unpaired Student’s T-test in PROC TTEST. For the amino acid and the fatty acid profiles, a logistic regression approach was used with PROC LOGISTIC to identify subsets of variables that best discriminate wild and captive spawns, and low and high quality spawns respectively. Models were computed using a stepwise algorithm allowing variable entry and retention at the default significance level $P=0.15$.

Results

Comparison between Spawns from Captive and Wild Females

Proximate composition of ova, muscle, and liver. Summary statistics for ova (unfertilized eggs), muscle, and liver tissues sampled from wild and captive females and their spawns are presented in Table 6. The moisture, crude protein,
and lipid content did not differ significantly between ova samples from wild and captive females. In contrast, significant differences in moisture, crude protein, and crude lipid contents of muscle and liver tissues of females were detected between the two groups. The muscle tissue of wild females contained significantly more water (79.2% versus 75.3%), less crude protein (19.3% versus 21.8%), and marginally less crude lipids than that of captive females. The liver of wild females contained significantly more water (77.6% versus 61.6%), fewer lipids (5.4% versus 17.8%), and marginally less crude protein than that of captive females.

Table 6

Proximate composition (moisture, crude protein and crude lipid content given as mean ± SD) of ova and tissue samples (muscle and liver) from 24 red snapper females, and results of unpaired Student T test comparing values of wild and captive groups.

<table>
<thead>
<tr>
<th>Ova</th>
<th>All (n=24)</th>
<th>Wild (n=17)</th>
<th>Captive (n=7)</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>94.18±0.57</td>
<td>94.15±0.65</td>
<td>94.26±0.36</td>
<td>0.6434</td>
</tr>
<tr>
<td>Crude protein</td>
<td>3.67±0.47</td>
<td>3.72±0.53</td>
<td>3.57±0.29</td>
<td>0.4679</td>
</tr>
<tr>
<td>Crude lipid</td>
<td>1.43±0.38</td>
<td>1.43±0.36</td>
<td>1.45±0.46</td>
<td>0.4190</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Muscle</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>78.07±2.31</td>
<td>79.20±1.43</td>
<td>75.31±1.59</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Crude protein</td>
<td>20.04±1.51</td>
<td>19.33±1.12</td>
<td>21.76±0.72</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Crude lipid</td>
<td>2.12±0.65</td>
<td>2.05±0.62</td>
<td>2.29±0.74</td>
<td>0.4219</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Liver</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>72.93±9.05</td>
<td>77.58±1.28</td>
<td>61.61±9.94</td>
<td>0.0053</td>
</tr>
<tr>
<td>Crude protein</td>
<td>16.54±1.65</td>
<td>16.85±1.26</td>
<td>15.76±2.29</td>
<td>0.1387</td>
</tr>
<tr>
<td>Crude lipid</td>
<td>9.05±8.25</td>
<td>5.43±0.83</td>
<td>17.84±11.49</td>
<td>0.0010</td>
</tr>
</tbody>
</table>

Fatty acid profile of ova Mean value ± SD of fatty acids in ova for spawns of wild and captive fish are reported in % of wet weight and in % of the total fatty acid content in Table 7. The γ-Linolenic acid (18:3n-6) was detected in the ova of wild females (0.03±0.06%) but not in any of the ova sampled from captive
females. The three most abundant fatty acids were Palmitic acid (16:0), DHA (22:6n-3), and Oleic acid (18:1n-9) regardless of the female origin (wild or captive). Similarly, the three fatty acids least abundant in the unfertilized eggs were γ-Linolenic acid (18:3n-6), Eicosatrienoic acid (20:3n-6), and Arachidic acid (20:0). Unfertilized eggs from captive and wild females did not differ significantly for their fatty acid contents except for the saturated fatty acid 20:0 ($P=0.0066$). Ova sampled from wild fish contained less fatty acid 20:0 than those obtained from captive fish (0.20±0.16% and 0.34±10%, respectively). Stepwise logistic regression failed to detect a combination of fatty acid predicting the captive or wild origin based on the fatty acid profile of the ova.

**Amino acid profiles.** Summary statistics for the 10 essential and 15 non-essential amino acids detected in ova and female muscle and liver tissues are presented in Table 8 for the two groups (wild and captive females). The abundance of the various amino acids paralleled the trend observed for crude protein content with higher contents in muscle (mean 76.7 mg g$^{-1}$) than in liver (50.5 mg g$^{-1}$) and lower content in eggs (14.1 mg g$^{-1}$). The non-essential amino acid (NEAA) Citrulline was not detected in any of the tissues examined. The three most abundant amino acids in the ova were Leucine, Alanine, and the combination of Glutamine and Glutamic acid (Glu+Glu), regardless of the fish origin. Three amino acids were not detected in ova (Hydroxyproline: found in muscle and liver, 1-Methylhisitine: only found in muscle in low concentration, and Ornithine: only found in low concentration in liver) while two others (Cystine and Anserine) were found at the minimum detection threshold.
Table 7

Total fatty acid content expressed as % of wet weight (%WW ± SD) or % total fatty acids (%TFA ± SD) in ova for the 17 wild and 7 captive spawns. Bold font denotes significant differences between the two groups during Student's T-test, and larger fatty acid value designed by *P<0.05, **P<0.01, or ***P<0.001.

<table>
<thead>
<tr>
<th></th>
<th>% WW ± SD</th>
<th>% TFA ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild (n=17)</td>
<td>Captive (n=7)</td>
</tr>
<tr>
<td></td>
<td>Wild (n=17)</td>
<td>Captive (n=7)</td>
</tr>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C 14:0</td>
<td>4.63±1.08</td>
<td>4.44±1.19</td>
</tr>
<tr>
<td>C 15:0</td>
<td>1.06±0.36</td>
<td>1.12±0.53</td>
</tr>
<tr>
<td>C 16:0</td>
<td>17.17±2.97</td>
<td>17.06±2.19</td>
</tr>
<tr>
<td>C 17:0</td>
<td>1.31±0.39</td>
<td>1.41±0.45</td>
</tr>
<tr>
<td>C 18:0</td>
<td>7.53±1.19</td>
<td>7.57±0.94</td>
</tr>
<tr>
<td>C 20:0</td>
<td>0.20±0.16</td>
<td>0.34±0.10**</td>
</tr>
<tr>
<td>Σ saturated</td>
<td>31.90±4.22</td>
<td>31.95±3.73</td>
</tr>
<tr>
<td>Mono-Unsaturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C 14:1</td>
<td>0.24±0.20</td>
<td>0.26±0.15</td>
</tr>
<tr>
<td>C 16:1</td>
<td>2.91±0.40</td>
<td>2.82±0.31</td>
</tr>
<tr>
<td>C 18:1 n-9</td>
<td>8.97±2.58</td>
<td>8.34±2.41</td>
</tr>
<tr>
<td>C 18:1 n-11</td>
<td>2.52±0.45</td>
<td>2.46±0.32</td>
</tr>
<tr>
<td>C 18:1 t</td>
<td>0.64±0.30</td>
<td>0.77±0.11</td>
</tr>
<tr>
<td>C 20:1</td>
<td>0.50±0.22</td>
<td>0.51±0.14</td>
</tr>
<tr>
<td>Σ Mono Unsaturated</td>
<td>15.78±3.01</td>
<td>15.18±2.58</td>
</tr>
<tr>
<td>Poly Unsaturated n-6</td>
<td></td>
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</tr>
<tr>
<td>C 18:2 n-6 (LA)</td>
<td>1.15±0.34</td>
<td>1.39±0.33</td>
</tr>
<tr>
<td>C 18:3 n-6</td>
<td>0.03±0.06</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>C 20:3 n-6</td>
<td>0.03±0.08</td>
<td>0.02±0.06</td>
</tr>
<tr>
<td>C 20:4 n-6 (ARA)</td>
<td>2.67±0.79</td>
<td>2.37±0.47</td>
</tr>
<tr>
<td>Σ n-6</td>
<td>3.88±0.90</td>
<td>3.78±0.63</td>
</tr>
<tr>
<td>n-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C 18:3 n-3 (LAN)</td>
<td>0.54±0.18</td>
<td>0.65±0.21</td>
</tr>
<tr>
<td>C 20:2 n-3</td>
<td>0.50±0.12</td>
<td>0.38±0.21</td>
</tr>
<tr>
<td>C 20:3 n-3</td>
<td>0.52±0.15</td>
<td>0.45±0.05</td>
</tr>
<tr>
<td>C 20:5 n-3 (EPA)</td>
<td>3.73±0.74</td>
<td>3.91±0.82</td>
</tr>
<tr>
<td>C 22:5 n-3</td>
<td>1.42±0.32</td>
<td>1.31±0.27</td>
</tr>
<tr>
<td>C 22:6 n-3 (DHA)</td>
<td>16.48±3.44</td>
<td>17.37±2.84</td>
</tr>
<tr>
<td>Σ n-3</td>
<td>23.19±3.97</td>
<td>24.08±2.76</td>
</tr>
<tr>
<td>n-3/n-6</td>
<td>6.21±1.45</td>
<td>6.45±0.81</td>
</tr>
<tr>
<td>Σ 18C PUFA</td>
<td>1.72±0.46</td>
<td>2.04±0.52</td>
</tr>
<tr>
<td>Σ PUFA</td>
<td>27.07±4.44</td>
<td>27.86±3.29</td>
</tr>
<tr>
<td>EPA/ARA</td>
<td>1.51±0.56</td>
<td>1.77±0.76</td>
</tr>
<tr>
<td>DHA/EPA</td>
<td>4.46±0.71</td>
<td>4.64±1.27</td>
</tr>
</tbody>
</table>
The absolute concentration of amino acids of ova did not differ significantly between captive and wild groups for any of the amino acids surveyed. The relative content of amino acids (expressed as % Total Amino Acids) was very similar between the two groups for most of the amino acids surveyed (mean difference measured as mean wild – mean captive/(mean (wild+captive)) = 5.1\%). Significant differences were observed for 3-Methylhistidine, Leucine, Valine, and Tyrosine (Leucine and Valine higher in wild spawns, 3-Methylhistidine and Tyrosine higher in captive spawns).

In the muscle, the amino acid content of captive and wild females paralleled the crude protein content, with greater values in captive females for almost all amino acids surveyed (Table 8). The difference between the two groups was significant for 60% (17 out of 25) of the amino acids examined and impacted both essential and non-essential amino acids. Once amino acid contents were standardized to the total amino acid content, significant differences were observed for only one amino acid (Taurine, mean difference 14\%), and the average magnitude of the difference between the two groups across all amino acids was only 1.7\%.
<table>
<thead>
<tr>
<th>Table 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (protein bound + free) amino acid content expressed as % of wet weight (%WW ± SD) or % total amino acids (%TAA ± SD) in ova, and female muscle and liver tissues for 17 wild and 7 captive red snapper females and their spawns. Bold font denotes significant differences between the two groups during Student’s T-test and the group with larger amino acid content is identified with asterisks (*P&lt;0.05, **P&lt;0.01, or ***P&lt;0.001).</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Essential AA</th>
<th>Ova</th>
<th></th>
<th></th>
<th>Muscle</th>
<th></th>
<th></th>
<th>Liver</th>
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<tbody>
<tr>
<td></td>
<td>%WW ± SD</td>
<td>%TAA ± SD</td>
<td>%WW ± SD</td>
<td>%TAA ± SD</td>
<td>%WW ± SD</td>
<td>%TAA ± SD</td>
<td>%WW ± SD</td>
<td>%TAA ± SD</td>
</tr>
<tr>
<td></td>
<td>Wild</td>
<td>Captive</td>
<td>Wild</td>
<td>Captive</td>
<td>Wild</td>
<td>Captive</td>
<td>Wild</td>
<td>Captive</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.23±0.07</td>
<td>0.26±0.08</td>
<td>6.70±0.22</td>
<td>7.87±0.47</td>
<td>1.21±0.07</td>
<td>2.13±0.33**</td>
<td>0.80±0.12*</td>
<td>0.71±0.20</td>
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<td>Histidine</td>
<td>0.11±0.03</td>
<td>0.13±0.04</td>
<td>3.34±0.25</td>
<td>3.40±0.15</td>
<td>0.51±0.05</td>
<td>0.56±0.04*</td>
<td>1.05±0.10</td>
<td>2.64±0.11**</td>
</tr>
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<td>1 Meth histidine</td>
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<td>0.10±0.00</td>
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<td>0.00±0.00</td>
<td>0.02±0.01</td>
<td>0.01±0.01</td>
<td>0.07±0.05</td>
<td>0.00±0.00</td>
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<tr>
<td>3 Meth histidine</td>
<td>0.06±0.03</td>
<td>0.09±0.03</td>
<td>1.64±0.89</td>
<td>2.32±0.43*</td>
<td>0.44±0.12</td>
<td>0.49±0.11</td>
<td>2.38±0.60</td>
<td>2.33±0.46</td>
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<td>Isoleucine</td>
<td>0.22±0.06</td>
<td>0.24±0.07</td>
<td>6.50±0.26</td>
<td>6.56±0.22</td>
<td>0.98±0.06</td>
<td>1.09±0.04***</td>
<td>5.30±0.12</td>
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<td>Leucine</td>
<td>0.36±0.10</td>
<td>0.39±0.11</td>
<td>10.64±0.30*</td>
<td>10.39±0.16</td>
<td>1.71±0.10</td>
<td>1.89±0.06***</td>
<td>9.25±0.11</td>
<td>9.11±0.24</td>
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<td>Lysine</td>
<td>0.25±0.09</td>
<td>0.24±0.09</td>
<td>6.96±0.07</td>
<td>6.68±0.80</td>
<td>1.86±0.22</td>
<td>2.09±0.13*</td>
<td>10.14±1.00</td>
<td>10.07±0.78</td>
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<td>Methionine</td>
<td>0.08±0.04</td>
<td>0.10±0.03</td>
<td>2.36±0.70</td>
<td>2.53±0.27</td>
<td>0.62±0.05</td>
<td>0.68±0.04**</td>
<td>3.35±0.12</td>
<td>3.29±0.11</td>
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<td>Phenylalanine</td>
<td>0.17±0.07</td>
<td>0.19±0.05</td>
<td>4.82±1.29</td>
<td>5.19±0.27</td>
<td>0.94±0.12</td>
<td>0.98±0.25</td>
<td>0.96±0.49</td>
<td>4.81±0.08</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.18±0.06</td>
<td>0.19±0.06</td>
<td>5.24±0.23</td>
<td>5.13±0.23</td>
<td>0.95±0.06</td>
<td>1.06±0.05***</td>
<td>5.15±0.13</td>
<td>5.12±0.10</td>
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<td>Valine</td>
<td>0.26±0.07</td>
<td>0.27±0.08</td>
<td>7.72±0.57*</td>
<td>7.36±0.20</td>
<td>1.06±0.07</td>
<td>1.22±0.05***</td>
<td>5.83±0.08</td>
<td>5.86±0.11</td>
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<tr>
<td>Non-essential AA</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>Alanine</td>
<td>0.28±0.09</td>
<td>0.30±0.09</td>
<td>8.12±0.49</td>
<td>8.69±0.34</td>
<td>1.19±0.09</td>
<td>1.36±0.44***</td>
<td>6.45±0.18</td>
<td>6.53±0.20</td>
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<td>B-Alanine</td>
<td>0.03±0.01</td>
<td>0.04±0.01</td>
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<td>1.03±0.34</td>
<td>0.04±0.03</td>
<td>0.05±0.02</td>
<td>0.22±0.15</td>
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<td>Asparagine</td>
<td>0.01±0.02</td>
<td>0.02±0.04</td>
<td>0.17±0.54</td>
<td>0.30±0.52</td>
<td>0.21±0.11</td>
<td>0.22±0.06</td>
<td>1.15±0.57</td>
<td>10.7±0.26</td>
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<tr>
<td>Asp + Asp</td>
<td>0.14±0.04</td>
<td>0.14±0.05</td>
<td>0.42±0.36</td>
<td>0.37±0.35</td>
<td>1.37±0.11</td>
<td>1.55±0.55***</td>
<td>7.40±0.40</td>
<td>7.46±0.30</td>
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<tr>
<td>Citrulline</td>
<td>0.00±0.00</td>
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<td>0.00±0.00</td>
<td>0.00±0.00</td>
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<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.01±0.01</td>
<td>0.01±0.01</td>
<td>0.28±0.17</td>
<td>0.30±0.17</td>
<td>0.06±0.01</td>
<td>0.07±0.01**</td>
<td>0.32±0.03</td>
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<tr>
<td>Glu + Glu</td>
<td>0.27±0.09</td>
<td>0.29±0.10</td>
<td>7.78±0.59</td>
<td>7.65±0.71</td>
<td>1.98±0.14</td>
<td>2.16±0.06**</td>
<td>10.72±0.56</td>
<td>10.42±0.37</td>
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<td>Glycine</td>
<td>0.12±0.03</td>
<td>0.13±0.04</td>
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<td>3.51±0.15</td>
<td>0.96±0.10</td>
<td>1.13±0.13**</td>
<td>5.19±0.29</td>
<td>5.4±0.55</td>
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<tr>
<td>Ornithine</td>
<td>0.00±0.00</td>
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<td>0.00±0.00</td>
<td>0.00±0.00</td>
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<tr>
<td>Proline</td>
<td>0.23±0.07</td>
<td>0.25±0.07</td>
<td>6.71±0.30</td>
<td>6.83±0.40</td>
<td>0.71±0.06</td>
<td>0.80±0.06**</td>
<td>3.84±0.12</td>
<td>3.85±0.26</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.04±0.02</td>
<td>0.06±0.05</td>
<td>0.20±0.11</td>
<td>0.27±0.02</td>
</tr>
<tr>
<td>Serine</td>
<td>0.21±0.07</td>
<td>0.22±0.07</td>
<td>6.03±0.22</td>
<td>5.90±0.20</td>
<td>0.80±0.05</td>
<td>0.90±0.04***</td>
<td>4.35±0.07</td>
<td>4.30±0.08</td>
</tr>
<tr>
<td>Taurine</td>
<td>0.04±0.02</td>
<td>0.05±0.02</td>
<td>1.21±0.33</td>
<td>1.27±0.13</td>
<td>0.12±0.07</td>
<td>0.24±0.10</td>
<td>0.62±0.33</td>
<td>1.15±0.46*</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.15±0.05</td>
<td>0.21±0.06</td>
<td>4.77±0.66</td>
<td>5.52±0.89*</td>
<td>0.69±0.09</td>
<td>0.80±0.10*</td>
<td>2.71±0.34</td>
<td>3.25±0.43</td>
</tr>
</tbody>
</table>
The liver of wild and captive females differed significantly for their content in 12 amino acids (Table 8), and the average difference between the two groups was 22.1%. In all cases except for ß-Alanine, the livers of wild females had greater amino acid content than those of captive females. Once standardized to the total amino acid content, ten amino acids still differed significantly between wild and captive females, but the average magnitude of the difference was only 4.4%.

**Correlations between Nutrient Content of Ova and Egg Quality Parameters of Wild Fish**

*Relative fecundity*. The relative fecundity of wild fish was positively correlated with the ova concentration of fatty acid 14:0 ($r=0.58$, $P<0.05$) and negatively correlated to that of 20:0 ($r=-0.49$, $P<0.05$) and 20:3n-6 ($r=-0.63$, $P<0.01$). None of the proximate composition parameters or amino acid contents measured in ova, muscle, and liver tissues were correlated to the relative fecundity. The ova concentrations of two fatty acids were selected during stepwise regression analysis giving the following prediction equation:

$$\text{Relative fecundity} = 3.73 \times 14:0 - 6.63 \times 20:3n6 + 4.19 \quad P=0.0055 \quad R^2=0.52$$

*Fertility*. The fertilization rate of spawns from wild females was negatively correlated with the moisture content of ova ($r=-0.56$, $P<0.05$) and positively correlated with their protein content ($r=0.53$, $P<0.05$). Moderate positive correlations (ranging from 0.48 to 0.50, $0.01<P<0.05$) were detected between fertility and the ova content in Histidine, Arginine, Proline, and Serine. No correlation was detected between the nutrient contents in the reserves tissues
(muscle and liver) of the female and the fertilization rate. The stepwise regression analysis selected four fatty acids for best prediction of the fertilization rate according to the equation below.

\[ \text{Fertility} = 3.61 \times 14:1 - 3.68 \times 15:0 - 4.33 \times 20:1 - 4.35 \times 20:0 + 2.00 \]

\[ P=0.0003 \quad r^2=0.81 \]

*Standardized hatch.* No significant correlation was detected between standardized hatch rate and nutrient contents (proximate composition, amino acid and fatty acid profiles) of ova, muscle, or liver of wild fish. Accordingly, no parameter was selected for inclusion in a multiple linear regression model predicting this parameter during stepwise regression analysis.

*Survival duration.* The muscle concentration of crude proteins and Glutamine and Glutamate (Glu+Glu) were both significantly negatively correlated to survival duration \( (r=-0.59 \text{ and } r=-0.49, \text{ respectively}) \). Survival duration was also negatively correlated with the liver concentration of the EAA 3-Methylhistidine \( (r=-0.5 \text{ to } -0.51, \quad P<0.05) \) and the NEAA Phenylalanine \( (r=-0.50, \quad P<0.05) \). No correlation was detected between survival duration and proximate composition parameters or any of the amino acid or fatty acid concentrations measured in ova. The combination of three amino acids and ARA was selected during stepwise multiple regression analysis giving the predicting equation below.

\[ \text{Survival} = 80.92 \times \text{Taurine} + 54.45 \times \text{ß-Alanine} - 54.43 \times \text{Lysine} - 7.48 \times \text{ARA} + 4.18 \times \text{Sum Amino Acids} \]

\[ P=0.0009 \quad r^2=0.81 \]

*Quality parameter.* The nutrient concentrations measured in samples of ova, muscle, and liver were compared between spawns classified in the high quality and low quality groups based on the quality score defined in Chapter II.
None of the proximate composition parameters differed significantly between the two groups except for the crude protein content of the muscle. Crude protein content was slightly lower in muscle tissues when the quality was higher (19.0±1.1% and 20.2±2.1%, for high and low quality groups, respectively, \( P=0.0295 \)).

No significant difference between the two groups defined by the quality score was detected for the amino and fatty acid concentrations of ova. However, the stepwise logistic regression model selected the fatty acid 20:4 n-6 (ARA) for prediction of the quality score.

Significant correlations were also found between muscle and liver nutrient contents and the quality score of the spawns. The two quality groups differed significantly \( (P<0.05) \) for the muscle concentration of two EAA (1-Methylhistidine and Lysine) and two NEAA (Asp+Asp and Glu+Glu). All four amino acids were at lower concentrations in the muscle of females that produced larvae with high survival at first feeding (quality=1). Lysine, Glu+Glu, and Asp+Asp were among the four most abundant amino acids in the muscle tissue (mean, 1.37±0.11 to 2.03±0.15%). The Lysine content of muscle was the only nutrient content parameter selected during a stepwise logistic regression predicting the quality parameter.

Only one amino acid (3-Methylhistidine, \( P=0.0362 \)) differed significantly in liver concentration between high and low quality groups. However, the liver concentration of another amino acid (Anserine) was selected during logistic
regression analysis for the prediction of the quality parameter. Anserine was found at very low concentration in all groups (0.09±0.03% WW, Table 8).

**Association with Pre-Spawning Parameters**

The proximate composition parameters and nutrient concentrations that differed significantly between the captive and wild groups or showed a significant association with at least one of the quality parameters were examined for correlation with the suite of measurements taken during hormonal induction in order to characterize females and spawns (see Chapter III).

None of the parameters correlated to egg quality or female origin was significantly associated with the measured pre-spawning parameters except for female age and latency time of ovulation. The ova moisture and crude protein content were correlated (negatively and positively, respectively) with both the fertilization rate and the female age. The crude protein concentration of the muscle was negatively correlated with survival duration, quality, and latency time of ovulation.

**Discussion**

The first objective of this work was to characterize the nutrient content of red snapper ova and compare nutrient profile between ova of captive and wild females. The proximate composition of ova and their amino acid and fatty acid profiles did not differ significantly between the two groups. Few studies have compared the nutrient content of eggs from wild and captive fish. Studies in farmed and wild Atlantic salmon *Salmo salar* showed that captive and wild females produced eggs of similar composition; in particular, eggs from captive
females contained suitable amounts of PUFAs (Migaud et al. 2013). The eggs from captive striped trumpeter \textit{Latris lineata} fed chopped fish had similar proximate composition to those of wild fish, but differences in some fatty acids including EPA, DHA and other PUFAs were observed (Morehead et al. 2001). In Atlantic cod, Salze et al. (2005) observed similar nutrient profiles between eggs of captive and wild groups, but significant differences in the content of some phospholipids were detected together with a lower ARA content in the spawns from captive females, leading to substantially higher EPA/ARA ratio in that group.

In several species, the nutrient content of the eggs has been shown to be influenced by the composition of the broodstock diet (Cahu et al. 2004; Fuiman and Faulk 2013; Salze et al. 2011) and is a primary factor discussed to explain differences among broodstocks in the composition of egg reserves (Migaud et al. 2013). In this work the nutrient content of the diet fed to the captive broodstock was not characterized, and the diet of wild fish could not be characterized either. The diet of wild caught fish is often considered a reference high quality diet (Almansa et al. 1999; Izquierdo et al. 2001), and the wild females used in our spawning trials were generally in good condition and showed high fecundity, suggesting that nutrient availability was not an issue for most of the wild spawns. Accordingly our results suggest that the captive diet provided sufficient quantities of fatty acids and amino acids to support the production of eggs with profiles comparable to those of wild brood fish in good spawning condition.

Studies of the nutritional requirements of fish eggs and larvae are rendered difficult by the large array of nutrients that need to be considered and
the logistical difficulties inherent to implementing experiments testing diets with varying content in a specific amino acid or fatty acid (Kamler 2008). The critical role of long chain polyunsaturated fatty acids (≥C20) has, however, been demonstrated in marine species (Fernández-Palacios et al. 2011), and high quantities of PUFAs are therefore recommended for marine broodstock and larval diets (Sargent et al. 1999; Tocher 2003). Within the fatty acid pool, the relative content of specific fatty acids, especially the long chain n-6 and n-3 PUFAs, and in particular DHA and ARA and EPA, respectively have also been shown to be of particular importance in order to achieve high survival of embryos and larvae (Bell and Sargent 2003; Henrotte et al. 2011; Migaud et al. 2013; Ohs et al. 2013). Our captive diet included a supplement that incorporated fish oils rich in PUFAs (including DHA, EPA and ARA) which likely contributed to balance the diet if the frozen fishmeal was deficient in those fatty acids. The supplemented diet thus appeared to provide sufficient amounts of lipid and fatty acids including PUFAs to achieve appropriate lipid content in the ova. While a similar result was obtained during the comparison of amino acid profiles of ova from captive and wild caught females, interpretation needs to be more cautious because the total content of amino acids (protein-bound + free amino acids) was measured in this study. The composition of the free pool of amino acids has been shown to be of high importance to embryonic and larval development (Buentello et al. 2011; Finn and Fyhn 2010). Amino acids are delivered into oocytes in the form of lipoproteins and hydrolyzed into amino acids during oogenesis (prior to fertilization in marine fishes, Finn and Fyhn 2010). They are used for anabolism
but also as a source of energy during embryonic development and thus the content and composition of the free pool of amino acid is an important metric for diagnosing the nutrient content of a spawn and its viability potential. Our data did not distinguish between the abundance and composition of the free pool of amino acids from those of the protein-bound fraction, and consequently spawns could show high total amino acid content but a reduced content in free amino acids available for energy and growth. A reduced fraction of free amino acids in captive spawns would involve, for example, a less effective hydrolysis of yolk protein in those spawns or an increased oxidation of amino acids. In such circumstances, the profiling of the sum of free and protein bound fraction would be misleading regarding on the actual availability of amino acids. The metabolism of yolk proteins is still largely unknown (Fabra et al. 2006) and the likelihood that differences in proteolysis or amino acid degradation rates occur among spawns due to factors resulting from captive holding is difficult to evaluate. As a precautionary measure, further assessment of spawns should therefore include characterization of the free pool of amino acids.

The condition and nutritional status of the broodstock was characterized by measuring the nutrient content of reserve tissues (muscle and liver) to evaluate if these parameters acted as limiting factors contributing to the very low fecundity and lower hatch rate of spawns from captive females. However, the condition coefficient of captive females was higher than that of wild females as discussed in Chapter III. Better condition of captive females was also suggested by analyses of the nutrient content of the reserve tissues. The muscle tissue of
captive females had significantly more protein (and amino acid) than that of wild females, and their liver had substantially higher levels of lipids. The liver is the central organ controlling the metabolism of protein and lipids (Sheridan 1994). Lipids are mobilized from reserve tissues and delivered to the oocytes in the form of lipoproteins (Wiegand 1996). The high HSI of captive females along with the extremely high lipid content of the liver suggests that large amounts of lipids were available but were not incorporated into oocytes or used for other functions. This result may signal an excess of lipids in the diet but may also be due in part to the lack of spontaneous spawning activity throughout the spawning season and the low fecundity observed following hormonal induction. Lipids obtained from the diet remained in the liver, instead of being used for reproduction or energy. The higher protein and amino acid content of the muscle is also consistent with this observation (i.e., proteins and amino acids in the muscle would not have been mobilized as much in captive females, Cahu et al. 2004).

The distribution of amino acids in muscle and liver tissues of captive and wild females were relatively similar. The profiles of fatty acids in particular PUFAs in muscle and liver were not characterized and might have differed between the two groups due to differing diets. If such differences occurred, they were not reflected in the ova of captive versus wild females as discussed above. However, captive spawns were very small, and an inadequate lipid balance in reserve tissues resulting from dietary profiles may be revealed in spawns from captive females once a higher fecundity is achieved in culture conditions. Monitoring of the nutrient content of broodstock diets in future studies along with that of eggs
(and possibly reserve tissues) will thus be important to evaluate the adequacy of experimental diets.

Disruption of spawning activity due to behavioral, endocrine, and stress issues as discussed in Chapter III are most likely involved in the low spawning activity, which resulted in the accumulation of reserves in captive red snapper female tissues. Food availability may also be higher in captive conditions where feeding rates are matched with the biomass of stocks while availability of food items in the wild may vary as wild red snapper broodstock are competing with each other and with other reef fishes for a limited supply. The wild females were actively spawning at the time of hormonal induction and also had a much higher fecundity during spawning trials reported here which likely contributed to partial exhaustion of their reserves, reflected in lower nutrient contents measured in tissues of that group. The broodstock feeding schedule was determined based on optimum feeding rates in other marine tropical species (e.g., red drum, Gatlin 2002) and assumes that active spawning is occurring and thus that females utilize the lipids and proteins of the diet for egg production. Considering that red snapper in these experiments did not utilize the protein and energy of the diet to produce eggs, an excess of nutrient is expected and was reflected by fat stores in the liver and larger protein and amino acid content in the muscle. However, red snapper in the size range of our study are still in a rapid growth phase of their life cycle (Porch 2007) and would thus require more energy and proteins than red drum or spotted sea trout (Cynoscion nebulosus) spawners that are typically used as broodstock once they have reached a slower somatic growth phase.
Optimum feeding rates will therefore need to be investigated in future studies, accounting for age, size, and expected somatic growth rate of broodstock in order to optimize egg production.

The second objective of this study was to examine the relationship between egg nutrient contents and measures of egg quality developed in Chapter II. The general finding of this work is that correlations between spawn quality parameters and ova nutrient content were infrequent and weakly supported. In most cases, correlations involved fatty acids or amino acids found in low concentrations close to the detection threshold, therefore the correlations observed may reflect artifacts due to the limited sensitivity of the assay and will minimally need to be verified by using larger sample sizes.

Fecundity was correlated to the abundance of three fatty acids (14:0, 20:0 and 20:3n-6), suggesting the balance of fatty acids may be impacted when a large spawn is produced and some fatty acids may be limited in reserve tissues. However, two of the three fatty acids significantly correlated with fecundity were found at trace concentration in the ova suggesting these correlations may be artifacts as discussed above and need to be taken with caution. As discussed in Chapter III, there was no significant correlation between fecundity and any of the other spawn quality parameters evaluated in this work, which suggests that these minor differences in fatty acid concentrations among spawns of varying sizes did not significantly impact spawn quality.

The fertilization rate was moderately positively correlated to the concentration of proteins and four amino acids in the ova. To date there is no
available data on the influence of amino acids and protein on the fertilization potential of ova in fish. Proteins are hydrolyzed in oocytes prior to fertilization to form the free amino acid pool available to the embryo. Post-ovulatory aging impacts several intracellular components including protein and lipids via oxidative stress (Lord and Aitken 2013). Lower fertilization rates reflecting post ovulatory aging may thus be associated with reduced protein and amino acid contents. The age of the female parent was also positively correlated to the crude protein content of the ova. Older females tended to display higher success at fertilization and were hypothesized to have undergone higher active spawning activity prior to the trial as discussed in Chapter III. This prior activity might have led to a more effective investment of proteins in the oocytes and their hydrolysis following hormonal induction and explain the higher fertilization potential to the ova produced from older females.

The hatch rate was unrelated to any proximate composition parameter or nutrient content. Previous studies focused largely on effects of egg nutrient content on larval survival with few reports of effects on hatch. Available data linked larval viability and normal development of eggs to appropriate content and balance of essential fatty acids (Izquierdo et al. 2001; Bobe and Labbé 2010). The lack of correlation of hatch rate with egg nutrient content in the present work thus suggests that most of the studied spawns had appropriate content and balance of fatty acids to ensure development to hatch. The content in vitamins (in particular A, E, and C) has also been linked to success rates during embryonic
development (Bobe and Labbé 2010) and may need to be investigated in future work.

While no variable was significantly associated with survival duration during pairwise correlation analysis, the multiple regression model retained the ova concentrations in ARA (20:4n-6, negative relationship) and three amino acids (Lysine, Alanine, and Taurine). ARA was also retained as a variable predicting the quality parameter (i.e., differentiating spawns showing high viability to first feeding). This fatty acid is hypothesized to be of primary importance to embryos and larvae due to its biological functions including its role in the production of eicosanoids, prostaglandins, and by extension in the process of ovulation (Henrotte et al. 2010; Migaud et al. 2013; Rainuzzo et al. 1997; Stacey 2003; Tocher 2003). ARA has been shown to stimulate oocyte maturation in European sea bass (*Dicentrarchus labrax*, Sorbera et al. 2001) and in Eurasian perch (Henrotte et al. 2010). Increased concentration of ARA in eggs of wild Arctic charr *Salvelinus alpinus* was related to higher fertility in comparison to eggs of captive charr (Pickova and Brännäs 2006). ARA requirements appear to be linked to other PUFAs in particular that of EPA and DHA, thus, the negative relationship observed in this work may reveal inadequate balance of PUFAs in spawns. Indeed, the EPA/ARA ratio has been hypothesized to influence the efficacy of many physiological processes during growth and development of fish embryos and larvae (Izquierdo and Koven 2011), and low values of this ratio have been hypothesized to reduce embryo and larval survival potential (Henrotte et al. 2010). In this work, when all 24 spawns were examined, the ratio EPA/ARA
was significantly lower in ova of spawns given a quality score of 1 (i.e., spawns characterized by high survival rate at first feeding). However, this trend should be considered with caution as it was estimated using pooled data from two different fish populations (captive and wild). Further investigation of the role of ARA and the EPA/ARA ratio in red snapper, and more generally the balance of EPA, ARA, and DHA (and maybe additional PUFAs) will need to be examined in the future.

The analysis of the survival duration parameter also suggested that the amino acids Taurine, Lysine, and Alanine were associated with spawns showing longer survival duration post hatch. Taurine is involved in several physiological functions including antioxidant processes, osmoregulation, modulation of neurotransmitters, regulation of calcium concentration in cells, hormone release (Fernández-Palacios et al. 2011), and enrichment of prey with this amino acid increased growth and survival of cobia larvae *Rachycentron canadum* (Salze et al. 2011). Alanine can be associated with Asparagine and Proline to form a specific motif within proteins of the major intrinsic proteins (MIP) family. These proteins are transmembrane protein channels creating the osmotic driving force during yolk hydrolysis (Lubzens et al. 2009). While Lysine is one of the essential amino acids for teleost fish (Li et al. 2008), the specific roles of this amino acid for egg and larval development are not documented to date and may deserve further investigation.

A recent study of changes in amino acid concentration during embryonic development of red snapper suggested that Valine and Isoleucine were of primary importance for red snapper early development based on the drastic
decrease of the abundance of these two amino acids over embryogenesis (Hastey et al. 2010). Valine is one of the most abundant amino acids in eggs of marine species with pelagic dispersal (Rønnestad et al. 1999), and the Valine concentration in fertilized eggs was the parameter showing highest correlation with both fertilization and hatching success of Atlantic halibut (Mommens et al. 2013). In this work, Valine and Isoleucine were detected in relatively high concentrations in all tissues but were not different between muscle and liver tissues or ova of wild and captive females. The concentrations of these amino acids (expressed in percent of wet weight) were not correlated to measures of egg quality in red snapper. However, the relative abundance (expressed in percent of the sum of all amino acids) of Valine and Leucine was slightly higher in the ova of spawns of wild females as compared to those from captive fish. Valine, Leucine, and Isoleucine account for 18 to 20% of the amino acids in animal proteins, and these essential amino acids might be involved in growth and immunity of adult fish but exhibit such large discrepancies in their metabolism among species that their potential specific role in larval fish is still unclear (Li et al. 2008). Studies of spawns showing varying content in Valine (i.e., through manipulation of dietary amounts of Valine) may be warranted in the future to evaluate the importance of this amino acid for early development of the red snapper.

Finally, the crude protein concentration in the female muscle was negatively correlated to survival and to the latency time between hormonal induction and ovulation. Reduced protein content in muscle tissue may reflect
exhaustion of reserves due to insufficient food and intense spawning activity in the wild or some other stressor including the stress induced by capture and transportation. In such a case, egg quality may be impacted due to a combination of factors not necessarily limited to nutrient availability in the muscle. Response to hormonal induction might also be delayed in weaker fish leading to the observed correlation between muscle protein content and latency. The relationships involved are likely complex considering that the latency duration was not correlated with survival duration as discussed in Chapter III.

In summary, this study was limited by the low number of spawns accessible and also the high correlation among nutrient contents, which largely limited assessment of the impact of variation of individual amino acids or fatty acids. Obtaining data on a larger number of spawns seems essential in order to reduce the risk of artifactual correlations when evaluating several variables for association with spawn quality parameters. Studies varying the nutrient content of diets would also be helpful in order to better understand the nutritional requirements of red snapper broodstock, embryos, and larvae. Tentatively, the current diet seems to provide the nutrients necessary for captive females, to match the composition of the eggs produced by wild females and this diet could serve as a base (control) for comparison with test diets varying the content in specific amino acids or fatty acids in experiments. However, the lack of spawning of captive broodstocks leads to the accumulation of large amount of nutrient reserves (lipids and protein) that are not incorporated into gametes. Therefore, an immediate priority for future work is to improve captive maturation, spawning
activity and fecundity of captive broodstock and evaluate the nutrient content of actively spawning red snapper females. While studies testing diets with varying concentrations of specific nutrients and evaluating the outcome of these treatments on egg viability are very challenging in a species like the red snapper (Papanikos et al. 2008), assessment of the role and requirement of nutrients could be improved by assaying the spawn content for vitamins and also by characterizing their free amino acid pool.

Finally, the ARA concentration in ova and the EPA/ARA ratio seem to play a role in the early survival potential of red snapper, and the protein and amino acid content of the female muscle appeared correlated to the latency time and fertilization rate of spawns. The value of these parameters as indicators of egg quality may be further investigated using larger sample sizes or by implementing test diets varying the contents of these nutrients. If confirmed this information will also be useful for the development of appropriate prepared diets for captive broodstocks.
CHAPTER V

ENDOGENOUS FACTORS RELATED TO RED SNAPPER EGG QUALITY:

PART B – TRANSCRIPTOME ANALYSIS OF OVA

Introduction

The viability of an egg and the fitness of the early larvae ensuing from it are determined in large part by the intrinsic properties of the egg itself (Brooks et al. 1997). These endogenous properties are fully acquired at the time of fertilization with the addition of the paternal genome, but they depend for a very large part on the characteristics of the ovulated oocyte or ovum. The ovum contains half of the future zygote genome (maternal gamete) but also several cytoplasmic components that have been shown to be critical to the successful development of an egg, including in particular the maternal RNAs and proteins, some maternal hormones, and the nutrient components of the yolk (Lubzens et al. 2010). Research to date has largely focused on the role of vitellin reserves, and the impacts of the nutrient composition of the yolk on developmental success have been extensively studied in cultivated fish species (Kamler 2008; Finn and Fyhn 2010; Kaushik and Seiliez 2010; Rainuzzo et al. 1997; Rønnestad et al. 1999; for reviews). Other determinants of fish egg quality such as parental genomic material have recently received increasing attention (Bobe and Labbé 2010; Brooks et al. 1997). The maternal RNAs present in eggs at fertilization play an important role in early development, particularly in the regulation of early cell cycles (Dworkin and Dworkin-Rastl 1990).
In teleost, these maternal RNAs are accumulated into the oocyte during oogenesis (Evsikov et al. 2006; Pelegri 2003; Tadros and Lipshitz 2009). At ovulation, the zygote genome is considered silent, and the transcriptome consists by definition of the maternal transcripts (Vesterlund et al. 2011). Studies on zebrafish showed that maternal transcripts play a central role in controlling the initial embryonic development until the mid-blastula transition (MBT). During that period, maternal mRNAs direct the initial mitotic divisions and specify the fate and patterning of embryonic cells (Tadros and Lipshitz 2009) before being eliminated by degradation at the MBT as the first cohort of zygotic transcriptions are activated (Kleppe et al. 2012). The importance of the composition of the maternal RNA pool was recently demonstrated in Atlantic halibut by Mommens et al. (2010) who reported significant correlations between the expression of specific maternal genes and egg viability at hatch. In rainbow trout, the abundance of prohibitin 2 mRNAs in oocytes was significantly correlated to hatching success (Bonnet et al. 2007). More recently, Chapman et al. (2014) identified a suite of 233 genes in the ovary transcriptome potentially explaining more than 90% of the variance in embryonic survival in striped bass. These findings were greatly facilitated by the improved transcriptomic reference database in this species developed by Reading et al. (2012). Over recent decades, the drastic improvement of high throughput genomic technologies greatly enhanced the characterization of transcriptomes. A major improvement provided by these methods was the ability to survey the whole transcriptome of tissues or organisms differing in phenotype or exposed to different treatments.
without a priori information on the genes potentially impacting the characters of interest (or those involved in response to treatments).

Recent work applied these principles to studies of the maternal mRNAs in fish oocytes in relation to egg quality using homologous micro-arrays (Bonnet et al. 2007; Chapman et al. 2014). The micro-array approach employed in these studies allow surveying differential expression of a large number of transcripts but is limited to a pre-determined set of probes that do not necessarily reflect gene regulations occurring in a specific experiment (Wang et al. 2009). Also, the development of a homologous micro-array requires access to transcriptome or genome sequence databases, which are usually not available in non-model species. An alternative approach for the study of non-model species is brought about by the increased availability and reduced cost of Next Generation Sequencing (NGS) through RNA-sequencing. The RNA-sequencing approach relies on direct sequencing of the RNA (or cDNA obtained from RNA) in samples using high throughput sequencing (e.g., using the Illumina Hi-seq platform). This method is especially advantageous for non-model species because all transcripts present in samples can be quantified as opposed to a predetermined set of reference sequences surveyed in micro-arrays as discussed above (Wang et al. 2009). Thus, the RNA-seq approach allows directly surveying the entire transcriptome of experimental groups without requiring any a priori knowledge of transcribed regions (Wilhelm and Landry 2009). RNA-seq analysis relies on proper mapping and annotation of transcripts to a reference genome or transcriptome and is significantly affected by the quality of the reference used for
mapping (Costa et al. 2010), highlighting the importance of developing those tools for species of interest. Achieving high assay sensitivity during RNA quantification requires deep coverage during sequencing, which can currently be achieved at reasonable costs through multiplexing (Liu et al. 2012; Singhal 2013).

Oocytes and ova have a very dynamic gene expression pattern compared to somatic tissues (Aanes et al. 2011; Mathavan et al. 2005; Sirard, 2012). Therefore, standardization of the sampling procedure, in particular its timing during oogenesis and the spawning process, is essential in order to allow robust comparison of experimental groups. Collecting ova immediately at the detection of ovulation and prior to activation by spermatozoa appears suitable as the maternal RNAs have all been incorporated at that stage, and assuming ova are collected before any degradation (i.e., over-ripening) occurs, the RNA profiling of obtained samples represents a standard measure of the maternal transcriptome that can be compared among experimental groups.

In this chapter, the transcriptome of ova was analyzed to compare gene expression levels between spawns from captive and wild females, and between spawns showing high and low quality as measured by the various criteria defined in Chapter II (relative fecundity, fertilization, hatching rate, and survival duration). Tests of the significance of the association between gene expression (transcript abundance) and egg quality variables, as well as parameters measured on females and their spawns are conducted to identify early transcriptomic signatures linked to egg and larval quality.
Material and Methods

The transcriptome of ova was characterized in 24 red snapper induced spawns studied in the previous chapters. The 24 spawns were obtained from 17 wild and 7 captive red snapper females. Wild females were caught offshore of the Alabama coastline during the course of the 2011 and 2012 spawning seasons. Five spawns were obtained in May, 9 in July, and three in August-September, corresponding to the beginning, peak and end of the natural spawning season for the red snapper in the northern Gulf of Mexico (Collins et al. 1996). Wild females were brought to the Thad Cochran Marine Aquaculture Center (TCMAC) and hormonally induced within 8 hours of capture. Captive females were held at the TCMAC in 10-m³ tanks connected to thermo-regulated recirculating systems for at least a year prior to hormonal induction. Rearing conditions are described in detail in Chapter II. The photoperiod and temperature in rearing tanks followed the natural cycle in Mississippi coastal waters. Water quality was controlled weekly, and parameters were maintained within the range of values routinely applied for the management of broodstocks at the TCMAC (NO₃<50 mg L⁻¹, NO₂<5 mg L⁻¹, NH₃<0.5 mg L⁻¹, dissolved oxygen > 80% saturation). Fish were fed a diet composed of frozen fish, shrimp, and squid at 3% body weight 3 times a week. The diet was partially substituted twice a week with a vitamin and fish oil supplement described in Chapter II.

For spawning induction, females were anesthetized, and an ovarian biopsy sample was taken using a catheter to allow staging of oocytes. Females with fully grown oocytes (>300 μm) received an intramuscular injection of
1,100 IU hCG kg\(^{-1}\) and were allocated to 400-L aquaria for monitoring during the induction period at 26±1°C and a salinity of 30±1 psu. When ovulation was detected, ova were collected by manual striping, and a subsample was immediately snap frozen by direct immersion in liquid nitrogen. Samples were preserved at -80°C until RNA extraction.

A suite of parameters were recorded on females and spawns as described in Chapters II and III. Spawn quality was characterized as relative fecundity, fertilization and hatching rate, survival duration post hatch determined as described in Chapter II, and the quality score (1 if no significant mortality is observed until 4 dph, 0 otherwise). Females were characterized by their age determined by otolith increment analysis, weight, length, condition coefficient (Fulton’s K), hepato-somatic index (HSI), and captive or wild origin. Spawn parameters included the date of spawn, the duration of the latency phase between hormone injection and ovulation, the oocyte stage at the time of induction, and the pH of the ova.

Total RNA was extracted from preserved ova samples using the RNA STAT-60 kit (amsbio®) following protocols recommended by the manufacturer. Briefly, samples were homogenized in the RNA STAT-60 reagent for a period of 60 sec. A mixture of chloroform and RNA STAT60 was then used to extract nucleic acids, followed by precipitation of the RNA in isopropanol at -80°C overnight. Following a treatment with Dnase I (New England BioLabs® Inc.), total RNA was eluted in 50 µl of RNA storage solution (Ambion®). RNA quality and integrity was measured using an RNA nano chip on an Agilent 2100 Bioanalyzer.
RNA Samples with a RNA Integrity Number (RIN) greater than 7.7 and a concentration greater than 600 ng µL⁻¹ were sent to the University of Colorado, School of Medicine, Denver, CO, for subsequent steps of library preparation and sequencing.

Normalized starting quantities of total RNA (200 ng) were used to prepare the 24 individual Illumina paired-end sequencing libraries with the TruSeq (Illumina®) RNA sample preparation kit following the manufacturer’s instructions. Briefly, total RNA was purified and fragmented to synthesize double stranded cDNA. Adapters were ligated to the obtained fragments and incorporated a unique barcode to each individual library to allow multiplex-sequencing and a Unique Molecular Identifier (UMI) consisting of a bar-code of 8 randomized bases to allow identification of PCR duplicates resulting from subsequent PCR amplification steps. The libraries were enriched by PCR and analyzed for size and integrity using a 2100 Bioanalyzer (Agilent Technologies). The 24 libraries were pooled and sequenced (100 cycles run) on two lanes of the Illumina HiSeq 2000 platform, producing 100 bp paired-end reads.

**Bioinformatics Treatment**

A draft reference genome sequence for red snapper previously developed in our group using genomic DNA from one red snapper specimen was used in this study to map RNA-seq reads. The draft genome assembly includes 75,690 genome contigs over 500 bp with an average contig size of 10,181 bp (minimum 500 bp, maximum 157,915 bp) and an average coverage of 25x.
Raw RNA-seq reads were quality checked, and adapter sequences and low quality data (Phred scores Q<20, 99% accuracy) were removed. Only reads with at least 75 consecutive bases with Phred scores>20 were kept for further analysis. Quality controlled reads from the two sequencing lanes were combined for each individual, and paired reads were joined. Reads were aligned on the reference genome using GSNAP (Genomic Short read Nucleotide Alignment Program, Wu and Nacu 2010), allowing for 6% mismatch. The package Cufflinks was used to create a de novo annotation file using successfully mapped reads in order to identify the placement of genes from the transcripts on the reference genome for each individual sample (Trapnell et al. 2012). A consensus annotation file across all samples was then generated in the Cuffcompare program of the Cufflinks package. Next, quantification of differential expression was performed in Cufflinks using the consensus annotation file for all samples (Roberts et al. 2011). In this second run of Cufflinks, read counts were normalized for each gene accounting for the length of the transcript and the total coverage of each sample. The resulting FPKM (Fragments per Kilobase of transcripts per Millions of fragments mapped) provide a measure of transcriptional activity (Trapnell et al. 2009) that can be compared across all individuals within the experiment (Costa et al. 2010).

FPKM data were log2 transformed for further analysis. Comparison of transcript abundance (log2 FPKM) between spawns from captive and wild females and between spawns showing low and high quality scores was performed using one way ANOVAs in the R package. Probabilities were
corrected using the Benjamini-Hochberg (1995) procedure to control the false discovery rate (FDR) at a rate of \(q<0.05\). Fold changes between groups were calculated as the difference of \(\log_2\) FPKMs.

The association of gene expression and spawn quality variables (relative fecundity, fertilization, hatch rates, and survival duration) and parameters measured on females and spawns (spawning date, age, weight, length, \(K\), HSI, latency duration, oocyte stage, and ova pH) was analyzed using simple linear regression analysis in R. Differences were considered significant when the FDR-corrected probability value of ANOVAs comparing captive versus wild groups or when testing the significance of the effect of independent variables in regression analysis was less than 0.05.

Transcripts where significant differential expression was inferred as a function of one of the studied factors (female origin, or parameters measured on spawns or females) were used as input for a Modulated Modularity Clustering (MMC) analysis to characterize correlation patterns among selected genes. Pairwise relationships between genes were quantified using Pearson’s correlation coefficients (Stone and Ayroles 2009). Relationships among samples were explored using data from genes showing significant association with tested factors in ANOVAs above using hierarchical clustering in Cluster 3.0. Average linkage clustering was performed using a similarity matrix based on Pearson’s correlations. Resulting dendrograms were visualized with Java TreeView (Saldanha 2004).
A bootstrap resampling approach was used to test whether the proportion of genes significantly associated with two of the studied factors was greater than that expected by random chance (i.e., if the distributions of genes impacted by the two factors were independent). Random sub-samples of the total transcriptome, matching the number of significant genes identified for each factor during ANOVA and regression analyses above, were generated without replacement. The procedure was run independently for each tested factor, and the number of transcripts shared between the two factors was recorded. One thousand random subsamples were generated and the 95% upper bound of the distribution of the number of shared genes in a given pair of factors was used in a one tailed test (i.e., the observed number of shared genes is significant if it lays beyond the 95% upper bound of the simulated distribution). The test was run for all possible pairs of factors (including female origin, predictor variables, and spawn quality parameters).

Reads showing significant association with female origin or spawn quality parameters were analyzed in Blast2GO version 2.7.1 using the default parameters (nr database, Expected value $1.0 \times 10^{-3}$, number of Blast hits 100. Parameters for GO annotation were E value hit filter $1.0 \times 10^{-6}$, annotation cutoff 55, weight 5 and Hsp hit coverage cutoff 0) in order to identify homologous sequences in the non-redundant (nr) database and to perform functional annotations. Briefly, significant transcripts were input into Blastx to find homolog sequences in the NCBI database and were annotated according to the Gene Ontology (GO) Consortium standardized nomenclature. Affected enzymatic
Pathways were retrieved by using KEGG pathways analysis. Tree maps for the annotated sequences at $P<0.05$ were created using ReViGO (Supek et al. 2011) to remove redundant annotation, using default parameters (similarity=0.7, database=whole UniProt, and semantic similarity=SimRel), and implemented with GO-terms from Blast2GO and their associated sequences minimum E-value as input.

Finally, the $\log_2$ FPKM of significant genes were entered into stepwise multiple linear regression models using PROC REG of SAS 9.3 to select a subset of variables that best predict the variability of the egg quality parameters of wild spawns (relative fecundity, fertility, hatch, and survival duration). As in the previous chapters, multiple regression analysis was performed using only data from wild females (n=17) considering the differences detected in spawning parameters between wild and captive females. Models were computed using a stepwise algorithm allowing variable entry at the significance level $P=0.01$ to ensure the collinearity between variables retained in the model was kept at acceptable levels (Variance Inflation Factor <10 and Condition Index <30; Belsley et al. 1980). The multiple regression model was also run using 12 spawns randomly selected among the 17 wild spawns. The obtained equations for the partial dataset were used to predict the spawn variables of the remaining 5 samples in order to test the accuracy of the prediction. Transcripts discriminating captive and wild spawns, or high and low quality eggs (quality scores of 1 and 0, respectively), were determined by discriminant analysis (PROC STEPDISC and PROC DISCRIM). The thresholds for variable entry and
retention were also set to $P=0.01$ in order to ensure collinearity among transcripts retained in the final model was kept within acceptable values. As above, model parameters were estimated using 12 of the 17 wild spawns and 5 of the 7 captive spawns for inference on captive versus wild origin. For the discriminant function predicting high versus low egg quality, 9 of the 12 spawns with score 1 were used as well as 3 of the 5 spawns with score 0. The obtained models were used to classify the remaining (test) samples as captive or wild or high versus low quality score.

**Results**

An average $43.3 \times 10^6$ raw reads per sample were obtained with the two lanes of sequencing (range $18.9 \times 10^6$ – $79.6 \times 10^6$). After quality checking, 58.8% of the reads could be mapped on contigs of the draft genome assembly, yielding $21.9 \times 10^6$ (range $9 \times 10^6$ - $45.1 \times 10^6$) reads available per sample for differential gene expression analysis. The reference annotation file generated in Cufflinks included 33,540 transcripts. On average 899 transcripts (range 355 - 1,934, Table 9) showed a significant association with the studied spawn quality parameters or with characteristics of females and spawns measured during spawning trials. **Comparison of the Ova Transcriptome between Captive and Wild Spawns**

A total of 1,349 transcripts differed significantly in abundance between the ova of wild ($n=17$) and captive ($n=7$) females (Table 9). More than 85% of these transcripts were more abundant in the ova of captive spawns. Although the magnitude of differential expression was large for a few transcripts with absolute fold changes between the two groups as high as 1,696, the fold change of 94.7%
of the significant genes were between -5 and 5 (mean 3±57, Table 9). Also, the range of values of the log₂ FPKM in captive and wild spawns overlapped for all 1,349 significant transcripts.

Table 9

Number of transcripts (n) significantly associated to factors, percentage of positive relationships, fold changes between treatments (captive vs wild groups, high vs low quality groups) or regression slope between studied parameters and log₂ FPKM, and associated coefficient of determination (r²) obtained during regression analysis. Data presented as means ± SD (range).

<table>
<thead>
<tr>
<th>Factors</th>
<th>N</th>
<th>% positive relationships</th>
<th>Slope (range)</th>
<th>Fold change (range)</th>
<th>r² (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Captivity*</td>
<td>1,349</td>
<td>85% x</td>
<td>3±57 (-1,696 – 719)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1,934</td>
<td>90%</td>
<td>-5,431 – 6,642</td>
<td>0.35±0.08</td>
<td></td>
</tr>
<tr>
<td>Date</td>
<td>906</td>
<td>91%</td>
<td>-763 – 519</td>
<td>0.32±0.08</td>
<td></td>
</tr>
<tr>
<td>HSI</td>
<td>480</td>
<td>35%</td>
<td>-1,293 – 2,239</td>
<td>0.32±0.06</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>355</td>
<td>32%</td>
<td>1,454±16,790</td>
<td>0.30±0.06</td>
<td></td>
</tr>
<tr>
<td>Latency</td>
<td>1,313</td>
<td>94%</td>
<td>-503 – 55</td>
<td>0.32±0.08</td>
<td></td>
</tr>
<tr>
<td>Ova pH</td>
<td>1,230</td>
<td>88%</td>
<td>-765±5,264</td>
<td>0.31±0.07</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>496</td>
<td>84%</td>
<td>-4,224 – 4,309</td>
<td>0.30±0.06</td>
<td></td>
</tr>
<tr>
<td>TL</td>
<td>1,098</td>
<td>53%</td>
<td>-4,669 – 5,662</td>
<td>0.25±0.72</td>
<td></td>
</tr>
<tr>
<td>Wt</td>
<td>891</td>
<td>54%</td>
<td>7±1,371 (-38,514 – 11,044)</td>
<td>0.31±0.07</td>
<td></td>
</tr>
<tr>
<td>Rfecundity</td>
<td>392</td>
<td>50%</td>
<td>-123±1,097 (-13,231 – 7,188)</td>
<td>0.31±0.07</td>
<td></td>
</tr>
<tr>
<td>Fertility</td>
<td>539</td>
<td>95%</td>
<td>206±1,727 (-15,048 – 16,088)</td>
<td>0.31±0.07</td>
<td></td>
</tr>
<tr>
<td>Std Hatch</td>
<td>1,204</td>
<td>56%</td>
<td>-18±1,072 (-9,019 – 24,158)</td>
<td>0.30±0.07</td>
<td></td>
</tr>
<tr>
<td>Survival duration</td>
<td>604</td>
<td>49%</td>
<td>-39±793 (-10,082 – 6,852)</td>
<td>0.29±0.06</td>
<td></td>
</tr>
<tr>
<td>Quality*</td>
<td>696</td>
<td>73% y</td>
<td>3±102 (-304 – 1,830)</td>
<td>0.31±0.01</td>
<td></td>
</tr>
<tr>
<td>Mean±SD</td>
<td>899±450</td>
<td>69%±22% (32% - 95%)</td>
<td>50.7±411.0 (-57,010 – 218,942)</td>
<td>0.31±0.01</td>
<td></td>
</tr>
</tbody>
</table>

*: ANOVA, #: n=17 wild spawns versus n=7 captive spawns, x: higher expression in the captive group, y: higher expression in the high quality group.
Figure 4. Partial representation of hierarchical clustering based on transcripts showing significantly different expression in captive (C) and wild (W) spawns; transcripts (reference ID on the side) were included if the probability of homogeneity of log$_2$ FPKM distributions between the two groups during ANOVA was (A) $P<0.05$ and (B) $P<0.01$. Green/red color indicates that the abundance (log$_2$ FPKM) of a transcript (raw) in a sample (column) is lower/higher than the mean abundance of the transcript across individuals.

The results of hierarchical clustering performed on the 1,349 transcripts showing significantly different expression levels between ova of captive and wild females ($P<0.05$) are presented in Figure 4 A. Spawns were poorly grouped by fish origin (captive or wild). When a more restrictive criterion was applied to select transcripts included in hierarchical clustering (i.e., by including transcripts only if the probability of significance during ANOVA was $P<0.01$ instead of $P<0.05$), 434 transcripts remained and allowed a better discrimination of captive and wild spawns (Figure 4 B). All but one spawn from wild females were included in two related clusters and showed greater similarity with each other than with samples from the captive group, except for the captive spawn C2 that was
included in one of the two clusters of wild female spawns. Similarly, five of the seven spawns from captive females were included within the same hierarchical cluster (Figure 4 B).

Five hundred and twenty seven (39%) of the 1,349 transcripts could be annotated by Gene Ontology (GO) in Blast2GO (Table 10). The summarized annotations obtained after removal of redundant GO terms in ReViGO were synthesized in Table 12. Biological processes differentially expressed in spawns from captive and wild females were identified as fat cell differentiation (70% of all processes) and Glycosyl Phosphatidylinositol (GPI) anchor proteins (19%). Histone deacetylase binding and sulfuric ester hydrolase activity each represented about 20% of all biological functions impacted by captivity, and most of the annotated cellular components were in the histone acetyl transferase complex (81%).

Table 10

<table>
<thead>
<tr>
<th>Factors</th>
<th>Total</th>
<th>Without blast results</th>
<th>Without blast hits</th>
<th>With Blast results</th>
<th>Mapped</th>
<th>Annotated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Captivity*</td>
<td>1,349</td>
<td>23.4%</td>
<td>28.4%</td>
<td>5.2%</td>
<td>4.0%</td>
<td>39.1%</td>
</tr>
<tr>
<td>Age</td>
<td>1,934</td>
<td>23.1%</td>
<td>11.8%</td>
<td>4.4%</td>
<td>4.7%</td>
<td>55.9%</td>
</tr>
<tr>
<td>Date</td>
<td>906</td>
<td>34.9%</td>
<td>14.1%</td>
<td>2.5%</td>
<td>3.5%</td>
<td>44.9%</td>
</tr>
<tr>
<td>HSI</td>
<td>480</td>
<td>25.2%</td>
<td>23.3%</td>
<td>3.3%</td>
<td>2.9%</td>
<td>45.2%</td>
</tr>
<tr>
<td>K</td>
<td>355</td>
<td>20.6%</td>
<td>26.2%</td>
<td>4.2%</td>
<td>3.7%</td>
<td>45.4%</td>
</tr>
<tr>
<td>Latency</td>
<td>1,313</td>
<td>34.0%</td>
<td>17.6%</td>
<td>4.2%</td>
<td>2.8%</td>
<td>41.4%</td>
</tr>
<tr>
<td>Ova pH</td>
<td>1,230</td>
<td>21.5%</td>
<td>19.0%</td>
<td>6.6%</td>
<td>4.6%</td>
<td>48.4%</td>
</tr>
<tr>
<td>Stage</td>
<td>496</td>
<td>27.4%</td>
<td>15.1%</td>
<td>7.7%</td>
<td>3.6%</td>
<td>46.2%</td>
</tr>
<tr>
<td>TL</td>
<td>1,098</td>
<td>25.8%</td>
<td>20.0%</td>
<td>4.6%</td>
<td>4.3%</td>
<td>45.3%</td>
</tr>
<tr>
<td>Wt</td>
<td>891</td>
<td>26.9%</td>
<td>19.4%</td>
<td>4.9%</td>
<td>5.1%</td>
<td>43.7%</td>
</tr>
</tbody>
</table>
More than 70% of enzymatic pathways affected by the origin of the female (wild versus captive) were related to fatty acid (42%) and amino acid (31%) metabolism based on KEGG pathway analysis of annotated sequences (data not shown). The 1,349 significant transcripts were grouped in 14 modules during Modulating Modularity Clustering (Figure 5). Eighty-four percent of these transcripts were included in 8 modules where the average Pearson’s correlation among transcripts within module $r_{av}$ was moderate (0.40 < $r_{av}$ < 0.70). Two modules containing 31 transcripts (2% of the 1,349) showed high connectivity ($r_{av}$ within module >0.95), and three modules consisted of singleton transcripts. Transcripts included in the 14th cluster (13.6% of the total) were weakly correlated to each other ($r_{av}$=0.24). Most of the modules that included more than 2 transcripts (nine out of 11) showed moderate positive correlation to other modules except module 1 and 8, which displayed negative correlations with all remaining modules (Figure 5).
Figure 5. Heat map illustrating the structure of the ordered correlation matrix obtained during Modulated Modularity Clustering of the 1,349 transcripts showing significantly different log$_2$ FPKM in ova from captive versus wild spawns ($P<0.05$). The correlation coefficients $r$ between transcripts are color-coded as shown on the side scale.

Four modules (modules 3, 5, 7 and 11) included more than 100 sequences (n=127 to 407), and the distribution of GO annotations in individual modules were compared to those in the overall set of 1,349 significant transcripts to evaluate if modules were enriched in specific functions or processes. The predominant processes inferred during annotation of three of the modules were histone acetylation (82% of module 3), microtubule anchoring (81% of module 7), and regulation of GTPase activity (40% of module 11). These predominant
processes were weakly represented in the annotation file of the overall dataset indicating enrichment of the three modules with transcripts involved in specific processes. The main process involving the transcripts clustered in module 5 was the same as that inferred for the overall dataset (fat cell differentiation, 37%), but the second most represented process (mRNA processing, 25%) for this module was not among the dominant processes annotated in the overall dataset.

Stepwise discriminant analysis identified six transcripts that allowed best discrimination of captive and wild spawns. Half of them could be annotated and were identified as potential GTPases involved in calcium transport, cytoskeletal binding proteins, and phospholipid binding proteins. When a partial data set was used to build a discriminant model, the obtained function allowed reclassifying correctly all 7 test samples (5 wild and 2 captive spawns) into their group of origin.

**Transcriptomic Signatures of Egg Quality and Spawning Parameters**

Distribution patterns of transcripts impacting egg quality and spawning parameters. Single linear regression analysis was used to test the significance of the relationship between ova transcript abundance and the continuous egg quality parameters (fecundity, fertilization rate, standardized hatch rate, and survival duration), as well as the parameters measured on females and spawns and evaluated as predictors of spawn characteristics (age, spawning date, HSI, K, latency time of ovulation, ova pH, oocyte stage, TL, and Wt).

The numbers of transcripts showing significant change in abundance as a function of egg quality or spawn predictor values are presented in Table 9. The
greatest number of significant associations between egg quality and transcript expression involved the standardized hatch rate (1,204 transcripts showed significant change in abundance as a function of spawn standardized hatch rate). Among the other parameters measured on females and spawns, the highest number of significant associations involved female age (1,931 transcripts), followed by the latency time of ovulation and the pH of ova (Table 9).

The slopes of regression models were predominantly positive indicating an increase of transcript expression when the egg quality parameter or predictor variable increased. The slopes were also highly variable (ranging from -57,010 to 218,942, Table 9), but average values were very low and the percentage of phenotypic variance explained by individual transcript expression was relatively low across parameters (mean $r^2$ 0.31, Table 9), indicating that most of the significant transcripts had a moderate influence on egg quality variables or were moderately influenced by predictor parameters measured on females and spawns. On average, the fraction of transcripts simultaneously influencing two of the studied parameters was relatively low (8%, Table 11). However, pairwise comparisons revealed that several parameters shared a greater number of associated transcripts with other parameters than would be expected by random chance. The highest percentage of co-associated transcripts was between hatch rate and age (40% of significant transcripts in common) and between female weight and length (73%). Also, more than 20% of the transcripts significantly associated with the quality parameter were also associated with female age.
Table 11

Percentage of transcripts jointly associated with two parameters measured on spawns of wild females (n=17). The numbers of transcripts significantly associated with each individual parameter are reported on the diagonal. Italicized numbers correspond to pairs of parameters where the number of co-associated transcripts does not differ significantly from that expected under random chance assuming independence as inferred during Monte Carlo simulations. Pairs of parameters sharing more than 30% transcripts are highlighted in boxes.

<table>
<thead>
<tr>
<th>N</th>
<th>Captivity*</th>
<th>Age</th>
<th>Date</th>
<th>HSI</th>
<th>K</th>
<th>Latency</th>
<th>OvapH</th>
<th>Stage</th>
<th>TL</th>
<th>WT</th>
<th>Rfec</th>
<th>Fert</th>
<th>Hatch</th>
<th>Survival</th>
<th>Quality</th>
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</thead>
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<td>9.3%</td>
<td>16.6%</td>
<td>10.2%</td>
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<td>5.3%</td>
<td>6.3%</td>
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<td>2.1%</td>
<td>1.2%</td>
<td>3.1%</td>
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</tr>
<tr>
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<td>15.8%</td>
<td>28.0%</td>
<td>9.3%</td>
<td>8.1%</td>
<td>22.4%</td>
<td>24.8%</td>
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<td>0.2%</td>
<td>3.5%</td>
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<td>4.2%</td>
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</tr>
<tr>
<td>K</td>
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<td>5.9%</td>
<td>1.1%</td>
<td>2.0%</td>
<td>14.4%</td>
<td>1.4%</td>
<td>1.7%</td>
<td>7.6%</td>
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<td>5.1%</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>2.7%</td>
<td>4.2%</td>
<td>3.1%</td>
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<td>3.7%</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>6.7%</td>
<td>7.9%</td>
<td>0.7%</td>
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<td>3.1%</td>
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<td></td>
</tr>
<tr>
<td>Stage</td>
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<td>2.6%</td>
<td>6.9%</td>
<td>1.5%</td>
<td>0.7%</td>
<td>0.5%</td>
<td>0.4%</td>
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<tr>
<td>TL</td>
<td>1098</td>
<td>73.1%</td>
<td>15.1%</td>
<td>6.5%</td>
<td>3.6%</td>
<td>13.4%</td>
<td>2.6%</td>
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<td></td>
<td></td>
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<tr>
<td>Wt</td>
<td>891</td>
<td>8.4%</td>
<td>2.6%</td>
<td>5.7%</td>
<td>7.6%</td>
<td>2.6%</td>
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<tr>
<td>Rfecundity</td>
<td>392</td>
<td>1.8%</td>
<td>1.0%</td>
<td>27.8%</td>
<td>10.7%</td>
<td></td>
<td></td>
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<tr>
<td>Fertilization</td>
<td>539</td>
<td>1.5%</td>
<td>0.0%</td>
<td>0.7%</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Std hatch</td>
<td>1204</td>
<td>7.6%</td>
<td>10.6%</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Survival</td>
<td>604</td>
<td>22.7%</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Quality</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>696</td>
<td></td>
</tr>
</tbody>
</table>

*: n=17 wild spawns vs n=7 captive spawns
Transcripts influencing quality or survival duration were also affected by fecundity (11% and 28%, respectively). The survival duration shared 20 and 13% of significantly associated transcripts with the parameters spawning date and female length, respectively. Finally, 20 to 28% of the transcripts associated with the latency interval between hormonal induction and ovulation were also significantly associated to spawning date, oocyte stage, ova pH, condition coefficient K, and HSI (Table 1).

The mean number of modules k generated during MMC clustering of the transcripts significantly associated with studied parameters was k=13 (Appendix). The mean pairwise Pearson’s correlation coefficients ($r_{av}$) of transcripts included in modules ranged between 0.4 and 0.9. In all cases, most of the transcripts were included in modules where $0.4 < r_{av} < 0.7$. However, modules of highly correlated transcripts ($r_{av} > 0.7$) were identified for all parameters, and notably, 14% and 25% of the transcripts significantly associated with hatch and survival duration, respectively were grouped in three to four clusters where $r_{av}$ was greater than 90%.

*Annotation of transcripts significantly associated with egg quality parameters.* The mean proportion of transcripts that could be annotated in Blast2GO was 47% and ranged from 41% for transcripts associated with the latency duration between induction and ovulation to 56% for those associated with female age (Table 1). Enzymes identified in transcriptome sets related to egg quality parameters during KEGG pathway analysis mainly pertained to amino acid and fatty acid metabolism. In particular, hatch rate was associated
with change in expression of enzymatic pathways involved in the degradation of several amino acids (Valine, Leucine, Isoleucine, Lysine, Arginine, and Proline).

The results of synthetic annotation conducted in ReViGO are described in Table 12. The transcripts significantly associated with the relative fecundity were involved in regulation of apoptotic processes (76% of annotated processes), and to a lesser extend in GTP catabolism (9%). Functions affected were mainly β-tubulin binding (24%) and GTPase activity (11%), and cellular components involved were principally the endoplasmic reticulum (ER, 40% of all components).

Only one module contained more than 100 transcripts related to fecundity (module 4, 70% of the 392 significant transcripts, Appendix). The average correlation coefficient among transcripts was 0.36, and the distribution of annotations in this module was very similar to the one obtained for the full data set, except that the transcripts were expected to be represented into the Golgi apparatus rather than the ER.

The major biological process involving transcripts associated with the fertilization rate was annotated as transmembrane receptor of protein-Serine/Threonine kinases signaling, while the molecular functions affected were Ral GTPase binding and amino acyl tRNA editing activity (Table 12). Among the 9 modules defined by MMC for fertility (Appendix), three had more than 100 transcripts. Module 2 (n=126 sequences, $r_{av}=0.70$) was enriched with transcripts involved in the process of purine biosynthesis, but the most frequent function (Ral GTPase binding) was the same as that inferred for the full data set. The two
other modules contained moderately correlated transcripts ($r_{av}=0.6$). Module 3 was enriched with transcripts involved in protein heterodimerization, while module 4 included transcripts affecting DNA repair process.

The most frequent annotations of transcripts related to the hatch rate were calmodulin-dependent protein phosphatase activity (23% of annotated functions) and induction of synaptic plasticity (61% of annotated processes). Three modules included more than 100 transcripts. One of them was enriched with anion transport processes (cluster 3, $n=129$, $r_{av}=0.86$) and a second one with intracellular signal transduction processes (cluster 4, $n=311$, $r_{av}=0.55$). The largest cluster (5) contained 721 transcripts moderately correlated to each other ($r_{av}<0.50$) and showed a similar annotation profile to that of the full set of transcripts significantly related to hatch.

Transcripts significantly associated with survival duration were mainly affecting the ATP hydrolysis proton transport (67%) and protein binding (19%). One cluster included more than 100 sequences weakly correlated to each other ($r_{av}=0.39$), and their synthetic annotations in ReViGO were similar to those of the full set of transcripts.
Table 12

Relative proportions of the two major non-redundant Gene Ontology functions, processes and cellular components synthesized by ReViGO tree maps for annotated transcripts expressed in red snapper ova and significantly associated ($P<0.05$) with egg quality parameters in spawns from wild-caught females ($n=17$) or differing significantly in abundance between spawns from captive ($n=7$) and wild females ($n=17$).

<table>
<thead>
<tr>
<th>Biological process</th>
<th>Molecular function</th>
<th>Cellular component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Captivity*</td>
<td>Fat cell differentiation (70%), Attachment of GPI anchor to protein (19%)</td>
<td>Histone acetyl-transferase complex (81%), Dendritic shaft (8%)</td>
</tr>
<tr>
<td>Relative fecundity</td>
<td>Negative regulation of neuron apoptotic process (76%), GTP catabolism (9%)</td>
<td>B-tubulin binding (24%), GTPase activity (11%)</td>
</tr>
<tr>
<td>Fertilization</td>
<td>Transmembrane receptor protein Serine/Threonine kinase signaling (66%), Vesicle-mediated transport (15%)</td>
<td>Ral GTPase binding (26%), amino-acyl-tRNA editing activity (26%)</td>
</tr>
<tr>
<td>Standardized hatch</td>
<td>Induction of synaptic plasticity by chemical substance (61%), Purine nucleobase biosynthesis (21%)</td>
<td>Calmodulin-dependent protein phosphatase activity (23%), Cytoskeletal regulatory protein binding (17%)</td>
</tr>
<tr>
<td>Survival duration</td>
<td>ATP hydrolysis coupled proton transport (67%), DNA topological change (24%)</td>
<td>Unfolded protein binding (19%), $\omega$-peptidase activity (11%)</td>
</tr>
<tr>
<td>Quality</td>
<td>Cell development (79%), Protein autoubiquination (12%)</td>
<td>Cytoskeletal protein binding (29%), Hydrolyase activity on glycosyl compounds (18%)</td>
</tr>
</tbody>
</table>

*: n=17 wild spawns vs n=7 captive spawns
Evaluation of the power of transcripts for predicting egg quality parameters by multiple regression analysis. Results of stepwise multiple regression analysis are presented in Table 13. Models explaining more than 90% of the variance could be built for all continuous parameters. However, the correlation between predicted values from equation developed using 12 of the 17 samples, and observed values in test samples were at best moderate for relative fecundity and fertilization rate ($r^2 = 0.51$ and 0.74, respectively) and extremely low for the hatch rate and survival duration parameters ($r^2 = 0.04$ and 0.09, respectively, Table 13).

**Quality Parameter**

A total of 696 transcripts differed significantly in abundance between ova from spawns classified as high versus low quality (Table 9). Seventy-three percent of these transcripts were more abundant in ova leading to larvae with high survival rate at first feeding (quality score of 1, Table 9). More than 95% of transcripts differed moderately in abundance between the two groups with fold change were between -20 and 20 (average $3 \pm 102$). A small percentage of transcripts showed very large difference in abundance as indicated by the range of fold change (-304 to 1,830, Table 9). Four of the 696 transcripts differing significantly in abundance between low and high quality spawns had non overlapping FPKM ranges between the two groups, three were always more abundant in the ova samples from high quality spawns (fold changes between 1.4 to 2.3), and one was substantially less abundant in high quality spawns (fold
change of -37.9), indicating that this transcript was specifically abundant in ova samples from low quality spawns.

All but one spawn with a quality score of 1 were included into a series of related clusters during hierarchical clustering based on the 110 transcripts for which the probability of significance during ANOVA comparing the two quality groups was $P<0.01$. Except for LoW3 and LoW15, the low quality spawns were not grouped in clusters or sets of related clusters, suggesting increased variability of transcript abundance in that group and the lack of common patterns (Figure 6). Clustering of high and low quality spawns was less effective when all 696 transcripts differing between groups at the level $P < 0.05$ were included in the analysis (data not shown).

Figure 6. Partial representation of hierarchical clustering based on transcripts showing significantly different expression in low (Low) and high (Hi) quality spawns; transcripts (reference ID on the side) were included if the probability of homogeneity of log$_2$ FPKM distributions between the two groups during ANOVA was $P<0.01$. Green/red color indicates that the abundance (log$_2$ FPKM) of a transcript (raw) in a sample (column) is higher/lower than the mean abundance of the transcript across individuals.
A large fraction of the transcripts significantly associated with the quality parameter were also associated with survival duration and with female age (20% of shared significant transcripts), relative fecundity (10% shared transcripts), and standardized hatch rate (10% shared transcripts, Table 11).

More than 54% of the 696 transcripts significantly associated with the quality score at $P<0.05$ could be annotated (Table 10). Cell development and protein auto-ubiquitination were the most frequent biological processes inferred in ReViGO, while cytoskeletal protein binding and hydrolase activity were the most frequent molecular functions (Table 12). Enzymatic pathways inferred in KEGG pathway analysis were involved in the metabolism of purine and pyrimidine, several amino acids (Valine, Leucine, Isoleucine, Glycine, Serine, Threonine, Alanine, Asparagine, and Glutamate), arachidonic acid, and glycerophospholipids, and sphingolipids.

MMC clustering identified three modules, one of which contained highly correlated transcripts ($r_{av}>0.9$) and accounted for 21% of the 696 transcripts affected by quality, Appendix). The main annotations of transcripts included in this module of highly correlated transcripts were cell redox homeostasis (process), protein hetero-dimerization (function), and nuclear chromosome (cellular component). These annotations differ from the main annotations recorded for the overall pool of transcripts related to quality and suggest that the module was enriched with specific processes and functions.
Table 13

Results of stepwise multiple linear regression analysis of egg quality parameters by transcript expression levels measured as log₂ FKM (transcripts are identified as X_ID in equations) in spawns from wild-caught females (n=17). Max VIF: Maximum Variance Inflation Factor, Max CI: Maximum Condition Index, Model $r^2$: model coefficient of determination, Test sample $r^2$: coefficient of determination of observed parameter by predicted value from the multiple regression model in test samples (n=5).

<table>
<thead>
<tr>
<th>Egg quality parameters</th>
<th>Prediction equation*</th>
<th>Max VIF</th>
<th>Max CI</th>
<th>Model $r^2$</th>
<th>Test samples $r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative fecundity</td>
<td>$0.012xX_{16840} - 0.010xX_{13414} - 0.06xX_{10856} - 0.003xX_{31574} + 5.295$</td>
<td>2.26</td>
<td>11.56</td>
<td>0.96</td>
<td>0.51</td>
</tr>
<tr>
<td>Fertilization</td>
<td>$0.031xX_{10543} - 0.007xX_{2576} - 0.00001xX_{6451} + 1.148$</td>
<td>1.16</td>
<td>9.28</td>
<td>0.93</td>
<td>0.74</td>
</tr>
<tr>
<td>Standardized hatch</td>
<td>$0.0360xX_{28621} - 0.020xX_{17502} - 0.011xX_{19918} - 0.011xX_{8905} - 0.007xX_{33443} - 0.001xX_{15885} + 0.003xX_{31589} + 1.2e^{-10}xX_{9671} + 0.967$</td>
<td>4.37</td>
<td>26.58</td>
<td>0.99</td>
<td>0.04</td>
</tr>
<tr>
<td>Survival duration</td>
<td>$-0.057xX_{28743} - 0.029xX_{25732} - 0.015xX_{20934} + 1.459$</td>
<td>1.26</td>
<td>15.07</td>
<td>0.90</td>
<td>0.09</td>
</tr>
</tbody>
</table>

* Equation generated using 12 of the 17 samples, the remaining 5 samples serve as test samples
Stepwise discriminant analysis identified four transcripts that best distinguished the two quality groups. Only one of these transcripts could be annotated in Blast2Go as Histone Lysine n-methyl-transferase. The quality parameter was predicted with an estimated 100% accuracy by five transcripts during partial stepwise discriminant analyses. The five test-samples were all correctly classified as high or low quality score by the inferred discriminant function (data not shown).

Discussion

The objective of this work was to apply Illumina sequencing and the RNA-seq methodology to compare transcriptome profiles in ova (unfertilized eggs) of captive and wild females and study the relationships between transcript abundance in ova and (1) spawn quality criteria and (2) parameters measured on females and spawns at the time of spawning and evaluated as potential factors influencing egg characteristics and quality. Mapping on the draft genome available in our laboratory resulted in an average coverage of $21.9 \times 10^6$ (minimum of $9 \times 10^6$) mapped reads per sample (58%). In a simulation study, Wang et al. (2009) concluded that using 75 bp reads, 10 million uniquely mapped reads per sample were sufficient to reflect the high correlation among technical replicates and recover 80% of annotated genes expressed in the studied transcriptome. In the context of their dataset increasing coverage to 20 million reads per sample led to minimal improvement of these results. The current dataset exceeded these suggested minimum limits and is therefore expected to provide a thorough profiling of the transcriptome of red snapper ova samples.
examined in the present work. However, a limitation of this dataset commonly encountered in non-model species such as the red snapper is the moderate percentage of reads that could be mapped and further quantified for differential expression analysis. In this study, the strategy was to map reads on a draft genome sequence available in our laboratory. An alternative strategy could have been to assemble the transcriptome and use it as a reference to map reads. This option was not chosen in this work due to the technical difficulties in obtaining reliable assemblies using short reads generated by Illumina sequencing and the high costs and efforts involved in acquiring long reads from RNA-sequencing.

The draft reference genome is currently being improved through acquisition of additional sequence data, which are expected to increase coverage to 80X in the next assembly. Moreover, a linkage mapping project is on-going in our laboratory. This improved genome sequence will be available in the near future and will allow mapping a higher percentage of reads and improving the reliability of inferences on differential transcript abundance.

The first objective of the analysis of gene expression was to compare the transcriptome of ova from captive and wild-caught females. A total of 1,349 transcripts differed significantly in abundance between the two groups. However, the magnitude of observed differences was moderate (less than 5 absolute fold changes for 95% of the transcripts), and the distributions of log2 FPKM values in captive and wild samples overlapped for all 1,349 transcripts. Accordingly, hierarchical clustering resulted in clusters involving both captive and wild spawns, failing to differentiate the profiles of the two groups. The profiles of
captive and wild females were found more distinct when hierarchical clustering was performed using only transcripts differing in abundance at a lower significance level ($P<0.01$). However, even in that case, one captive spawn showed a greater similarity to wild spawns and was included in one of the “wild” clusters and two spawns (one from a captive female and one from a wild female) were highly divergent from all other spawns and were not included in clusters. These results suggest that gene expression patterns in spawns within the captive and wild groups are heterogeneous, and transcripts differing between the two groups for their average abundance are not consistently differentially expressed as a function of the female origin, making it difficult to identify a signature of captivity.

The two most frequent annotated processes for the 1,349 genes as synthesized in ReViGO were labeled as fat cell differentiation and attachment of GPI anchor to protein. The latter is related to transduction signals across the plasma membrane (Yu et al. 2013). Interestingly, KEGG pathway analysis revealed pathways involved in amino acid or fatty acid metabolisms, reflecting the critical role of metabolic processes for early development as discussed in Chapter IV. Reading et al. (2012) and Schilling et al. (2014) used annotations from the Gene Ontology (GO) Consortium and described dominant biological processes (metabolic processes), molecular functions (proteins binding), or cellular components (cell, intracellular parts or organelles) impacting oocytes, similar to those found during annotation of the transcripts differentially abundant
in captive and wild groups, but also during annotation of most of the sets of transcripts found significantly associated with egg quality parameters.

A general difficulty encountered during annotation was the low proportion of transcripts that could be annotated. As discussed above, the draft red snapper genome is in the process of being improved, and efforts in the next few months will focus on annotation, which will likely improve annotation of sequences and inferences on processes and pathways affected by differentially expressed transcripts. A second problem encountered was the relatively low specificity of reported function or processes of annotated sequences and the high redundancy of GO terms. Annotation remains an important issue, especially for functional genomics studies in non-model organisms (Schunter et al. 2014; Singhal 2013; Smith et al. 2013). Annotations and inferences on processes, functions, cell compartment, and enzymatic pathways are based on the analysis of sequence similarity in Blast2GO. Because they are based on similarity, these annotations need to be considered as hypotheses that must be tested directly in vivo (Evsikov et al. 2006), e.g., through protein knock out studies (Sirard 2012). Mutational and gene knock-down experiments have been used to validate the role of numerous maternal mRNAs initially discovered during transcriptome studies in the zebrafish (e.g., Traverso et al. 2011).

Modulated modularity clustering applied to the 1,349 transcripts found to differ significantly in abundance between captive and wild spawns revealed 14 modules of correlated transcripts. Empirical data examined by Stone and Ayroles (2009) showed that clusters identified during MMC tend to be enriched with
transcripts involved in specific and distinct functions and processes. The most frequently annotated processes within MMC modules in this study generally differed from those identified in the overall dataset, suggesting that modules were enriched with transcripts involved in specific processes (module 3: histone acetylation and nuclear-transcribed mRNA catabolism, module 7: microtubule anchoring, module 11: GTPase activity). These results are thus consistent with the suggestion by Stone and Ayroles (2009) that modules of correlated transcripts may correspond to distinct pathways as reflected by (1) the enrichment in specific functions and processes with regards to the overall population of transcripts differentially expressed between groups, and (2) the relative independence of the corresponding sets of transcripts as showed by the moderate or complete lack of correlation between modules. These modules can therefore serve as a base for further exploration of processes differentially regulated in spawns form captive and wild females.

The second objective of this work was to examine the relationship between transcript abundance, egg quality parameters, and parameters measured on females and spawns before or during spawning. The number of transcripts showing significant co-variation with egg quality parameters varied between 392 and 1,204. Hatch rate variation was associated with change in the abundance of the largest number of transcripts (1,204) while other egg quality parameters co-varied with relatively moderate numbers of transcripts (392 to 696). The finding of a broader relationship between ova transcriptome profile and hatch rate is suggested by other studies of the ova mRNAs that indicated a major
role of maternal mRNAs on early embryonic development (Chapman et al. 2014; Reading et al. 2012; Traverso et al. 2011). The maternal mRNAs support the embryonic development until the activation of the zygotic genome (Pelegri 2003), and different expression levels potentially signaling deficiencies of the maternal mRNAs would be expected to directly influence the early developmental phases (before the blastula transition) and translate here into differences in hatching success. Later development events such as survival at first feeding may be better predicted by the transcriptome of the zygote in future studies. However, considering that the transcriptome is expected to be dynamic and evolve rapidly as the embryo goes through several developmental events within a short time span, care should be taken to ensure that the zygote transcriptome is assessed at a standardized developmental stage to allow comparison among experimental groups. Sampling embryos shortly after the mid-blastula transition may be suitable in order to determine if the zygote transcriptions were initiated correctly and attempt to diagnose subsequent viability.

As in the case of the comparison of captive and wild spawns above, a high proportion of transcripts significantly differentially expressed in spawns of varying quality could not be annotated. Various forms of protein binding were among the most frequently annotated functions for all quality parameters, a result also consistent with findings in striped bass (Chapman et al. 2014; Reading et al. 2012). Predominant processes found associated with spawn quality differed among quality parameters with an emphasis on membrane receptors and signaling processes associated with fertility, cell development, and energetic
metabolism for transcripts impacting the quality parameter, or synaptic plasticity and mRNA processing for those associated with hatch rate. As discussed above, MMC clustering identified modules of correlated transcripts within the sets of transcripts associated with each quality parameter, and the clusters appeared enriched with transcripts annotated to specific functions that can be used to build hypotheses regarding the pathways and mechanisms involved in egg quality.

A bootstrap resampling approach was used to determine if transcripts significantly associated with a specific egg quality variable were preferentially associated with other spawn quality parameters. The percentage of transcripts associated with multiple quality parameters was in general low, and in several cases did not differ from that expected under random chance. Notably, the percentage of transcripts co-varying with both fecundity and survival duration/quality was greater than expected if the two traits were independent. Similarly, a significantly large number of transcripts were associated with both hatch rate and survival post hatch. An approach for future studies could be to determine if modules of correlated transcripts are associated with multiple traits in order to identify critical pathways broadly influencing egg quality.

Among the parameters measured on females and spawns during the spawning process, the age of the female was found to co-vary with the largest number of transcripts (1,934), and a high proportion of those transcripts (significantly higher than expected under random chance) were also impacting the egg quality parameters fertility (10%), hatch rate (40%), and quality (21%). These results are consistent with phenotypic correlations estimated in Chapter
III: positive relationships were detected between the age of the female and the hatch rate of embryos, two parameters that share the largest proportion of associated transcripts. Older fish in this study were likely invested in reproduction before the spawning trials. Endocrine processes, possibly involved in mRNA accumulation in the cytoplasm of oocytes, may have differed between older fish actively spawning and younger fish or first spawners. This possible relationship between maternal mRNAs content of ova and age will require further investigation. Relatively high numbers of transcripts were also found associated with ova pH (1,230) and latency duration between hormonal induction and ovulation (1,313), and significantly high proportions of these transcripts were shared between these two parameters (22%) and with those impacting the hatch rate (17% and 8% respectively). Transcripts differentially expressed as a function of the latency duration were also more likely to affect fertility, survival duration, and the quality parameter.

Stepwise multiple regression analysis was used to attempt to determine if subsets of transcripts could be used to classify spawns as wild or captive or to predict spawn quality parameters. Initial runs of stepwise discriminant analysis and multiple regressions indicated a predicted strong success in differentiating spawns of wild versus captive origin and predicting egg quality parameters ($r^2>0.91$). Therefore, we used an approach similar to that of Chapman et al. (2014) and estimated parameters of discriminant functions (for categorical variables: captive versus wild origin, quality score of 0 versus 1) or multiple regression models (predicting relative fecundity, fertilization rate, standardized
hatching rate, or survival duration) using only a subset of the data (17 of the 24 available spawns, 71%). The equations obtained with this partial dataset were then used to predict the quality scores of test samples not used in parameter estimation and naïve to the estimated functions. All test samples were classified correctly in the appropriate category (wild or captive female origin; quality score of 0 or 1), suggesting that the function was indeed effective and that the few transcripts included could be used as indicators/predictors of these characteristics. These results suggest that a signature of the potential of eggs to survive through first feeding may be present in the ovum transcriptome. Indeed, four of the 696 transcripts differing significantly in abundance between high and low quality spawns did not overlap in distribution between the two groups. Hierarchical clustering suggested that most of the high quality spawns had similar profiles at the transcripts differing significantly between the two groups while low quality spawns were more variable and lacked common patterns. This observation is consistent with the notion that a specific transcriptome profile is associated to a high survival potential of ova, and departure from this profile leads to reduced survival potential. This hypothesis and the potential signature of a high survival potential to first feeding in ova will require further investigations using larger sample sizes.

In contrast, prediction of continuous variables in test samples was much less effective with an $r^2=0.7$ at the most for fertilization and $r^2$'s as low as 0.08 for survival duration. These results suggest that the prediction of categorical variables might be more effective than that of continuous variables in the context
of our dataset. The parameters of prediction equations based on only a small number of transcripts are subjected to relatively large sampling variance considering our small sample sizes. This small sample size could explain the failure to predict continuous parameters while better results were obtained when predicting categorical parameters. These categorical parameters are best differentiated using variables showing extreme values in the two groups to be discriminated. Chapman et al. (2014) determined that accurate prediction of egg survival potential required a large number of transcripts (with >200) each individually showing only subtle (and often non-significant) variations between spawns of high and low quality. This suggests that any determination of prediction equations will be likely challenging as the regression coefficients of transcripts only affected moderately by egg quality would be more impacted by artifacts occurring during quantification of expression in samples. Minimally, large numbers of samples may be required in order to achieve robust parameter estimates, which is a priori difficult to implement considering the large effort and cost associated with generating and analyzing large scale transcriptome data. Most transcripts involved in egg characteristics only showed moderate fold change between spawns of different quality or origins, a result consistent with Chapman et al.’s (2014) hypothesis. The need to account for a large number of transcripts in order to explain and predict differences in egg quality is also consistent with the observation of modules (or clusters) that included large numbers of correlated transcripts during MMC in this work. These clusters likely
reflect pathways involved in the realization of multiple and likely complex steps of early development.

In summary, major efforts need to be oriented towards improving the draft genome in order to enhance mapping and annotation and assist in the formulation of hypotheses regarding processes and pathways involved in egg quality. The results obtained during this study are consistent with the hypothesis proposed by Chapman et al. (2014) that the transcriptome of ova is very informative on the potential of an egg to develop successfully. Additional samples are needed to examine more rigorously the predictive power of the transcriptome. Alternative analytical approaches allowing to better account for correlation of transcript expression during evaluation of the relationship between gene expression and phenotypic characters such as egg quality are warranted. This work focused on maternal RNAs present in ova. These mRNA are likely to impact early development steps such as fertilization and hatch rate as illustrated by the larger number of transcripts significantly associated with hatch rate. Although a possible early signature of viability to first feeding was suggested by the results of discriminant analysis, the characterization of the zygote transcriptome should be useful to further evaluate gene expression impacting the survival potential of hatched larvae.

The necessary decomposition of egg quality in multiple parameters highlighted in this work (Chapter II) increases the complexity of the prediction of survival to first feeding (the ultimate objective). The analysis of transcriptomic
signatures associated with each individual parameter may be key to improve our understanding of mechanisms involved in this complex character.
CHAPTER VI
EFFECTS OF ENVIRONMENTAL STRESSORS (EXOGENOUS FACTORS) ON THE VIABILITY OF RED SNAPPER EMBRYOS AND EARLY LARVAE

Introduction

Achieving a reliable supply of viable fry for hatchery production is one of the main current challenges faced by the developing marine aquaculture industry. The viability of embryos and pre-feeding larvae is particularly difficult to control because it is determined by several factors (Brooks et al. 1997). To date, studies have largely focused on the maternal determinants of egg characteristics, survival potential, and the modulation of these maternal effects by parameters such as female age, size, condition or the nutritional and stress status prior to spawning (Bobé and Labbé 2010). However, environmental factors can induce stress and affect the viability of eggs and larvae post fertilization (Brooks et al. 1997). In the wild, marine species generally release their gametes into an optimal buffered environment that ensures maximal survival and effective development of their offspring during the early larval period (Rijnsdorp et al. 2009). On another hand, hatchery conditions usually differ from the natural environment, and exogenous stressors in intensive aquaculture are unavoidable, especially when high densities are employed to promote high productivity (Ashley 2007).

Incubation at high density facilitates the harvest and stocking of newly hatched larvae from incubators into larval rearing tanks. However, high density during incubation can lead to rapid deterioration of the water quality. Fluctuation of environmental parameters such as the depletion of dissolved oxygen (DO) and
increase of ammonia concentration during embryonic development are expected at high stocking densities. These factors have been linked to a reduction in hatch and early larval survival rates and also to long term effects on offspring fitness such as increased rates of deformities in exposed groups (Holt and Arnold 1983; Shang and Wu 2004). In aquaculture, these water quality parameters could reach levels that embryos and larvae would never experience in the wild, exceeding their tolerance limits and resulting in post-fertilization stress. In such a situation, egg quality could be high at fertilization, but if the embryos encounter such stressors, the viability and fitness of fry may be reduced. Despite the potential importance of incubation conditions in determining survival and fitness of offspring, information on the effects of such exogenous factors on egg and larval quality is still relatively limited in marine species (Yúfera and Darias 2007).

Fish embryos depend exclusively on cutaneous respiration, and their surface area for gas exchange is limited (Elshout et al. 2013). Therefore, fish embryos are assumed to be more sensitive to hypoxia than adults and juveniles, a hypothesis that is supported by empirical data across several freshwater species (Elshout et al. 2013).

Oxygen depletion, or hypoxia, is expected to result from high oxygen demand of embryos stocked at high density in incubators and aerobic processes involved in the degradation of dead and unfertilized eggs. In marine environments, hypoxia has been defined as the condition whereby dissolved oxygen levels in the water fall below 2.8 mg DO L\(^{-1}\) (Wu 2002). This definition, however, is difficult to use to characterize hypoxia across studies because the
availability of oxygen strongly depends on other parameters, particularly water temperature, salinity, and depth. Characterization of hypoxia based on the oxygen saturation level is a more reliable approach (Chabot and Claireaux 2008). European sea bass and Atlantic cod juveniles avoid waters where oxygen saturation is less than 45-50% (Claireaux et al. 2000; Schurmann et al. 1998) which is considered to be a level of moderate hypoxia (Chabot and Claireaux 2008). Severe hypoxia (between 15-20% saturation) results in rapid mortality of Atlantic cod juveniles (Claireaux et al. 2000). The effects of hypoxia also depend on the type of exposure (e.g., chronic or acute) and the physiological status of the affected organisms (e.g., active swimming, digestion, stress) which determines their oxygen demand. To date, data are still very limited for embryos and larvae of marine fish species, and this topic deserves specific investigation due to their limited respiratory and avoidance capacity. Sub lethal levels of hypoxia (0.8 mg L⁻¹) have been shown to affect several physiological processes of the embryonic and larval development of zebrafish such as slowing and delaying development, altering the normal process of apoptosis, causing malformations, impairing primordial germ cell migration, and inducing an unbalance of the sex hormones (Shang and Wu 2004; Lo et al. 2011). Demand for oxygen during early embryonic stages is fulfilled in part by oxygen stores in the yolk and perivitelline fluid (Czerkies et al. 2002). As a result, anoxic external conditions will not inhibit development until gastrulation (Bunn et al. 2000). After this stage, the embryo is unable to regulate its oxygen uptake, which largely depends upon oxygen diffusion through the surface of the egg (Reardon and
Chapman 2012). As the embryo grows, its oxygen requirements will increase while the respiratory surface area remains constant (Nilsson and Ostlund-Nilsson 2008) which is expected to lead to a steady decrease in the ability to tolerate hypoxia until hatching, although embryos of some species appear to survive hypoxia by slowing down development, or almost totally shutting down metabolism (Podrabsky et al. 2007). Nonetheless, the critical oxygen concentration, or lowest level at which fish can maintain, increases with time during the embryonic development (Kamler 1992). In hatched embryos and small larvae, oxygen uptake still occurs primarily through the skin, while the predominant function of gill anlagen is ion exchange (Fu et al. 2010). However, small fish larvae are more tolerant to low oxygen concentration than eggs and embryos, probably due to their advantageous surface-to-volume ratio in comparison to fish eggs and by the absence of the chorion (egg envelope) which has been hypothesized to act as a barrier for gas exchange (Elshout et al. 2013). To our knowledge, there is no data on the tolerance of red snapper embryos and larvae to hypoxia.

Another water quality parameter affecting fish embryos and larvae under culture conditions is the level of dissolved ammonia. After oxygen, ammonia is considered one of the most important water quality parameters, potentially impacting fish condition and fitness, especially in intensive aquaculture (Francis-Floyd et al. 2012). Ammonia concentration is expected to increase in aquaculture tanks through two main routes: (1) ammonia is the main nitrogenous metabolic waste product excreted by teleost fish and thus is naturally produced in a healthy
aquaculture tank, and (2) the degradation of unfertilized eggs and dead embryos also generates ammonia during proteolysis. The combination of these two processes can lead to reaching sub-lethal or lethal levels due to accumulation over time unless water is renewed at high rates or dissolved ammonia is actively removed by use of biofilters in recirculating aquaculture (Francis-Floyd et al. 2012). Total ammonia nitrogen (TA-N) consists of the highly toxic un-ionized form of ammonia (UIA-N, or NH₃) in equilibrium with the relatively non-toxic form (NH₄⁺, Aubrey et al. 2014). The concentration of the two forms of ammonia is regulated primarily by water pH and temperature. Maximum levels of UIA-N between 50 and 200 µg L⁻¹ were recommended for marine finfish species by Person-Le Ruyet et al. (1997) and Lemarié et al. (2004), but these recommendations were based on juvenile and adult fish while information on embryos and larvae is still very limited. Available data suggest that the development of embryos can proceed to hatch even in the presence of elevated concentrations of UIA-N in the water, but newly hatched larvae seem much less tolerant to ammonia than eggs (Chen et al. 2012). The higher tolerance of teleost eggs may be due in part to their chorion that provides a potential barrier preventing diffusion of toxic molecules to the embryos (Harcke and Daniels 1999), although recent studies showed that ammonia can penetrate the chorion of zebrafish (Braun et al. 2009). An alternative explanation for the higher tolerance of eggs is related to the urea synthesis during embryogenesis that has been hypothesized to protect the embryo from toxic effects of ammonia (Bucking et al. 2013). Eggs of spotted sea trout hatched at 6.0 mg L⁻¹ of UIA-N, but newly
hatched larvae only tolerated 0.28 mg UIA-N L\(^{-1}\) (Daniels et al. 1987). Results of this study also indicated that larval tolerance was not affected by prior exposure of eggs to elevated ammonia concentration. Similar results were obtained in red drum where high hatch rate was reported at 7.2 mg L\(^{-1}\) of UIA-N, while elevated mortality of newly hatched larvae was observed when UIA-N concentration increased from 0.31 mg L\(^{-1}\) to 0.55 mg L\(^{-1}\) (Holt and Arnold 1983). To date, the tolerance of red snapper embryos and larvae to elevated levels of ammonia remains unstudied.

The current routine hatchery protocol for red snapper involves stocking embryos at a density of 1 egg mL\(^{-1}\) in 120-L incubators under gentle aeration and moderate water turnover (<20% h\(^{-1}\)). Larvae are typically transferred 24 hours post hatch to larval tanks where rearing density is lowered to 0.1 larva mL\(^{-1}\) or less. Stocking density of 2 eggs mL\(^{-1}\) or higher for incubation was shown to be detrimental to larval survival estimated 36 hours post hatch (Bourque and Phelps, 2007), but the actual changes of water quality parameters such as levels of dissolved oxygen and un-ionized ammonia induced by elevated density were not reported in this experiment. In addition, this study did not document the kinetics of mortality during development, which prevented evaluating the viability of larvae surviving the early phases of exposures and determining the cumulative effects of prolonged exposures.

The objective of this chapter was to evaluate the tolerance of the red snapper eggs and larvae to individual environmental stressors resulting from high stocking density in intensive aquaculture. The author focused on the two main
water quality parameters discussed above (concentration of dissolved oxygen and total ammonia) and studied the kinetics of mortality during exposures to provide preliminary data for the management of water quality during incubation and early larval rearing.

Materials and Methods

All experiments were performed using embryos from three different wild-caught females serving as biological replicates. Females were induced for gamete maturation with human Chorionic Gonadotropin (hCG) following a protocol based on the method developed by Minton et al. (1983) and fully described in Chapter II. Eggs were collected from each female by manual stripping at ovulation, and immediately fertilized in vitro with the sperm of one or two wild-caught males. Conditions at fertilization were temperature 26±0.5°C, salinity 30±1 psu, dissolved oxygen at 85% saturation or greater (>6 mg L⁻¹), and total ammonia (TA-N) 0 mg L⁻¹. At 1 h post-fertilization (hpf), random subsamples of each spawn were transferred to experimental 1-L beakers for challenges. Artificial seawater (BIOSEA® Marinemix, Aqua Craft, Hayward, CA) was prepared using deionized water (Mako RO system, Aquatic Ecosystems) for all experiments. For both tested factors, challenges were aimed to identify sub-lethal levels or levels leading to moderate mortality as needed to provide recommendations for the management of water quality in hatcheries. In all trials, treatments were evaluated with reference to control groups.
Hypoxic Challenges

Exposures of embryos to hypoxic conditions were performed in an oxygen chamber I-Glove incubator glovebox (Biospherix, Lacona, NY), which allowed uninterrupted control of dissolved oxygen (DO) levels in the water. A gas control module PROOX model 360 was used to lower dissolved oxygen concentration in the water to the desired level by injecting nitrogen gas into the oxygen chamber. Infusion of gas exactly matched chamber leakage to hold oxygen level constant. Accordingly, only one reduced (hypoxic) oxygen concentration could be tested per trial.

Three hypoxic challenges were performed exposing embryos and larvae to different levels of DO. For each challenge, the eggs from three females were transported in separate closed container at a density of 1 egg mL\(^{-1}\) to the location of the oxygen chamber where they were immediately stocked in experimental beakers (approximately 100 eggs estimated volumetrically were stocked in each 1-L beaker). Experimental beakers were maintained under test conditions (control or hypoxic) until the end of the trial.

During the first two challenges, embryos were immediately exposed to the target hypoxic concentration (exposed eggs). To do so, a preliminary trial determined the rate of oxygen depletion in the water contained in experimental beakers and the time needed to reach the desired hypoxic concentration (2 and 3 mg DO L\(^{-1}\)); the target hypoxic conditions were reached within 24 hours in the chamber. Based on these results, beakers were placed in the chamber 24 hours prior to introducing eggs to ensure that the desired oxygen concentration was
reached at the time of stocking (i.e., eggs were transferred directly from water at >85% saturation to the tested hypoxic conditions). The first trial compared the survival of embryos and larvae exposed to a severe hypoxia (2 mg DO L⁻¹, 29% saturation) to those of controls maintained at ambient air (>85% saturation). The second trial compared the survival of embryos exposed to moderate hypoxia (3 mg DO L⁻¹, 43% saturation) to controls. The last hypoxic challenge aimed to expose newly hatched larvae from eggs that had not been exposed to hypoxia (non-exposed eggs) to a moderately hypoxic treatment (3 mg DO L⁻¹, 43% saturation). To achieve this objective, eggs were stocked in experimental beakers at 1 hpf as for the previous two challenges, but oxygen decrease was only initiated at 36 hpf and reached the target hypoxic concentration (3 mg L⁻¹) at 2 dpf. Therefore, oxygen depletion was progressive and ultimately exposed larvae to a moderate hypoxia beginning at 2 dpf. In all three experiments, control beakers were set beside the chamber at ambient air condition, (25°C, >85% saturation). The water temperature was maintained at 25±1°C by heated water baths for all groups.

Larval survival was determined at three different developmental stages in all experiments: hatching (36 hours post fertilization, hpf), 3 days post fertilization (dpf, 84 hpf), and 5 dpf (132 hpf). At each of these three time-points, the number of live and dead embryos/larvae was determined in two replicate beakers for each female in each treatment (hypoxic and control), i.e., 3 females x 2 treatments x 2 replicates beakers were assessed for survival rate per time point. Because of the potential stress inflicted on the larvae by the process of counting,
replicate beakers were counted once and then disposed. Subsequent survival counts were performed on other duplicate beakers treated identically throughout the experiment. The level of DO and the temperature were monitored in three additional beakers in each treatment at each sampling date using an optical DO meter (YSI®). Distinct beakers were used for these measurements in order to avoid disturbing embryos and larvae in experimental beakers by immersing an oxygen and temperature probe.

*Ammonia Challenge*

The three concentrations of TA-N tested were generated by adding ammonium chloride (NH₄Cl) to artificial salt water (30 psu) maintained at 26±1°C. Concentrations tested were 0 (control), 1, 10, and 50 mg TA-N L⁻¹. Stock solutions for each concentration were prepared in 20-L containers at least 12 hours prior to experiments to ensure TA-N levels were stable and to adjust them prior to stocking of eggs when necessary. The control stock solution (salt water, 0 mg TA-N L⁻¹) was handled identically, except that no ammonium chloride was added to the water. After the determination of the fertilization rate at 1 hpf, eggs were introduced into 1-L beakers filled with stock solutions that had been equilibrated at the desired TA-N concentration beforehand. The number of live and dead embryos/larvae was determined in two replicate beakers per ammonia concentration for each female every 24±2 h until total mortality was recorded in all groups. As in the previous challenge, replicate beakers for a given experimental group were counted only once and subsequent survival counts were performed on other duplicate beakers treated identically.
Temperature, TA-N concentration, pH, and alkalinity were measured daily from one replicate beaker per ammonia dose for each female once survival in that beaker had been determined. TA-N concentrations in seawater was determined with a precision of 0.01 mg L\(^{-1}\) using a spectrophotometer (Hach DR2800) and the salicylate method (Hach, Method 8155 powder pillows) according to protocols provided by the manufacturer. UIA-N concentrations (Table 14) were determined from the TA-N concentrations using the table of Francis-Floyd et al. (2012), which gives the ratio of UIA-N to TA-N as a function of pH and temperature. UIA-N and TA-N concentrations were expressed in mg L\(^{-1}\), and pH was read with a pH pen-meter (YSI, pH-100 with piercing electrode). Alkalinity was determined by titration (Hach, alkalinity test kit AL-AP) and was >180 mg of calcium carbonate L\(^{-1}\) for the whole experiment.

**Statistical Analyses**

The percentage of surviving eggs/larvae at each time point in each experiment was arcsine square root-transformed before analyses in SAS® 9.3 with PROC GLM. Three way ANOVAs were performed accounting for the fixed factors time and treatment, the random factor female, and their respective interactions. Post-hoc comparisons were performed in PROC GLM using the Tukey procedure to compare treatment levels and treatment x time interaction levels when these factors were found significant.
Measured total ammonia (TA-N) concentrations, pH and estimated unionized ammonia (UIA-N) concentrations in experimental groups exposed to various levels of TA-N. Data from three female spawns and 7 days of exposure are summarized as mean ± SD.

<table>
<thead>
<tr>
<th>TA-N concentration (mg L⁻¹)</th>
<th>UIA-N concentration (mg L⁻¹)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Targeted</td>
<td>Measured</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.15±0.09</td>
<td>0.012±0.020</td>
</tr>
<tr>
<td>1</td>
<td>1.28±0.26</td>
<td>0.050±0.018</td>
</tr>
<tr>
<td>10</td>
<td>11.25±2.63</td>
<td>0.477±0.137</td>
</tr>
<tr>
<td>50</td>
<td>46.67±2.89</td>
<td>1.727±0.107</td>
</tr>
</tbody>
</table>

Results

Hypoxic Challenges

In the first challenge, red snapper embryos were exposed to severe hypoxia (2 mg DO L⁻¹, or 29% saturation). Total embryonic mortality was observed at 1 dpf (none of the embryos hatched), while survival rates in controls averaged 81, 73, and 69% at 1, 3, and 5 dpf respectively.

The next two challenges exposed red snapper embryos or newly hatched larvae to moderate hypoxia (3 mg DO L⁻¹, 43% saturation). The results of these challenges are presented in Tables 15 and 16.
Table 15

Percentages of live larvae (%Live) in experimental groups of embryos exposed to moderate hypoxia (target 3 mg L\(^{-1}\) DO, 45% saturation) beginning 1 hour post fertilization (exposed eggs) and in controls. Data from three females (F1 to 3) recorded at 1, 3 and 5 dpf in two replicates (Rep A and B) per experimental group; DO target: targeted oxygen concentration, Ctr.: control treatment (ambient air), DO meas.: measured oxygen concentration, Temp.: temperature. n: sample size.

<table>
<thead>
<tr>
<th>dpf</th>
<th>DO target</th>
<th>DO meas</th>
<th>Temp (°C)</th>
<th>%Live (n)</th>
<th>%Live (n)</th>
<th>%Live (n)</th>
<th>%Live (n)</th>
<th>%Live (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>F1 Rep A</td>
<td>F1 Rep B</td>
<td>F2 Rep A</td>
<td>F2 Rep B</td>
<td>F3 Rep A</td>
</tr>
<tr>
<td>1</td>
<td>Ctr</td>
<td>±0.04</td>
<td>±0.25</td>
<td>6.59</td>
<td>24.17</td>
<td>75.0</td>
<td>57.1</td>
<td>87.6</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>±0.23</td>
<td>±1.01</td>
<td>3.19</td>
<td>25.87</td>
<td>58.3</td>
<td>51.2</td>
<td>69.7</td>
</tr>
<tr>
<td>3</td>
<td>Ctr</td>
<td>±0.07</td>
<td>±0.31</td>
<td>6.57</td>
<td>24.43</td>
<td>67.9</td>
<td>28.0</td>
<td>82.4</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>±0.38</td>
<td>±0.68</td>
<td>2.96</td>
<td>25.07</td>
<td>27.2</td>
<td>41.3</td>
<td>44.0</td>
</tr>
<tr>
<td>5</td>
<td>Ctr</td>
<td>±0.05</td>
<td>±1.04</td>
<td>6.39</td>
<td>24.70</td>
<td>7.5 (67)</td>
<td>54.7</td>
<td>84.9</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>±0.40</td>
<td>±0.84</td>
<td>2.93</td>
<td>25.57</td>
<td>5.1</td>
<td>4.4</td>
<td>15.0</td>
</tr>
</tbody>
</table>

Table 16

Percentages of live larvae (%Live) in experimental groups of embryos exposed to moderate hypoxia (target 3 mg L\(^{-1}\) DO, 45% saturation) beginning 1 day post fertilization (non-exposed eggs, see text for detailed protocol) and in controls. Data from three females (F4 to 6) recorded at 1, 3 and 5 dpf in two replicates (Rep A and B) per experimental group; DO target: targeted oxygen concentration, Ctr.: control treatment (ambient air), DO meas.: measured oxygen concentration, Temperature was 25±1°C throughout the challenge. n: sample size.

<table>
<thead>
<tr>
<th>dpf</th>
<th>DO target</th>
<th>DO meas</th>
<th>%Live (n)</th>
<th>%Live (n)</th>
<th>%Live (n)</th>
<th>%Live (n)</th>
<th>%Live (n)</th>
<th>%Live (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>F4 Rep A</td>
<td>F4 Rep B</td>
<td>F5 Rep A</td>
<td>F5 Rep B</td>
<td>F6 Rep A</td>
<td>F6 Rep B</td>
</tr>
<tr>
<td>1</td>
<td>Ctr</td>
<td>5.87</td>
<td>81.2</td>
<td>92.4</td>
<td>72.3</td>
<td>65.5</td>
<td>21.2</td>
<td>20.8</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>5.81</td>
<td>73.5</td>
<td>87.6</td>
<td>61.0</td>
<td>52.6</td>
<td>31.1</td>
<td>27.0</td>
</tr>
</tbody>
</table>
The first moderate hypoxia challenge exposed embryos to 3.02±0.33 mg DO L⁻¹ (43% saturation on average) beginning 1 hour post fertilization. Measured oxygen concentration in control groups kept outside the chamber under ambient air averaged 6.01±0.10 mg DO L⁻¹ (94% saturation, Table 15, Figure 7 A). The survival of exposed embryos was significantly lower than that in the control treatment and decreased with time (P<0.0001, Table 17). The interaction between treatment and time was also significant (P=0.0207, Table 17). No significant difference in survival at hatching (1 dpf) was observed between exposed and control groups. However, survival in the hypoxic treatment was lower at the two subsequent sampling dates, and the magnitude of the difference between the two treatments increased over time from 10% at 1 dpf to 45% at 5 dpf (Figure 7 A). The survival of embryos and larvae also differed significantly between spawns (Table 15) and interestingly, the three spawns responded differently to hypoxic conditions as revealed by the significant interaction Oxygen x Female (P=0.0468, Table 17), denoting a difference in sensitivity to hypoxia among progeny from different females.
Table 17

Results of three way ANOVAs conducted for two moderate hypoxia challenges (see text for protocols applied in the two challenges).

<table>
<thead>
<tr>
<th>Factor</th>
<th>DF</th>
<th>F-value</th>
<th>P-value</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen</td>
<td>1</td>
<td>43.17</td>
<td>&lt;0.0001</td>
<td>0.64</td>
<td>0.4350</td>
</tr>
<tr>
<td>Time</td>
<td>2</td>
<td>19.11</td>
<td>&lt;0.0001</td>
<td>151.77</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Female</td>
<td>2</td>
<td>21.88</td>
<td>&lt;0.0001</td>
<td>38.46</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Oxygen x Time</td>
<td>2</td>
<td>4.85</td>
<td>0.0207</td>
<td>1.21</td>
<td>0.3219</td>
</tr>
<tr>
<td>Oxygen x Female</td>
<td>2</td>
<td>3.65</td>
<td>0.0468</td>
<td>0.20</td>
<td>0.8172</td>
</tr>
<tr>
<td>Female x Time</td>
<td>4</td>
<td>0.64</td>
<td>0.6384</td>
<td>9.80</td>
<td>0.0002</td>
</tr>
<tr>
<td>Oxygen x Female x Time</td>
<td>4</td>
<td>0.89</td>
<td>0.4895</td>
<td>1.74</td>
<td>0.1844</td>
</tr>
</tbody>
</table>

![Graph showing survival rate over days post fertilization with labels a and b indicating significance levels.](attachment:image.png)
Figure 7. Mean survival of red snapper larvae (±SD) exposed to moderate hypoxia (circles) and controls (squares) beginning at 1 hpf (A) or 1 dpf (B). Data averaged across 3 females with 2 replicate groups per female. Letters (a, b) denote homogeneous groups identified during post-hoc tests.

In the second hypoxic challenge, embryos were transferred to the hypoxic chamber at 1 hpf but were not immediately exposed to hypoxia. Beginning after hatch (at 1 dpf), oxygen concentration in the chamber was progressively lowered to reach the target level of hypoxia at 2 dpf (mean measured DO in the treated group 2.77±0.45 mg DO L⁻¹, 40% saturation, Table 16). The proportion of live embryos and larvae did not differ between the control and treated groups throughout this experiment (P=0.4609, Table 17). The rapid decrease in survival between 3 and 5 dpf was observed in both control and exposed larvae (Figure 7 B), and likely reflected low endogenous egg quality of the three spawns used in this test. A significant interaction between female and time was detected indicating that mortality kinetics differed between females (interaction Female x
Time $P=0.0002$, Table 17) but was unrelated to the treatment (non-significant interaction Female x Oxygen $P=0.8172$, Table 17).

**Elevated Ammonia Challenges**

Ammonia concentration significantly affected embryonic and larval survival ($P<0.0001$, Table 18). Survival of embryos and larvae did not differ significantly between the control group (no ammonia added, 0.01±0.02 mg UIA-N L$^{-1}$) and the group exposed to 1 mg TA-N L$^{-1}$ (1.28±0.26 mg UIA-N L$^{-1}$) throughout the entire duration of the experiment (Table 19, Figure 8). However, at 10 mg TA-N L$^{-1}$ larval survival was lower beginning at hatch (Figure 8) and the magnitude of the difference between this treatment and the control amplified rapidly with a complete mortality of larvae exposed to 10 mg L$^{-1}$ at 4 dpf, reflecting a significant interaction between time and treatment levels ($P<0.0001$, Table 18). Red snapper embryos exposed to 50 mg TA-N L$^{-1}$ (1.727±0.107 mg UIA-N L$^{-1}$, Table 19) did not hatch.
Figure 8. Survival of red snapper larvae exposed to three ammonia concentrations (0, 1, and 10 mg L$^{-1}$) during the first week of life. Data are presented as the mean ($\pm$SD) of three spawns with 2 replicates per spawn. Letters (a, b, and c) denote homogeneous groups identified during post-hoc tests.

Hatch rates and subsequent survival of larvae differed significantly among spawns from the three females ($P<0.0001$, Table 18). Hatch rates ranged from 59 to 92% on average per female in the control treatment (Table 19). No significant mortality was detected in the control and 1 mg L$^{-1}$ exposure groups until 6 dpf (Figure 8). Survival of these two groups remained above 75 % on average at this time and a significant mortality peak was then recorded at 6 dpf, reflecting starvation and exhaustion of vitellin reserves. Total mortality was observed at 7 dpf and marked the end of the experiment.

The magnitude of the difference among females varied with the ammonia treatment level (interaction Ammonia x Female $P<0.0001$, Table 18), suggesting
differences in tolerance among spawns. Differences were noticeable as early as hatch where the larvae from one of the females (female 1) were less affected by the 10 mg L$^{-1}$ treatment than the larvae from the other two females (Table 19). Subsequently, mortality occurred more rapidly in females 1 and 3 than in female 2, reflecting a significant interaction of female x time x treatment ($P=0.0004$, Tables 18 and 19).

Table 18

*Results of three way ANOVAs accounting for the ammonia treatments ($NH_3$), duration of exposure (time), female parent and their interactions.*

<table>
<thead>
<tr>
<th>Effects and interactions</th>
<th>DF</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$NH_3$</td>
<td>2</td>
<td>984.33</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Time</td>
<td>6</td>
<td>887.29</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Female</td>
<td>2</td>
<td>137.06</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>$NH_3$ * Time</td>
<td>12</td>
<td>110.53</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>$NH_3$ * Female</td>
<td>4</td>
<td>40.10</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Female * Time</td>
<td>12</td>
<td>7.24</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>$NH_3$ * Female * Time</td>
<td>24</td>
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Table 19

Percentages of live larvae (%Live) in experimental groups of embryos exposed to three total ammonia concentrations (TA-N, 1, 10 and 50 mg L⁻¹) beginning 1 hour post fertilization and in controls (no ammonia added). Data from three females (F1 to 3) recorded during 7 days of exposure in two replicates (Rep A and B) per experimental group. TANt: targeted TA-N, TANm: measured TA-N, UIA-N: estimated concentration of unionized ammonia in mg L⁻¹, pH: measured pH, n: sample size.

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**Discussion**

In this work, the tolerance of red snapper embryos and newly hatched larvae to two potential stressors resulting from intensive culture was investigated. The transfer of embryos to a DO level of 2 mg L⁻¹ (29% saturation) induced complete mortality of embryos in all three tested spawns before hatching, indicating that this hypoxic level is lower than the range tolerated by red snapper eggs, a result consistent with observations in other fishes (Elshout et al. 2013; Mejri et al. 2012; Wexler et al. 2011).

Groups transferred to moderately hypoxic conditions (3 mg L⁻¹, 45% saturation) showed slightly lower hatch rates than controls (10% difference), but the magnitude of the difference in survival rate between control and exposed groups increased over time. Survival of exposed groups was 50% that of controls at 3 dpf and only 30% at 5 dpf. The increase of the magnitude of the difference between control and treated groups over time could reflect either that there is a cumulative effect of the prolonged exposure to hypoxic conditions or that hatched larvae are more sensitive to hypoxia than embryos. Interestingly, when the decrease in DO was progressive and reached the same hypoxic condition (44% saturation) at 2 dpf in the second moderate hypoxia challenge, no significant difference in survival rate was detected between control and treated groups.
However, in that experiment, almost complete mortality was observed in both the control and the exposed group 3 days after the exposure level was reached (5 dpf), likely reflecting a lower quality of spawns used in this trial and preventing evaluation of treatment effects at that date. After 1 day of exposure (1 dpf for the first challenge, 3 dpf for the second challenge), the magnitude of the difference between control and exposed groups was similar in the two experiments (10% and 8.3%, respectively). Mortality of exposed larvae became significant and substantial only after 3 days of exposure in the challenge initiated at 1 hpf and accordingly would have been expected to reach significant levels between 3 and 5 dpf in the second challenge. Unfortunately, the two treatments could not be contrasted at 5 dpf in the latter experiment because of heavy mortality in all groups as discussed above. Therefore, additional data needs to be collected using spawns showing higher viability after 3 dpf to further determine if mortality patterns differ when hypoxia is induced on larvae versus embryos.

Overall, the two exposures conducted at 3 mg L\(^{-1}\) led to moderate and non-significant mortality after 1 day of exposure, and some larvae survived after 3 days of hypoxia (even 5 days in the first experiment). The DO level tested corresponds to 45% saturation at the temperature of our experiments and is considered a moderate level of hypoxia for juvenile and adult fish in other species (Claireaux et al. 2000). Few studies have examined hypoxia effects on embryo and newly hatched larvae of marine fishes with pelagic larvae such as the red snapper. Eggs and larvae of these species are less likely to encounter low oxygen concentrations in their natural habitat and have been hypothesized to
be more sensitive to hypoxic conditions (Miller et al. 2002). Eggs of Greenland halibut (Reinhardtius hippoglossoides), a cold-water species, hatched at DO levels as low as 20% of oxygen saturation (Mejri et al. 2012). In contrast, the estuarine black bream (Acanthopagrus butcheri), a relatively warm-water species, showed a delayed embryonic development at 45% saturation (3.1 mg DO L\(^{-1}\)), a treatment that also resulted in 100% deformed larvae (Hassell et al. 2008). Newly hatched larvae of the tropical yellow fin tuna (Thunnus albacares) died rapidly when exposed to 2.2 mg DO L\(^{-1}\) (34% saturation at 28°C) and showed a moderate reduction of survival to first feeding (23% lower than control) when exposed to 2.65 mg DO L\(^{-1}\) or 40% saturation (Wexler et al. 2011).

Available data on warm-water species are consistent with our results on red snapper where moderate mortality was observed at or close to 45% saturation.

Overall, these results suggest that the minimum level of dissolved oxygen compatible with survival of red snapper embryos and larvae is higher than 45% saturation under the conditions of this experiment. At 45% saturation, larval survival was less than 30% of controls at 5 dpf. Thus, a weak fraction of the larvae was still alive during the transitional feeding period (between 3 and 5 dpf), and considering the increased rates of mortality already detected at 3 dpf, the few larvae still alive at that point were likely stressed and not in good condition to initiate successfully exogenous feeding. Additional studies targeting higher intermediate levels of hypoxia will be needed to clarify the range of oxygen concentrations suitable for hatchery production. Further work is also needed to determine effects of hypoxia on the developmental rate including timing of hatch
of embryos and compare the sensitivity of embryos to that of newly hatched larvae as discussed above. In these studies, challenges exposing embryos but restoring normoxia shortly after hatch appear the most relevant to determine the potential impacts of production protocols in the hatchery where eggs are typically maintained at high density during the incubation period and then transferred to low density tanks after hatch, with low likelihood of experiencing hypoxia.

The results at 3 mg L\(^{-1}\) (minimal effect at hatch but major effects on survival at day 3 and 5) also highlight the need to monitor treatments during incubation and early larval life when refining the minimum DO level tolerable for embryos. Indeed, hypoxic treatments between 3 and 4 mg DO L\(^{-1}\) may reveal no significant effect on hatch rate but could induce significant mortality during the first few days of life if carryover effects occur as previously reported in brown trout *Salmo trutta* (Roussel 2007). The occurrence of such carryover effects in red snapper remain to be tested.

Finally, significant differences among spawns in sensitivity to hypoxia were detected during the first moderate challenge. The potential mechanisms involved in these differences are multiple and include genetic and maternal factors. The interaction pattern between the viability of spawns under normoxia and their sensitivity to hypoxia seems complex. During this experiment, the spawn with highest viability (under normoxia) showed highest tolerance to hypoxia, but the second best spawn appeared more sensitive to the treatment than the spawn displaying lowest quality. Thus, improving the viability of spawns, as measured under normoxic conditions, would not necessarily improve their
tolerance to hypoxia. Minimum threshold DO levels for incubation should thereby be determined during challenges involving several spawns to ensure identification of safe levels for all spawns.

The challenge evaluating the tolerance of embryos and newly hatched larvae to ammonia was based on a similar principle. Three doses of TA-N were tested by immersing embryos directly from water where TA-N was ~0 mg L\(^{-1}\) to different concentration levels at 1 hpf, and maintaining them unfed in the tested static condition until complete mortality was observed. Survival of embryos and larvae exposed to a TA-N concentration of 1 mg L\(^{-1}\) did not differ from those in control groups throughout the monitoring period, and no significant mortality was detected until 6 dpf in both groups. Hatch rates and subsequent survival rates were significantly lower in the groups exposed to 10 mg TA-N L\(^{-1}\), where complete mortality was observed at 4 dpf versus 7 dpf for the control and 1 mg TA-N L\(^{-1}\) groups. Complete mortality before hatch was observed in all groups exposed to 50 mg TA-N L\(^{-1}\). Thus, in our experimental conditions, exposure had no detectable effects on survival effects at 1 mg L\(^{-1}\) but significant reduction in survival at the 10 mg L\(^{-1}\) dose. In the latter group, survival was 54 % of that of controls when larvae are expected to initiate exogenous feeding (3 dpf), and complete mortality was recorded in this treatment the next day, indicating that larvae would not have initiated feeding successfully in this treatment.

Interpretation of these results in terms of range of tolerance for red snapper embryos and larvae need to account for the actual concentration of unionized
ammonia (UIA-N), which is the toxic form of ammonia found in equilibrium with the non-ionized (and non-toxic) form (Bower and Bidwell 1978).

In the current experiment, the pH of the exposure water was 7.8. At this pH, the expected concentration of UIA-N in the 1 mg L$^{-1}$ treatment was estimated to be 0.05 mg L$^{-1}$. Concentrations of UIA-N between 0.02 and 0.05 mg L$^{-1}$ have been shown to be harmless to first feeding marine larvae (Brownell 1980), consistent with the lack of detectable effect at this dosage in this challenge. In contrast, the 10 mg L$^{-1}$ TA-N treatment corresponded to 0.42 mg L$^{-1}$, a value that exceeds the upper range of toxicity tolerance for most aquatic organisms (Francis-Floyd et al. 2009). However, most exposure results reviewed by Francis-Floyd et al. (2009) were based on the tolerance of juveniles and adults of studied species, and few studies reported the effect of ammonia on embryos and larvae of marine fishes, even though available data suggest that larvae seem to be the most sensitive life history stage to UIA-N (Harcke and Daniels 1999). At 26±1°C, more than 90% of spotted sea trout and red drum eggs hatched at 6.0 mg UIA-N L$^{-1}$ or more, but significant mortality of larvae occurred in both species at concentrations as low as 0.26 mg UIA-N L$^{-1}$ (Daniels et al. 1987; Holt and Arnold 1983). In red snapper, 0.42 mg UIA-N L$^{-1}$ affected embryonic survival resulting in lower hatch rate, denoting a higher sensitivity of embryonic development to ammonia exposure than the estuarine Sciaenidae. The subsequent increase in mortality rate at this exposure concentration suggests that hatched larvae are more sensitive to UIA-N and/or that effects of the prolonged exposure to ammonia might be cumulative.
The causes for an increased sensitivity of red snapper embryos to ammonia are unclear. While some authors argued that the structure and function of the chorion could provide a high degree of protection to the embryo against ammonia (Bucking et al. 2013), others’ studies suggested that ammonia can penetrate the chorion (Braun et al. 2009; Steele et al. 2001). Also, very early in development, urea synthesis has been hypothesized to protect teleost embryos from toxic nitrogenous end-products produced during yolk absorption (LeMoine and Walsh 2013; Wright and Land 1998). Thus, increased sensitivity of red snapper embryos to ammonia could be related to a higher permeability of the chorion to ammonia than in other species or a lower efficiency of mechanisms protecting embryos from ammonia such as urea synthesis.

The sensitivity of red snapper embryos and larvae to ammonia UIA-N between 0.05 and 0.42 mg UIA-N L\(^{-1}\) should be further investigated to refine sublethal conditions and maximum thresholds tolerable during incubation and larval rearing in aquaculture. As for hypoxia, long term effects such as deformities could result from an acute exposure during early development (including embryonic development), and/or cumulative effects could lead to worsening mortality under a given dosage if the exposure is prolonged. In consequence, treatments need to be evaluated several days after hatch as was done in this study in all experiments (even if exposure is ended at hatch) and may also include evaluation of larval growth and deformities.

A consideration specific to ammonia challenges is the importance of the pH as this parameter directly influences the concentration of toxic UIA-N. In the
current challenges, if the pH had been 8.6, the proportion of UIA-N would be expected to reach 0.20 mg L\(^{-1}\) in the 1 mg TA-N L\(^{-1}\) treatment. This exposure is in the upper range of tolerance for a variety of organisms, and considering the major effects detected at 0.42 mg UIA-N L\(^{-1}\) in this study, could lead to significant mortality before the transitional feeding period. Monitoring and control of pH is therefore essential when designing and interpreting future experiments and, most importantly, when characterizing safe levels for incubation and larval rearing of the red snapper.

As in the study of hypoxia, significant maternal effects on the sensitivity to treatments were detected as revealed by a significant female x treatment interaction. The main aspect of this interaction is that the embryos from the female showing lower viability under control conditions (no ammonia) seemed more resilient to the 10 mg TA-N exposure treatment than the embryos from the other females that showed a significant reduction in survival at an earlier stage of the experiment (days 2 and 3). As discussed above, this indicates that improving the viability under normal conditions will probably not improve tolerance to stressful levels of ammonia. This variability among spawns will need to be accounted for by evaluating several spawns when designing protocols to identify a safe range of ammonia concentrations for red snapper embryos and larvae.

In summary, an immediate priority is to focus studies on the effects of exposures restricted to the incubation period until hatch, which represents the rearing phase where density is high and hypoxia and elevated ammonia levels are more likely to happen. Another aspect that needs to be evaluated is the
interaction between hypoxia and elevated ammonia stresses when applied simultaneously. Indeed, incubation at high density often results in both increase in ammonia and reduction of oxygen levels, and the combination of these two stressors may not be simply additive; thus, identification of safe levels may need to account for both oxygen and UIA-N concentrations.

The oxygen demand is expected to increase during the course of embryonic and larval development (Wright and Wood 2012) and will need to be quantified and accounted for when evaluating density treatments for aquaculture operations. Similarly, the kinetics of increase of ammonia in incubators due to embryo metabolic processes and to the degradation of dead and unfertilized eggs would need to be quantified to predict possible levels of UIA-N under different density scenarios. As discussed earlier, evaluation of exposures also needs to include onset of deformities and early growth as both of these effects were reported in studies of exposure to hypoxia (Buentello et al. 2000; Petersen and Pihl 1995; Tran-Duy et al. 2008) and ammonia (Holt and Arnold 1983; Lemarié et al. 2004; Schram et al. 2010).
CHAPTER VII

CONCLUSIONS AND PERSPECTIVES

The objective of this dissertation was to provide a better understanding of egg quality and its determinants in a marine species of commercial importance, the red snapper *Lutjanus campechanus*. This work was based on spawns of wild and captive red snapper females induced for oocyte maturation using human chorionic gonadotropin (hCG). The spawning protocol was standardized across all females and involved collecting ova by manual stripping as soon as ovulation was detected, according to the routine procedure for spawning of red snapper to seed culture trials. The high variability of the quality and viability of red snapper eggs obtained during spawning trials is a major challenge for red snapper aquaculture as most of the mortality of red snapper larvae in hatcheries occurs at very early stages (at or shortly after the time of first feeding). Elevated mortality due to uncharacterized variation of egg quality therefore prevents interpretation of the effects of experiments aiming to optimize husbandry practices for larvae. Efforts in this work focused on identifying factors influencing the quality of red snapper eggs and evaluating the value of parameters measured on females and spawns as predictors of the viability of embryos and newly hatched larvae. A better understanding of the role of factors related to the phenotype and condition of the female, broodstock husbandry, and those of endogenous characteristics of eggs such as their nutrient content or maternal RNAs is critical to provide assistance in the selection of high quality progeny for larval trials as well as for developing appropriate practices for broodstock management and spawning.
The egg quality is defined as the potential of an egg to produce viable fry characterized by high survival at first feeding and thus, high probability to successfully transition to autonomous life. This requires successful completion of several early developmental steps including fertilization, development to hatch, and further growth and ontogeny post hatch using endogenous vitellin reserves as the source of metabolites until exogenous nutrients can be successfully ingested and digested. Developmental failures can occur at any of these steps, possibly due to different causes and involving different mechanisms. Therefore, this work began by assessing the viability potential of eggs at various developmental stages including fertilization, hatch, and various times post-hatch. Surveys of survival post hatch focused in particular on determining the survival rates at the time when larvae would be expected to initiate exogenous feeding and the duration of their survival past that point, thus characterizing the transitional feeding period, a critical parameter to the successful initiation of exogenous feeding (Yúfera and Darias 2007). This decomposition of larval survival aimed to identify if multiple egg quality traits needed to be defined. Mortality events at specific developmental stages were weakly correlated to each other in this study, indicating that developmental failures at fertilization, hatch, or post hatch were in most cases independent and potentially involved multiple causes and mechanisms. A first implication of these results is that studies of factors impacting egg quality, in particular evaluating parameters to be used as early predictors, will need to account for potential effects at each of these developmental stages. Embryonic mortality was found to be a significant
component of overall spawn quality, in particular for spawns of captive females. Embryonic mortality prior to hatch in other species has been related to the early stages of the blastula period, where development is under the control of maternal RNAs. The understanding of embryonic mortality and factors impacting it in red snapper could be improved in the future by measuring mortality at the end of the mid-blastula transition to establish the importance of the early divisions for the overall success rate of development to hatch and further focus studies of the effects of factors hypothesized to influence egg quality.

This study revealed significant differences between reproductive parameters of captive and wild females including the characteristics of their spawns. Less than 20% of the captive females present in our broodstock were found at the spawning capable phase, and most of them were at early stages of the maturation process (stage II) at the time of hormonal induction. The response time to hormonal induction was on average 4 hours longer in captive females than that of wild ones, and their fecundity was three times lower. Finally, the author observed a total lack of spontaneous spawning of captive held females. All of these observations denote a severe disruption of oogenesis in captivity (Bardon-Albaret et al. in press). The causes of these inhibitions of maturation and spawning are still not understood but likely involved disruptions of the brain-pituitary-gonad axis through a lack of production or release of gonadotropin by the pituitary gland as reported in several other species (Mylonas and Zohar 2001). In addition, the author inferred that some captive females may have been undergoing their first reproductive cycle which may have contributed to poor
spawning results. Improving maturation, spawning activity, and fecundity of captive broodstock is therefore a priority for research in red snapper aquaculture. Studies based on a larger sample of captive females and varying husbandry parameters such as broodstock density and sex ratio, but also evaluating the effects of hormonal therapies would be very informative for that purpose. Once captive spawning is achieved, the egg quality could be compared between first time and multiple time spawner fish to determine if the egg quality differences (particularly the increased embryonic mortality rates in captive females) are related to the lack of spawning prior to these experiments or if they involve other factors.

Except for the hatch rate that may be improved with older females, egg quality parameters could not be predicted by the simple phenotype measurements obtained on females and their oocytes prior to hormonal induction in this study. While the detection of relationships between such variables and egg quality might be improved by using larger sample sizes, this finding is consistent with other studies that concluded that egg quality is very difficult to predict (Bobé and Labbé 2010; Brooks et al. 1997; Kjørsvik et al. 1990). It is therefore likely that other parameters such as the endogenous characteristics explored in Chapters IV and V will need to be included to achieve reliable prediction of the viability of spawns.

The nutrient content (proximate composition, amino acid, and fatty acid profiles) in ova of captive and wild females were very similar and did not differ significantly. However, nutrient profiles in the reserve tissues (muscle and liver)
did vary significantly between the two groups. The muscle tissue of captive females had more protein (and amino acid), and their liver contained more lipids than those of wild females, probably due to the low mobilization of resources invested for spawning and the access to a non-limiting food supply in captive conditions. The high HSI of captive females along with the high lipid content of their liver suggests that large amounts of lipids were available but were not incorporated into oocytes or used for other functions. This result may signal an excess of lipids in the diet but could also reflect the lack of spontaneous spawning activity throughout the spawning season along with the low fecundity observed following hormonal induction. Although the composition of reserve tissues differed between captive and wild fish, the ova nutrient concentrations were similar between the two groups, indicating that the captive diet provided the necessary nutrients to allow production of eggs of similar quality to those of wild females. This result may reflect in part the supplementation of the broodstock diet with vitamins and fish oils that ensured that all necessary fatty acids and vitamins were provided to brood fish. However, once captive reproduction of the red snapper is better controlled and spawning activity of captive fish increases, the reserve tissues may play a more important role in establishing vitellin reserves in oocytes. At that point, the altered nutrient profiles of reserve tissues in captive fish (possibly reflecting in part inappropriate nutrient balance of the diet) may more significantly impact egg quality. Thus, while the current diet can serve as a base for future studies, it should be analyzed for nutrient content. Nutrient content in tissues and ova of actively spawning captive females should
also be examined. These results can help identifying nutrient imbalance in the diet and assist in the formulation of the supplement. The current ova composition of wild females could also serve as a proxy to orient the formulation of industrially prepared food for red snapper broodstocks. The ova concentration in micro elements and vitamins was not surveyed during this study. Nutrients such as vitamins E and D, or hormones (Lubzens et al. 2010), proteases, prostaglandins, and carotenoid pigments which act as precursors of prostaglandins were shown beneficial to spawning and egg quality in other species (Izquierdo et al. 2001; Takahashi et al. 2013). Future efforts could incorporate characterization of samples for these nutrients during nutritional studies for a better assessment of egg viability.

An RNA-sequencing approach was used to compare transcriptome profiles in ova of wild and captive females. The magnitude of differences in standardized transcript abundance (log₂ FPKM) between ova of captive and wild females was moderate, and the distribution of log₂ FPKM values overlapped between the two groups for all transcripts. Hierarchical clustering based on the population of transcripts differing significantly between captive and wild females revealed a lot of variation in transcriptome profiles among spawns within the two groups, such that spawns could not be classified accurately as captive or wild. However, the results of stepwise discriminant analysis suggest that the two groups can be efficiently distinguished using signatures based on a subset of transcripts. This result is consistent with findings in Chapman et al. (2014) and will need to be explored further using larger sample sizes.
The second objective of the RNA-seq analysis of ova was to determine if maternal transcript profiles were correlated with measures of egg quality. Variation in hatching rate was associated with changes in the abundance of 1,204 transcripts while other egg quality variables were associated with the change of much lower numbers of transcripts (392 to 696). This result suggests that maternal transcripts had a major influence on the embryonic development as measured by success to hatch in our study. This hypothesis is also supported by reports in other species that indicated that maternal mRNAs support the embryonic development until the activation of the zygotic genome (Pelegri 2003; Abrams and Mullins 2009) at the end of the mid-blastula transition (Tadros and Lipshitz 2009). Predicting the survival potential of eggs after hatch, in particular the viability past the age of first feeding, is a primary objective for red snapper aquaculture. Results of discriminant analyses suggest that an early signature of this survival potential may occur in the transcriptome of ova although this result will need to be re-evaluated using larger sample sizes. Studies of gene expression in the zygote may be more effective for the purpose of predicting viability post hatch and could be evaluated in future studies. The importance of female age (likely reflecting differences in spawning activity prior to our trials) indicated by the significant correlation between hatch rate and female age in Chapter III was confirmed during analysis of the transcriptome that revealed that more than 40% of the transcripts found to co-vary with hatch rate, while also showing variation abundance as a function of female age. The differences in
transcriptome between females of different ages and different spawning history thus deserve further investigation.

The prediction of continuous egg quality parameters via multiple regressions was poor when test samples naïve to the prediction equations were evaluated. The current results and those of Chapman et al. (2014) suggest that transcripts showing moderate change in abundance are involved in complex patterns determining egg quality. In such a situation, robust estimation of a prediction equation is challenging using the small sample sizes used here and which are typical of RNA-seq studies. The distinction of the two levels of categorical variables (high versus low quality score) based on extreme values seemed more effective in the context of the current dataset and was also successful in the study of Chapman et al. (2014). These findings will need to be validated using larger sample sizes and could involve bootstrapping approaches to maximize the information drawn from available data. The analytical methods used to assess the relationship between transcript abundance and phenotype are still in development. Recent studies advocated the use of a negative binomial regression (Di et al. 2014) or rank transformation (Zwiener et al. 2014) during tests of differential expression. These approaches may provide improvement to differential expression analysis using RNA-seq datasets characterized by small sample sizes and high variances. Perhaps a major challenge in this and other similar studies was related to studying the effects of the combinations of several variables (i.e., the abundance of extremely large numbers of transcripts quantified for each sample) on characters. Chapman et al. (2014) used artificial
neural networks and support vector machines to study the relationship between ova transcriptome and egg quality in striped bass. The high efficiency reported in the current work (>90% of the variance in egg quality explained by maternal transcripts) is troubling considering that transcripts included in the model were in most cases weakly associated with egg viability with only subtle and non-significant observed changes in expression as a function of egg quality during single parameter analyses. Validation of these findings using larger samples is thus warranted. The modules identified during MMC analysis may be of assistance in the identification of robust groups of indicator transcripts. The enrichments in specific functions observed in this work strongly suggests that modules identified during MMC correspond to true sets of correlated genes within the panel of transcripts showing significant associations with a character of interest. Targeting genes or sets of genes within multiple modules may be a robust approach to develop reliable indicators in the future.

An average of 58% of quality controlled reads could be mapped on the draft red snapper genome, and on average ~50% of mapped reads could be annotated. Annotations based on similarity in Blast2GO were highly redundant and often not very specific, highlighting the need to develop the reference genome for red snapper. Increased coverage as is expected in the next assembly will be important to improve mapping of RNA-seq reads, and efforts should focus largely on annotation to improve interpretation of experiments such as those conducted in this work.
Modulated Modularity Clustering and ReViGO analyses revealed that sets of transcripts associated with egg quality included groups of correlated transcripts involved in distinct molecular functions, biological processes and cellular components. As suggested by Stone and Ayroles (2009), these groups of transcripts allow formulating hypotheses regarding the processes and functions contributing to the egg quality parameters studied. Further research on the mechanisms involved in egg quality could involve, for example, studies of modules of correlated transcripts associated with both egg quality and maternal traits such as age or other pre-spawning parameters studied in this work.

Finally, the prediction of survival to first feeding, the ultimate parameter of importance for red snapper aquaculture, could not be achieved except through promising results of discriminant analysis based on transcript abundance. This finding will need to be confirmed using larger sample sizes, and prediction may be improved through characterization of zygotic transcriptions as discussed above.

In Chapter VI, the author explored the effects of environmental stressors typical of aquaculture conditions on egg and larval viability. The tolerance of red snapper embryos and newly hatched larvae to degradations of water quality parameters (hypoxia and increased ammonia concentration) was studied. Results of challenge experiments revealed that dissolved oxygen levels need to remain higher than 3 mg L$^{-1}$, and unionized ammonia levels need to be kept below 0.2 mg L$^{-1}$. Changes in the sensitivity of red snapper between embryos and hatched larvae, will need to be evaluated. In particular, the sensitivity of red
snapper embryos to acute stresses involving these two parameters, characteristics of typical hatchery accidents, remains to be determined. The results of these studies will be useful to determine incubation and early larval rearing conditions that will ensure egg quality is not affected post fertilization and improve the reliability of a priori assessments of egg quality.

Egg quality is a complex biological phenomenon that has generated a lot of interest in aquaculture research during the past few decades due to the central importance of achieving a reliable supply of viable eggs when attempting to control the life cycle of cultured species. In red snapper, this work showed that understanding the determinants of the development potential of ovulated eggs requires evaluating viability at multiple time points because mortality events at different developmental stages were weakly correlated to each other, possibly reflecting different causes and mechanisms. Although egg quality ultimately reflects for a large part the recent history and phenotypic characteristics of the females, the prediction of egg quality based on simple measurements on the female parent was ineffective, reflecting the complexity and multiplicity of mechanisms regulating oogenesis. A significant effect of female age on hatch rate was, however, detected and will require further investigation. This work showed that the egg quality can be characterized by its intrinsic properties including the nutrient content of the yolk and the maternal mRNAs, both of which are incorporated into oocytes during oogenesis. Disruptions of the gametogenesis in captive red snapper led to a reduced response to hormonal induction, but the nutrient concentration of ova from captive females were similar
to those issues from wild females, indicating that the current diet of captive broodstock allowed for the production of suitable vitellin reserves to support embryo development to first feeding, although this result will need to be revisited when spawning activity of captive brood fish will be improved. The effect of the female age on hatching was confirmed with a high percentage of ova transcripts that were significantly influenced by the age of the female parent and also significantly correlated with the hatch rate of eggs. Encouraging results for the prediction of the viability to first feeding were also obtained but will need to be validated with larger sample sizes. The analysis of the transcriptome from a standardized zygotic stage may provide very useful information about the survival potential of red snapper larvae and mechanisms regulating this phenomenon.
Results of Modulated Modularity Clustering performed on ova transcripts from wild spawns (n=17) showing significant association (P < 0.05) with the studied factors: frequency distribution (k = number of clusters and percentage of n = the total number of significant transcripts, with minimum of two transcripts per cluster) of the mean Pearson’s correlations r among transcripts included in the same cluster. The r range that bears the largest proportion of transcripts is highlighted in bold.

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<th>OvapH</th>
<th>Stage</th>
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*: n=17 wild spawns vs n=7 captive spawns
REFERENCES


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