Optimisation of common snook *Centropomus undecimalis* broodstock management

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DECLARATION

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

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Yellow perch *Perca flavescens*

ABBREVIATIONS

ADF	Anterior dorsal fin
AF	Anal fin
AMI	Anterior median intestine
ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
0B	Buccal cavity
BPG	Brain-pituitary-gonad
BSA	Bovine serum albumin
C	Certahyal
cAMP	Cyclic adenosine monophosphate
ca	Cortical alveoli
CF	Caudal fin
CM	Cerebellum
CNB	Columnar nuclear bodies
CR	Cartilaginous ring
CY	Clearing yolk
cDNA	Complementary deoxyribonucleic acid
DA	Dopamine
DHP	17α, 20β-dihydroxy-4-pregnen-3-one
DNA	Deoxyribonucleic acid
DOM	Domperidone
DSW	Digestive system walls
dNTP	Deoxyribonucleotide triphosphate
DPH	Day post-hatch
DSG	Dividing spermatogonia
E	Eye
E_2	17β-estradiol
e.g.	For example
ELISA	Enzyme-linked immunosorbent assay
EST	Expressed sequence tag
FF	Fin fold
FL	Fork length

FSH	Follicle stimulating hormone
FWC	Florida Fish and Wildlife Conservation Commission
FWRI	Fish and Wildlife Research Institute
fy	Fluid yolk
GA	Golgi complex
GE	Germinal epithelium
GLM	General linear model
GnRH	Gonadotropin releasing hormone
GPR54	G-coupled protein receptor 54
GSI	Gonadosomatic index
GTH	Gonadotropin hormone
gv	Germinal vesicle
GVBD	Germinal vesicle breakdown
h	Hour
H	Heart
HS	Hyposynplectic cartilage
IgfI	Insulin-like growth factor I
IGL	Inner ganglion layer
IN	Intestine
Kiss	Kisspeptin
Kissr	Kisspeptin receptor
L	Liver
L	Luman
L	Lobule lumen
LH	Luteinising hormone
M	Moles
MC	Merkel's cartilage
M_H	Myomere height
MARP	Mote Aquaculture Research Park
Met	Metoclopramide
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
mRNA	Messanger ribonucleic acid
MV	Microvilli

n	Number
OC	Optic chiasma
od	Oil droplet
og	Oil globule
OLM	Outer limiting membrane
OMevg	Oocyte Maturation, eccentric germinal vesicle step
OMgvm	Oocyte Maturation, germinal vesicle migration step
OMpov	Oocyte Maturation, preovulatory step
NVR	Non-villous region
ON	Optic nerve
ONL	Outer nuclear layer
OPL	Outer plexiform layer
OT	Optic tectum
P	Pigments
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Pigment epithelium
PEC	Pigment epithelium cell granule
PGon	Primary Growth, one-nucleolus step
PGmn	Primary Growth, multiple nucleolus step
PGpn	Primary Growth, perinucleolar step
PGod	Primary Growth, oil droplet step
PIT	Passive integrated transponder
PiT	Pituitary extract
PM	Premaxilla
PR	Photopreceptor layer
PRES	Photoreceptor outer segment
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait locus
R	Ring oocyte
RACE	Rapid amplification of cDNA ends
RER	Rough endoplasmic reticulin
Res	Reserpine
RNA	Ribonucleic acid

SB	Swim bladder
SC	Spermatocytes
SD	Standard deviation
SE	Sertoli cells
SEM	Standard error of the mean
SEM	Scanning electron microscopy
SG	Spermatogonia
SGe	Secondary Growth, early step
SGl	Secondary Growth, late step
SGfg	Secondary Growth, full-grown
SL	Standard length
SP	Sperm
ST	Spermatids
T	Testosterone
TEM	Transmission electron microscopy
TW	Terminal web
UTR	Untranslated region
UV	Ultraviolet
V	Intestine-recto valve
VTG	Vitellogenesis
W	Weight
Y	Yolk
ZP	Zona pellucida
11KT	11-ketotestosterone

ABSTRACT

Advances in aquaculture technologies are being investigated to support the replenishment of local fisheries, develop marine food fish farming opportunities and to increase seafood production globally. In order to promote the expansion and development of aquaculture technologies required to raise new finfish species, a number of key bottlenecks restricting commercial-scale culture need to be addressed, including the ability to control fish reproduction in captivity and to produce high quality seeds. One candidate species for large-scale production, and the focus of this work, is common snook. Prized as a food fish in Mexico, Central and South America and as a popular game fish along the Gulf coast of the United States; common snook are economically important having both a high market value and recreational demand. Despite recent advances in captive spawning, a number of reproductive bottlenecks still need to be addressed such as lack of spontaneous spawning in captivity, poor fertilization rates and inconsistent production of high quality eggs and larvae. Therefore, the overall aim of this thesis was to better understand the reproductive biology of common snook in order to develop protocols to improve the reliability of captive spawning in closed recirculating aquaculture systems and the quality of eggs produced as a basis for commercial scale cultivation.

First, this PhD project described oocyte development in common snook and validated a non-invasive method for assessing reproductive condition in wild and captive stocks (Chapter 2). This was done by using a tiered and adaptable staging scheme to compare the wet mount technique with histological preparations of ovarian biopsies. When compared with histology, the wet mount provided an immediate and precise method for determining whether female broodstock were candidates for hormonal induction. In fishery biology, an understanding of fish reproductive success

and population reproductive potential is critical for designing and implementing effective fisheries management strategies. The wet mount technique provides a tool for non-lethal, low-cost determination of reproductive status in wild fish stocks.

The next research chapter focused on spawning induction of captive snook populations. The first trial compared the effects of slow and regular release GnRHa implants whereas the second trial investigated the effects of GnRHa, alone or in combination with the dopamine antagonist, pimozide (PIM), on milt characteristics and plasma steroid levels in captive male common snook broodstock (Chapter 3). In an effort to better enable reliable control of reproduction under captive conditions, the annual plasma sex steroid profile of captive male and female broodstock maintained under natural photo-thermal conditions was also examined. When possible, milt samples were collected pre and post implantation; sperm density, sperm motility and spermatocrit were documented among individual males. The assigned treatments appeared to have no or little effects on milt production in male broodstock although plasma steroid levels were found to be significantly elevated in individuals treated with GnRHa in combination with the dopamine antagonist, pimozide.

At the time this work was performed, no data on spawning dynamics, including individual spawning performance, had been reported for common snook in captivity. Mass spawning tanks are complex systems where fish are left to spawn naturally and fertilized eggs are collected with little or no control over the mating of the animals. Therefore, the third part of this thesis explored the potential of DNA profiling for monitoring mating outcomes in captive broodstock by employing eight microsatellite markers to detect and quantify individual parental contributions for 2,154 larvae obtained from the three broodstock tanks (Chapter 4). The panel of loci was generally robust and allowed unambiguous assignment of 89% of larvae to a single family.

Overall, spawn contribution data 1) provided a confirmation of GnRHa treatment efficacy in female snook with a minimum stage of oogenesis (late secondary growth-SGl) required for successful spawning, 2) identified a potential impact of handling on maturation and spawning of captive broodstock and 3) confirmed that, through photothermal conditioning, captive broodstock can spawn over consecutive days and several times per year including outside of their natural spawning season.

The exogenous cues that tropical species use to synchronize key life events like reproduction remain largely unstudied, therefore, my PhD project also investigated the influence of tidal cycle on reproductive activity in common snook (Chapter 5). Real-time quantitative RT-PCR assays were developed and validated to measure the temporal expression patterns of gonadotropin genes (*fshβ* and *lhβ*) during the reproductive cycle in males and females. These were evaluated in relation to sex steroid production, LH blood plasma levels, gonadal development and tidal cycle. The phylogenetic analysis of the deduced amino acid sequence of common snook for *fshβ and lhβ* revealed strong identity with other teleosts (75-90%). Additionally, the mRNA profiles of *fshβ* and *lhβ* in the pituitary of females displayed a clear pattern of expression concomitant with histological changes in oocyte development. Histological observations of gonads suggested a circa-tidal rhythm of follicular development. The findings, as a whole, provided new information supporting the role of tidal cycle on the entrainment of gametogenesis allowing for a better understanding of the environmental control of reproduction in common snook.

Although the primary research emphasis in this PhD was on broodstock spawning and gamete quality, the final chapter focuses on larval ontogeny. The goal of this research was to gain improve understanding of the early life history characteristics of common snook in order to improve larval culture technologies. To do so, a

combination of digital photography and histological techniques were used to document the embryonic and early larval development (0 to 14 days post hatch-DPH) of hatchery-reared individuals (Chapter 6). Larvae hatched 15 h after fertilization at 28°C, lacked pigmentation, had a rudimentary digestive tract and undeveloped visual system. Development was rapid and by 3 DPH larvae had almost doubled in length, the yolk sac was nearly exhausted, the mouth was open and eyes were pigmented with a well-structured retinal layer. The alimentary canal was differentiated into three distinct sections including the foregut, midgut and hindgut. Food was observed in the gut (rotifers) and structural epithelium organelles, such as the nucleus, mitochondria, and dark vesicles, were all present in high numbers. The swim bladder was formed and inflated. In summary, understanding early ontogenetic development in common snook can help provide information needed to address key bottlenecks seen in captive cultivation, such as the high incidence of larval mortality observed during the transition from endogenous to exogenous feeding.

Overall, this doctoral work 1) validated molecular and endocrine analytical tools for future studies of common snook reproductive physiology, 2) provided a better understanding of both broodfish requirements in tank systems as well as the endocrine control of reproduction and spawning at the level of the brain-pituitary-gonadal axis, 3) increased our knowledge in genetic management of captive broodstock, in terms of parentage assignment and 4) offered new insight into wild population reproductive strategy as well as how reproduction is entrained through environmental cues and the pathways leading to oocyte recruitment and maturation. The new information presented here can be used to conserve wild snook stocks through production of farm raised individuals as a sustainable source of seafood and for fisheries enhancement.

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1. GENERAL INTRODUCTION

1.1. Overview of common snook life history and relevance to aquaculture

1.1.1. Geographic distribution and biology

Among the 12 snook species from the genus *Centropomus*, six from the eastern Pacific and six from the western Atlantic and Caribbean, the common snook *Centropomus undecimalis* is the most common species of the family Centropomidae, Order: Perciformes. (Figure 1.1).

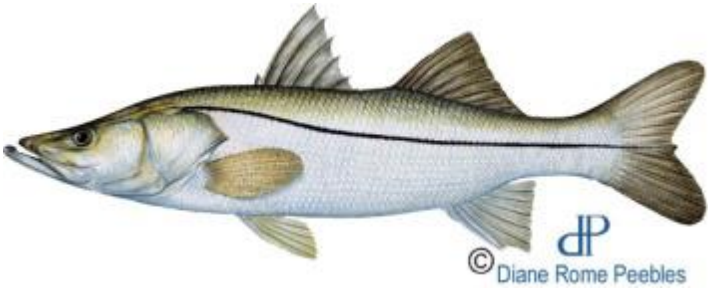

Figure 1.1 Common snook (*Centropomus undecimalis*). From http://myfwc.com/research/saltwater/stock-assessments/finfish/snook/

Common snook reside in tropical and subtropical rivers, estuaries, and oceanic habitats of North and South America and range from the mid-Atlantic coast to southern Brazil, including the insular and mainland margins of the Gulf of Mexico and the Caribbean Sea (Gilmore et al., 1983; Seaman and Collins, 1983) (Figure 1.2). They are most often found south of 28°N latitude (Rivas, 1986), as sudden or sustained exposure to water temperatures <14°C have resulted in behavioral changes (lethargy, feeding cessation) and even mortality (Howells and Sonski, 1990; Adams et al., 2012). Their range in Florida parallels that of mangroves, a primary component of their habitat throughout much of their life history (Marshall, 1958).

Figure 1.2 Global distribution of common snook *Centropomus undecimalis*. Many fish. Fewer fish.
http://commons.wikimedia.org/wiki/File:Centropomus_undecimalis_mapa.svg

Common snook is a euryhaline inshore species, capable of tolerating both salt and fresh water (Taylor et al., 2000). Juveniles are found in tidal ponds, creeks, and rivers (Stevens et al., 2007), whereas adults are often associated with mangroves, sea grass meadows, and other structured habitats in estuaries. Common snook are protandric hermaphrodites with males reaching sexual maturity during their first year at a fork length (FL) of 150-200 mm and some develop into females between 1 and 7 years of age (about 63 cm) (Taylor et al., 2000) although to date, the mechanisms for

initiating the sex inversion remains unknown. Wild females have been found to mature as early as 2.5 years (≥ 43 cm) and females smaller than about 500 mm fork length are uncommon (Muller and Taylor, 2012). Based on ageing techniques using sectioned otoliths, common snook have a maximum life span of approximately 20 years (Taylor et al., 2000). This oldest individual was identified as a male indicating that not every individual changes sex.

Although wild common snook has been studied for more than 50 years, the description of their reproductive biology and early life history is still incomplete. Similar to studies on wild common snook populations in Mexico (Parera-García et al., 2011), those conducted in Florida revealed snook have an annual reproductive cycle with peak spawning activity occurring in high salinity (>28 ppt) seawater during the summer months (May–August) when water temperatures (>24°C) and day length (≥ 13.5 h) are maximal (Volpe, 1959; Taylor et al., 1998; Yanes-Roca et al., 2009). They are primarily solitary or in loosely formed schools outside of the spawning season, however, aggregations of up to several thousand fish have been documented in known locations during the peak of the reproductive season (Lowerre-Barbieri et al., 2003). Early observations suggested that sexually mature adults travel from fresh and euryhaline waters to coastal marine areas to spawn in aggregations (Gilmore et al., 1993: Taylor et al., 1998). Improvements in technology including, but not limited to, the use of archival and real time data loggers, acoustic tagging methods (Trotter et al., 2012; Young et al., 2014), otolith chemistry (Campana, 2001; Rolls, 2014) and genetic markers (Seyoum et al., 2005) have allowed researchers to better track the migration patterns of common snook. Movement data from acoustic tagging has shown high intra-annual and inter-annual site fidelity to individual spawning sites along the

Atlantic (Lowerre-Barbieri et al., 2003) and Gulf (Adams et al., 2009) coasts of Florida in the United States. The available information shows spawning aggregations occur on both the east and west coasts of Florida near major inlets, river mouths, and around coastal islands, all characterized by strong currents, protective cover, and proximity to grass flats and deeper water (Taylor et al., 1998; Blewett et al., 2009; Adams et al., 2011; Trotter et al., 2012). New evidence using oceanic bio-modeling techniques suggests fertilized eggs and larvae would be swept inshore into vegetated shallow brackish tidal creeks, canals and lagoons in both low (riverine) and high-salinity (mangrove swamp and salt marsh) habitats (Yanes-Roca and Meyers, unpublished data). Collectively, this information has provided new insights into common snook migration patterns and their use of freshwater and marine habitats redefining our understanding of habitat residency and spawning schedules for snook.

1.1.2. Economic importance and brief history of management in Florida

Prized as a food fish in Mexico, Central and South America (Alvarez-Lajonchère and Taylor, 2003) and as a popular game fish along the Gulf coast of the United States (Winner et al. 2010); common snook are economically important having both a high market value and recreational demand (Alvarez-Lajonchère and Tsuzuki, 2008; Perera-García et al., 2011). Total snook landings in 2012 were 15,433 tonnes, mostly from Mexico (52%), Brazil (22.3%) and Venezuela (16.9%) (FAO, 2012). In the United States recreational fishing is a multibillion (US) dollar industry where the economic output from saltwater sport fishing in Florida alone was valued at over $4.9 billion in 2011 (American Sport Fishing Association, 2013). The American Sportfishing Association (2013) reported that 6 million saltwater anglers took 45

million fishing trips in 2010, an activity that contributes $41 billion (US) to the economy of the Gulf coast region annually and supports more than 300,000 jobs. As one of the three most popular inshore sport fish in Florida, common snook are a key part of a saltwater recreational fishing industry that contributes greatly to the economy. Their popularity, however, has a downside causing concern among stakeholders and resource managers. Declines in abundance due to fishing pressure, coastal habitat degradation, red tide events and periodic cold kills have led to the need for careful monitoring (Adams et al., 2012; Muller and Taylor 2012).

Florida has historically had one of the highest rates of human population increase in the United States (Campbell, 1997). With a large coast to land-area ratio, much associated activity and development is concentrated along Florida's coast, which can influence the coastal ecosystems (Bruger and Haddad, 1986; Lorenz and Sarafy, 2006). Habitat protection, fishery assessment and regulation have long been at the forefront of management efforts by the Florida Fish and Wildlife Conservation Commission (FWC). Fishing restrictions including bag limits and limitations on size and seasons for snook harvest have been implemented in Florida (Muller and Taylor 2012); where it has been illegal to buy or sell common snook since the collapse of the commercial fishing industry in the 1950's (Marshall, 1958).

During the early 1900's, commercial harvest via hook-and-line techniques and seine nets was permitted but, as demand for snook meat increased, seine nets were prohibited altogether (Marshall, 1958). Although commercial harvest of snook was never large in comparison to other fisheries, over 800,000 pounds of snook were harvested annually between 1941 and 1955 (Marshall, 1958). By 1957, it became apparent that snook populations were in serious decline and snook were granted 'game

fish' status that prohibited commercial harvest and sale, establishing hook-and-line as the only legal method of capture (Marshall, 1958). These measures were thought to be adequate for the protection of the population and from 1957 until 1974 nothing was done to monitor the condition of common snook populations in the state of Florida. By 1976, tagging studies in southwest Florida determined that local populations were decreasing at a dramatic rate, primarily as the result of increased recreational fishing pressure (Bruger and Haddad 1986) and the framework for an adaptable fisheries management plan was established.

The management of snook is complicated due to their reproductive physiology (Taylor et al., 1998). Current management strategies utilize a slot limit to provide males with an opportunity to change sex while maintaining larger females that presumably contribute greater batch fecundities. Regulations allow the harvest of only one snook per person per day, although slot size varies between 711.2 to 838.2 mm total length (TL) for fish caught on the Gulf coast of Florida and 711.2 to 812.8 mm TL for those captured on the Atlantic coast.. Stringent management measures are in place to protect the female spawning stock with a goal of maintaining a spawning potential ratio (SPR) at 40% or higher. SPR is calculated as the average fecundity of a stock that is caught divided by the average fecundity of a stock that is unfished. Since fishing regulations are based on stock assessments, a better understanding of snook reproductive strategies is fundamental for accurate stock assessments.

Today, traditional management measures are used to control harvest and harvest laws are continually adjusted to maintain resource levels in the face of recreational fishing popularity. Alongside traditional management practices, Florida's managers and stakeholders have directed efforts at an aquaculture-based fisheries enhancement

approach (Tringali et al., 2008) which seeks to augment the natural supply of juveniles and overcome recruitment limitation by releasing animals produced in aquaculture settings into the wild (Bell et al., 2008). This has led to funds being apportioned for the production of common snook fingerlings (Alvarez-Lajonchère and Taylor 2003) for use in studies related to the restoration of depleted stocks (Brennan et al., 2005; Brennan et al., 2006; Brennan et al., 2008).

1.1.3. Aquaculture prospects: current situation and challenges

Yields from capture fisheries have remained relatively constant for over a decade, and many fisheries are either fully exploited or overexploited, while others are recovering from historical overexploitation (FAO, 2012; Worm et al., 2009). Around the world, advances in aquaculture technologies are being investigated to support replenishment of local fisheries (Lorenzen et al., 2010; Leber, 2013; Lorenzen et al., 2013), develop marine food fish farming opportunities and increase seafood production globally (Parisi et al., 2014). A large number of high-value species are needed to meet seafood market demands; however, culture techniques have been developed for very few of these fish species (Fotedar and Phillips, 2011). Unfortunately, the production of new finfish species is currently restricted in relation to market demand, competition with traditional species, and the lack of standardized and reliable reproduction, weaning, feed, and/or on-growing techniques (Parisi et al., 2014).

To date, few reports exist about the life history and culture trials on most snooks. Among the 12 snook species from the genus *Centropomus*, the common snook and fat snook *Centropomus parallelus* are considered to be the most likely candidates

for aquaculture production possessing features such as fast growth, efficient food conversion ratios (FCRs) and the potential for high biomass yields per unit volume in nursery and grow-out systems (Alvarez-Lajonchère and Tsuzuki, 2008). Early trials to improve hatchery production of common and fat snook began by investigating the use of techniques successfully developed for the mass culture of barramundi *Lates calcarifer*, a phylogenetically close relative and former member of the Centropomidae family. Although significant interest in developing mass culture capabilities for snook began in the 1970's, efforts to identify reliable protocols for commercial scale farming are ongoing (Alvarez-Lajonchère and Tsuzuki, 2008; Muller and Taylor, 2012).

Initial success in breeding common snook relied on strip spawning. This involved the transportation of wild adults from the field to the laboratory followed by immediate hormonal induction of females with human chorionic gonadotropin; males were euthanized, and the testes were removed to collect milt (Neidig et al., 2000). In 2006, after more than 20 years of research trials at laboratories in Florida, Texas, Mexico and Brazil, wild-caught common snook broodstock were successfully matured and spawned volitionally using photothermal conditioning and hormonal induction (gonadotropin releasing hormone analogue, GnRHa) at Mote Aquaculture Research Park in Sarasota, Florida, USA. Despite this breakthrough, major reproductive bottlenecks involved in spawning common snook broodstock persist (Muller and Taylor, 2012). These include: 1) reduced milt production (≤ 100 µl) among captive maintained male broodstock when compared to males sampled from the wild population; 2) variable fertilization rates (20 to 90%) among spawns (Rhody, personal communication); 3) the failure of female broodstock, maintained under natural temperature (28°C) and photoperiod (14 L: 10D) conditions, to ovulate without

hormonal manipulation (Ibarra-Castro et al., 2011). Additional challenges include the sequential hermaphroditism exhibited in Centropomids which must be taken into consideration when establishing broodstock populations (Andrade et al., 2013).

Reliable hatchery fry production remains an essential pre-requisite for achieving large scale production of snook and a number of studies have been performed to improve larval rearing protocols. High mortality rates after the start of exogenous feeding (day 3) have been observed in hatchery trials with both common (Tucker. 1987; Wittenrich et al., 2009; Barón-Aguilar et al., 2013) and fat snook (Cerqueira, 1991; Cerqueira et al., 1995; Seiffert et al., 2001), resulting in < 10% survival by the end of 14 days, when the larvae begin notochord flexion. Some studies have investigated the effects of environmental parameters such as light intensity where 200 to 1500 lx resulted in increased survival for fat snook (Cerqueira and Brügger, 2001). Others showed the use of copepods (*Acartia tonsa*) during the early larval stages reduced mortality when administered alone or in combination with rotifers (Barrosa et al., 2014). Although progress continues to be made, high mortality during early larval rearing remains a bottleneck to the commercialization of common and fat snook.

Despite advances in broodstock spawning and larval rearing technologies, the mass production of common snook fingerlings for the food fish market or fisheries enhancement is still not successful, hence efforts to achieve this are ongoing (Yanes-Roca and Main, 2012). Improvements in our understanding of the reproductive physiology of complex marine fishes (i.e. protandric hermaphrodites like common snook) through the use of hormonal therapies, environmental cues and advanced molecular techniques will provide the tools needed to expand production of high-quality eggs, larvae and fingerlings.

1.2. Gametogenesis in fish

1.2.1. Nomenclature

A critical component for many studies of fish reproductive biology is an accurate assessment of the reproductive condition of individual fish. The importance of understanding reproductive success and population reproductive potential for the design and implementation of effective fisheries management has been summarized by Kjesbu (2009) and Lowerre-Barbieri (2009). In fields such as aquaculture, determining the reproductive condition of captive broodstock is essential for administering hormonal therapies and inducing ovulation and spawning (Mylonas et al., 2010). As the number of fish reproduction studies has proliferated, so has the number of gonadal classification schemes and terms (Brown-Peterson et al., 2011). Dodd's (1986) criticism that "ovarian terminology is confused and confusing" is still relevant today regarding the terminology used to describe reproductive development in females. However, classification terminology for testicular development is equally diverse and inconsistently used (Brown-Peterson et al., 2002; Schultz et al., 2010). Some of the staging schemes use letters (Grier et al., 2009a; Grier et al., 2012), while others use numbers (Núñez and Duponchelle, 2009) to describe and define the development of fish oocytes. The problems encountered using different numbered staging schemes to describe oocyte growth and maturation events in fish are well documented (West, 1990; Patiño and Sullivan, 2002; Brown-Peterson et al., 2011). The lack of generally accepted, standardized and consistent terminology has limited communication among scientists within and across research fields by making it difficult to conduct data comparisons (Grier et al., 2009a; Brown-Peterson et al., 2011). Bromley (2003) highlighted the problem with the terminology used in fisheries literature by citing the

incongruent staging (i.e., based on different numbered stages) used by fisheries biologists for the European plaice *Pleuronectes platessa*. The resulting data for plaice could not be compared within the same fishery, thus confirming the need for validated sampling methods as well as clear and consistent definitions of oocyte growth and maturity (Blazer, 2002). Research has indicated that ovarian germinal epithelia, cellular and structural aspects of folliculogenesis are emerging constants throughout the vertebrates (Parenti and Grier, 2004; Grier et al., 2007), thus indicating an important role for homology in the development of consistent nomenclature. In order to accommodate the diversity of teleost reproduction processes, a tiered scheme is needed; one where 1) concepts and terminology can adapt as new knowledge is generated; 2) is based on important developmental phases that all fish demonstrate within their annual reproductive cycles and 3) one which is applicable to species with differing reproductive strategies (e.g., determinate and indeterminate fecundity). Both Grier et al. (2009a) and Brown-Peterson et al. (2011) have recently addressed this problem by presenting recommendations for adopting a standardized or adaptive terminology, respectively, for describing reproductive development is fishes.

1.2.2. Female gonadal development in teleosts (oogenesis)

Oogenesis describes the morphological and functional processes by which oogonia transform into fertilizable eggs (Grier et al., 2009a). It begins as oogonia proliferate and enter into meiosis becoming oocytes, and ends with the ovulation of eggs (ova) capable of being fertilized (Lubzens et al., 2010). Oogenesis occurs during four periods of germ cell development: Active mitosis, active meiosis, arrested meiosis, and a second period of active meiosis (oocyte maturation) (Grier et al., 2009a). The

process of maturation is essential prior to ovulation with much of the growth of the teleost oocyte occurring during a relatively prolonged period of meiotic arrest. While studies on endocrine, cellular and ultra-structural aspects of oogenesis and oocyte growth (Wallace and Selman, 1981, 1990; Le Menn et al., 2007; Chapovetsky et al., 2007) have been strengthened in recent years with molecular aspects, including large scale transcriptomic and proteomic analyses (Cerdà et al., 2008), this information is still incomplete.

As described previously (Section 1.2.1), there is no set classification system for oocyte and follicular development due to the number of divisions throughout the maturation process varying between studies and species (Brown-Peterson et al., 2011). The staging used to describe ovarian development in common snook was based on histological analysis using a classification proposed by Grier et al. (2009a) where the stages of oogenesis, founded on the universal processes of mitosis and meiosis, include: Oogonial Proliferation, Chromatin Nucleolus, Primary Growth, Secondary Growth (vitellogenesis), Maturation and Ovulation (Table 1.1). Vitellogenesis, used synonymously with secondary growth, is a process in which the oocyte requires the nutritional reserves needed for the development of the embryo; beginning when vitellogenin-derived lipoprotein yolk globules start to accumulate in the ooplasm resulting in substantial growth (increase in size) of the oocyte (Le Menn et al., 2007; Grier et al., 2009a; Lubzens et al., 2010). At this period, the oocyte also accumulates RNA (known as maternal RNA) and completes the differentiation of its cellular and non-cellular envelopes (Lubzens et al., 2010). Additional markers of the oocyte growth process are characterized by the appearance of circumnuclear oil droplets and cortical alveoli in the ooplasm prior to the beginning of secondary growth. Oocyte maturation

(Figure 1.3) is marked by germinal vesicle or nuclear migration, germinal vesicle breakdown or nuclear envelop fragmentation and the resumption of meiosis (Grier et al., 2009a; França et al., 2010). Finally, at ovulation the egg is released from the follicular complex into the ovarian lumen (Lubzens et al., 2010).

Table 1.1 Oocyte development in fish during the reproductive cycle is divided into three levels: periods, stages and steps. The periods have five divisions that are common to all vertebrates and entail the processes of cell divisions, both mitosis and meiosis. The six stages are common to bony fish and vertebrates that produce yolked eggs. The codes, abbreviations for stages and steps are listed on the left of the table. Variable ooplasmic events are added in oocyte maturation, indicated with italic letters, which applies to those fish species whose pelagic eggs possess an oil globule (with permission, Grier et al., 2009a).

CODE	STEPS	STAGES	PERIODS
OP	FORMATION OF CELL NESTS	OOGONIA PROLIFERATION	MITOSIS
CNl	LEPTOTENE	CHROMATIN NUCLEOLUS (CN)	ACTIVE MEIOSIS I
CNz	ZYGOTENE		
CNp	PACHYTENE		
CNed	EARLY DIPLOTENE		
PGon	ONE-NUCLEOLUS	PRIMARY GROWTH, PREVITELLOGENESIS OR UNYOLKED OOCYTES (PG)	ARRESTED MEIOSIS IN LATE DIPLOTENE OF PROPHASE I
PGmn	MULTIPLE NUCLEOLI		
PGpn	PERINUCLEOLI		
PGod	OIL DROPLETS		
PGca	CORTICAL ALVEOLI		
SGe	EARLY SECONDARY GROWTH OR EARLY YOLKED OOCYTE	SECONDARY GROWTH, VITELLOGENESIS OR YOLKED OOCYTES (SG)	
SGl	LATE SECONDARY GROWTH OR LATE YOLKED OOCYTE		
SGfg	FULL-GROWN OOCYTE		
OMegv	ECCENTRIC GERMINAL VESICLE. *OIL DROPLETS COALESCING AND BECOMING ONE GLOBULE*	OOCYTE MATURATION (OM)	
OMgvm	GERMINAL VESICLE MIGRATION TO THE ANIMAL POLE. *OOCYTE HYDRATION*		
OMgvb	GERMINAL VESICLE BREAKDOWN. *OOCYTE HYDRATION ALMOST COMPLETE*		
OMmr	MEIOSIS RESUMES. 2ND ARREST. *OOCYTE HYDRATION COMPLETE*		ACTIVE MEIOSIS II & 2ND ARREST IN METAPHASE II
OV	OOCYTE EMERGING FROM THE FOLLICLE AND BECOMING AN EGG	OVULATION (OV)	

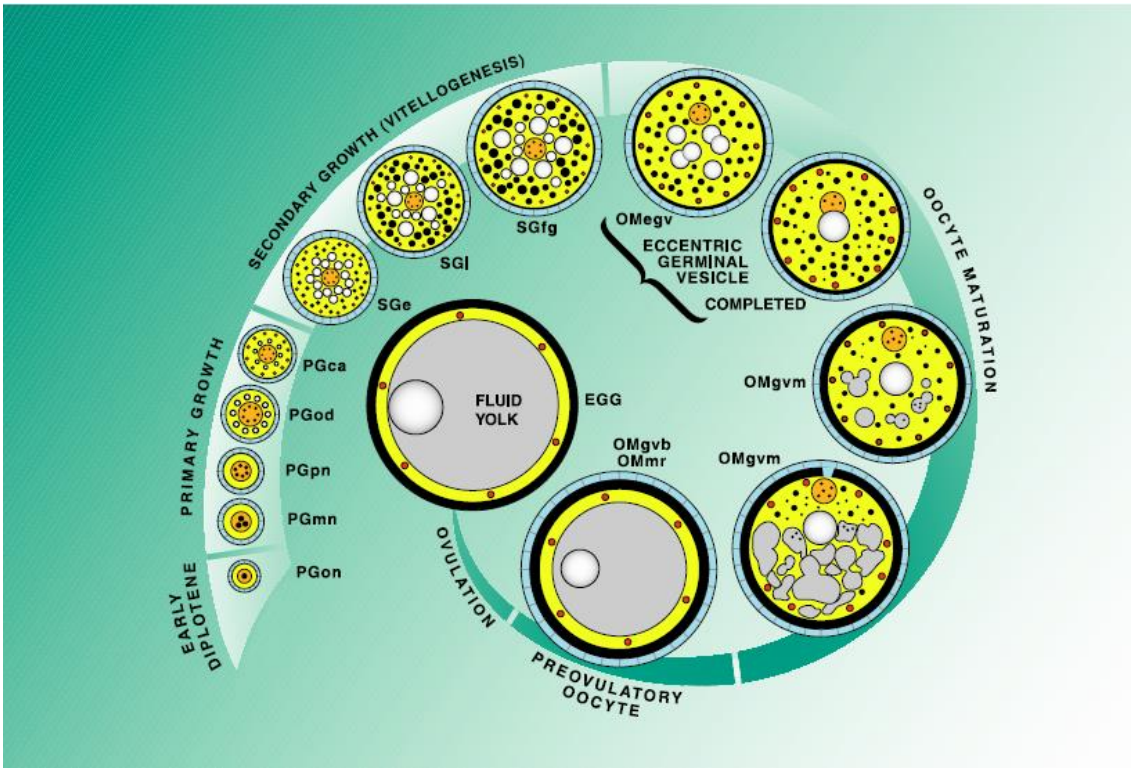

Figure 1.3 A schematic representing oocyte development in fish that produce pelagic eggs. Stages of development are represented around the outside of the spiral while steps are represented in the interior. Primary growth begins when the ooplasm of an early diplotene oocyte, having one nucleolus, becomes basophilic. In common snook the follicle becomes fully encompassed by a basement membrane, in the One Nucleolus Step of the Primary Growth Stage (PGon). The oocyte passes through the Multiple Nucleolus Step (PGmn) and proceeds to the Perinucleolar Step (PGpn). Prior to the initiation of vitellogenesis, oil droplets surround the germinal vesicle (PGod) and then cortical alveoli appear (PGca); these two latter steps are transitory steps just prior to secondary growth. During secondary growth yolk synthesis is ongoing. Early in secondary growth, the yolk globules are small (SGe), but as the oocyte grows some yolk globules reach maximum size and the Late Secondary growth Stage occurs (SGl). With more growth and yolk deposition, an oocyte becomes full grown (SGfg) and capable of responding to the gonadotropin surge, initiating oocyte maturation, the ooplasmic and germinal events that occur prior to ovulation. During maturation, oil globules begin to coalesce and displace the germinal vesicle; it becomes eccentric in position (OMegv). Meanwhile, yolk begins to coalesce at the vegetal pole and germinal vesicle migration ensues (OMgvm). Near the oocyte periphery, the germinal vesicle breaks down (OMgvb), and meiosis resumes (OMmr) followed by a second arrest in metaphase of the second meiotic division. Ovulation ensues. Original.

1.2.3. Patterns of ovarian development in teleosts

In most female teleosts, in terms of morphology, there are two ovaries suspended dorsally within the coelom by a mesovarium (Grier et al., 2009a). In some species, like medaka (*Oryzias laptis*) there is only one ovary which results from both ovaries fusing during embryological development (Strüssman and Nakamura, 2002). Three general patterns of ovarian development have been described in teleosts (Wallace and Selman; 1981; Blazer, 2002; Brown-Peterson et al., 2011): (a) synchronous, where all oocytes develop and ovulate at the same time, (b) group-synchronous, where at least two populations of oocytes can be recognized in the ovary throughout the reproductive season and (c) asynchronous, where a continuum of multiple stages of oogenesis are present simultaneously without a dominant population. Historically, common snook were described as having group-synchronous ovarian development (Taylor et al., 1998; Roberts et al., 1999) however; improvements to our understanding of oocyte histo-morphology suggest they in fact may have asynchronous ovarian development. Further analysis of histological preparations of ovarian biopsies obtained just prior to spawning and additionally at various time points up to 24 h afterward is required to investigate this hypothesis. What is known is that common snook are batch spawners with indeterminate fecundity (Taylor et al., 1998). Batch spawners are capable of spawning single batches of mature oocytes multiple times throughout a protracted spawning season (Brown-Peterson et al., 2011). Indeterminate fecundity indicates oocytes mature to vitellogenesis (yolk formation) throughout the spawning season, as opposed to determinate fecundity where the number of mature oocytes is fixed and are present in the ovary prior to the spawning season (Murua and Saborido-Rey, 2003; Brown-Peterson et al., 2011; Lowerre-Barbieri et al., 2011).

1.2.4. Male gonadal development in teleosts (spermatogenesis)

Spermatogenesis is a highly organized and coordinated process. It refers to the development of spermatogonial stem cells into mature spermatozoa and involves a process in which a small number of diploid spermatogonial stem cells produce a large number of highly differentiated spermatozoa carrying a haploid, recombined genome (Schultz et al., 2010) (Figure 1.4).

Morphologically, the teleost testis is classified into two types: tubular and lobular. Tubular testes are defined by germinal compartments that may be parallel to the testis surface, and the tubules typically branch repeatedly and also form anastomosing networks. In lobular testes, the germinal compartments end blindly at the testis periphery (Grier, 1993). Lobular testes are either "restricted" or "unrestricted" with regard to the distribution of spermatogonia; they may be restricted to the termini of lobules or be distributed along the lengths of lobules. During spermiation the spermatozoa are released into the lobular lumen continuing to the efferent ducts. While little information is available on testicular development in Centropomidae fish, this type of testis has been described in common snook and other higher teleosts such as cobia *Rachycentron canadum* and Mozambique tilapia *Oreochromis mossambicus* (Grier and Uribe-Arananzábal, 2009).

In most teleosts, testes are paired elongated organs which join caudally, converging in a central efferent duct system which is open to the urogenital pore. The testes are attached to the dorsal wall of the body by the mesorchium and germinal compartments are formed by germ cells and somatic cells. Germ cells are integrated at all stages of differentiation with a spermatocyst: spermatogonia, spermatocytes,

spermatids and sperm (Wilder et al., 2013). Histological examination shows testicular tissues form two compartments which are separated by a basement membrane: the germinal compartment and the interstitial compartment. The germinal compartment contains spermatocytes and is formed by the germinal epithelium which, in turn is composed of germ cells surrounded by somatic Sertoli cells (Grier et al., 2009b). Sertoli cells provide physical support and factors necessary for proliferation, differentiation and survival of the germ cells (Schulz et al., 2010). The interstitial compartment, integrated by connective tissues, contains nerve fibers, fibroblast cells, blood vessels, smooth muscle cells and Leydig cells (Koulish et al., 2002). Androgens including testosterone (T) and 11-ketotestosterone (11KT), produced in the Leydig cells within the testis, are the dominant sex steroids involved in spermatogenesis in male teleosts (Borg, 1994; Weltzien et al., 2004). Functions of teleost testes are basically conserved regarding the formation of spermatozoa during spermatogenesis, delivery to the efferent ducts during spermiation and the secretion of male sex steroids. Overall, the process of spermatogenesis can be categorized into three stages (Schulz et al., 2010). The first involves the mitotic proliferation of stem cells for the production of more stem cells and their differentiation into spermatogonia. The differentation of spermatogonia into primary then secondary spermatocytes by meiosis occurs in the second stage. In the third stage haploid spermatids differentiate into flagellated spermatozoa in the process known as spermiogenesis. Following these three events, spermiation takes place where the spermatocyts break open releasing spermatozoa into the sperm duct (Almeida et al., 2008; Grier et al., 2009b).

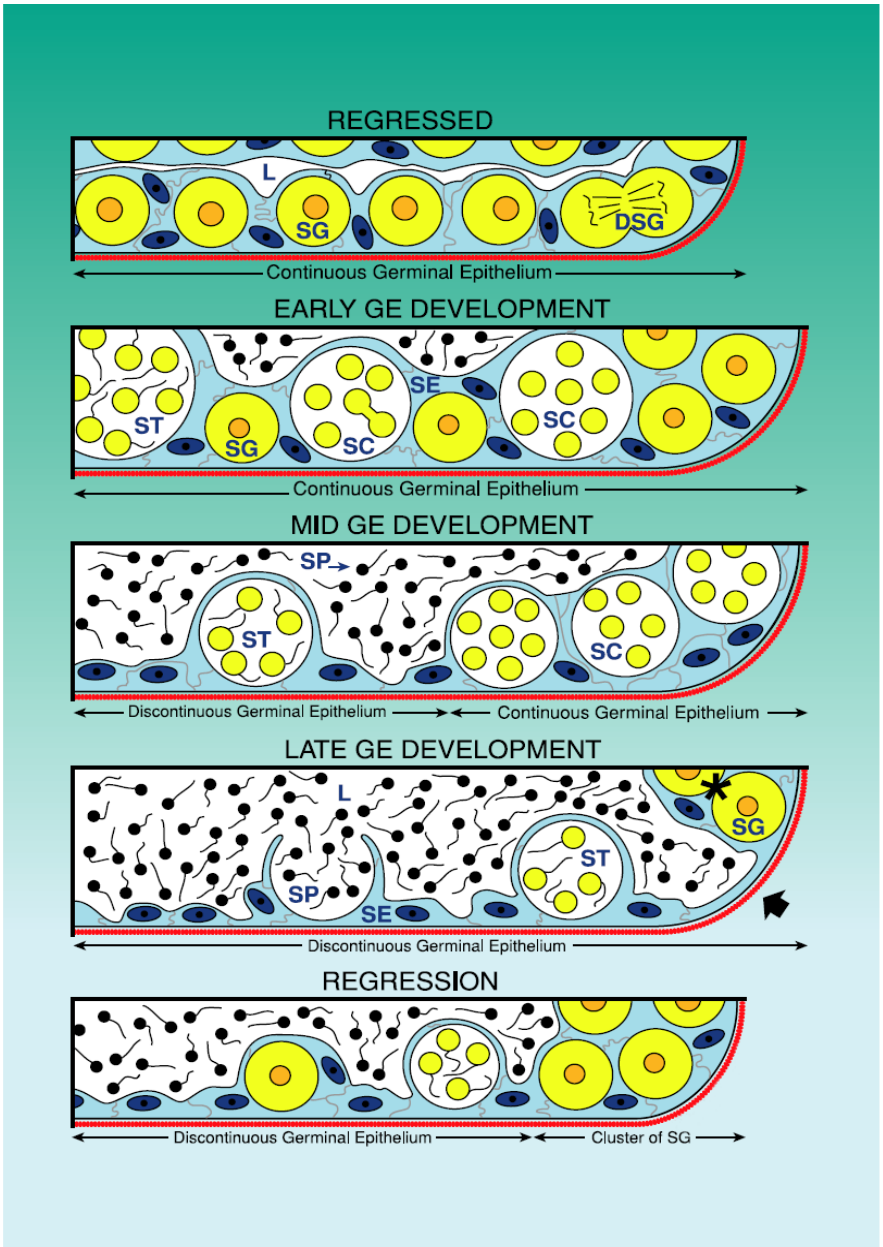

Figure 1.4 Schematic showing changes in testis lobules during spermatogenesis in fish. In regressed fish, lobules contain only spermatogonia that are associated with Sertoli cells. The spermatogonia and Sertoli cells form a continuum, a continuous germinal epithelium that is maintained in Early GE (germinal epithelium) Development when active germ cell divisions begin and spermatocysts form. As lobules grow in length, the germ cell component of the germinal epithelium is lost proximally, forming a discontinuous germinal epithelium, near the ducts signaling Mid GE Development. During the breeding season, and as lobules grow, some germ cells are also lost at the testis periphery, and this is Late Secondary Growth. At the end of the breeding season, regression ensues as sperm are lost due to declining spermatogenesis. Original. Dividing spermatogonia (DSG); Lobule lumen (L); Spermatocytes (SC); Sertoli cell (SE); Spermatogonia (SG); Spermatids (ST), Sperm (SP).

1.3. Neuroendocrine control of reproduction

In fish, as in other vertebrates, it is well known that reproduction is regulated by an intricate network of endocrine, paracrine and autocrine regulatory signals along the brain-pituitary gonadal (BPG) axis (Zohar et al., 2010). Among these, gonadotropin releasing hormone (GnRH) neurons have long been considered as the starting point of the BPG axis although other neuropeptides (e.g. kisspeptin) are clearly involved upstream (Tena-Sempere et al., 2012). Kisspeptin is vital for the neuroendocrine regulation of GnRH secretion and is now recognized as a central processor of a range of inputs that include energy balance signals, photoperiod and steroid feedback (Migaud et al., 2012; Zmora et al., 2012; De Bond and Smith, 2014). Processes along the BPG axis include the production of a series of hormones which are released into the blood circulation, in turn acting on target tissues within the brain, pituitary, liver and gonads to initiate and control sexual development (Zohar et al., 2010). The BPG axis is organized around the hypothalamus of the brain which releases neuropeptides and neurotransmitters that influence the pituitary (gonadotroph cells); which in turn synthesizes and releases gonadotropins (GtHs) including follicle stimulating hormone (FSH) and lutenizing hormone (LH) that are transported through the blood stream to stimulate sex steroid production (androgens, oestrogens and progestagens) in the gonads which act as endocrine signals controlling steroidogenesis, gametogenesis, ovulation and spermiation (Kah and Dufour, 2011; Diotel et al., 2011) (Figure 1.5). Endogenous cues have an influence throughout the system although many of their roles and interaction with the reproductive cascade remain unclear in most fish species. All regulators of the BPG axis integrate with energy and growth pathways such as leptin and growth hormone (IGF-1) to regulate reproductive processes in synchrony with life

stage and the surrounding environment to ensure spawning in the appropriate conditions (Migaud et al., 2010).

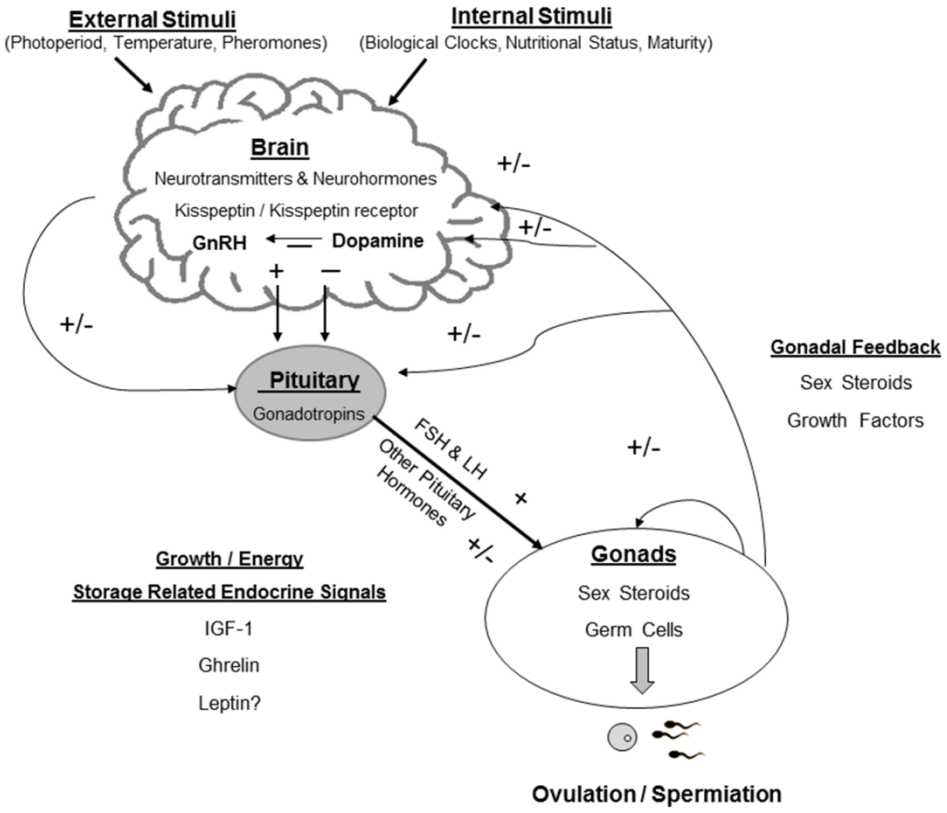

Figure 1.5 Schematic representations of the reproductive axis in fish and its environmental and endocrine control including regulatory pathways. GnRH, gonadotropin releasing hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone; IGF-1, insulin-like growth factor; GH, growth hormone.

1.3.1. Gonadotropin releasing hormone (GnRH)

First discovered in mammals in the early 1970's (Amoss et al., 1971; Matsuo 1971), gonadotropin-releasing hormone (GnRH) has an important role in vertebrate reproduction and thus, the characterization (identification, localization, pharmacology) of the GnRH system has received considerable attention (Chen and Fernald, 2008). GnRH is a decapeptide that is expressed and produced very early in development in olfactory neurons and migrates to three or four brain regions: the preoptic area (POA), the midbrain and the terminal nerve as well as some other areas in the forebrain (Yamamoto, 2003; Sherwood and Adams, 2005). It has a key role in the BPG axis of all vertebrates and, because of its use in fish farming, has consequently been studied in a range of teleosts (Kah and Dufour, 2011). Teleost fish represent the group with the highest number of different GnRH isoforms (Zohar et al., 2010) with data available on a range of species. A total of 24 distinct forms of GnRH have been identified, including eight variants purified and sequenced in teleosts (Table 1.2), the first of which was discovered in salmon (Guilgur et al., 2006; Van der Kraak et al., 2009).

Molecular phylogeny of GnRH ligands shows that there are three distinct forms, GnRH-1, GnRH-2 and GnRH-3, that arose from a common origin (Fernald and White, 1999). Almost all tetrapods investigated have at least two GnRH forms (GnRH-1 and GnRH-2) in the central nervous system. From distributional and functional studies in vertebrates, GnRH-1 neurons are known to be located in the preoptic area of the hypothalamus and project predominantly into the pituitary where they regulate reproduction via release (Schulz et al., 1993; White et al., 2002; Amano et al., 2004). Besides controlling reproductive activity, GnRH-1 is responsible for the release of

growth hormone from the pituitary (Marchant et al., 1989) as well as regulating prolactin. Produced in the midbrain tegmentum, GnRH-2 neurons project throughout the whole brain and have been shown to regulate energy balance and food intake (Temple et al., 2003). GnRH-3 has only been found in teleost fish (Zohar et al., 2010); it is located in the forebrain and known to be involved in sexual behavior (Ogawa et al., 2006) and spawning behavior (Volkoff and Peter, 1999). Taken together, all three GnRHs regulate reproduction however, GnRH-1 is the major form involved in the control of pituitary synthesis and release (Chen and Fernald, 2008). Through the use of isocratic and gradient high performance liquid chromatography (HPLC), the existence of the three forms of GnRH was identified in early work on common snook (Sherwood et al., 1993). These findings are similar to those of other perciform species (Zmora et al., 2002) as well as in most teleost orders (Andersson et al., 2001; Adams et al., 2002; Mohamed et al., 2007).

Table 1.2 Summary of the eight variants of GnRH identified in teleosts showing the amino acid sequence (Van Der Kraak, 2009).

Position	1	2	3	4	5	6	7	8	9	10
GnRH 1 clade										
Mammalian GnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-H2
Pejerrey GnRH	….	….	….	….	Phe	….	….	Ser	….	….
Sea bream GnRH	….	….	….	….	….	….	….	Ser	….	….
Catfish GnRH	….	….	….	….	His	….	….	Asn	….	….
Herring GnRH	….	….	….	….	His	….	….	Ser	….	….
Whitefish GnRH	….	….	….	….	….	….	Met	Asn	….	….
GnRH 2 clade	….	….	….	….	….	….	….	….	….	….
Chicken GnRH-II	….	….	….	….	His	….	Trp	Tyr	….	….
GnRH 3 clade	….	….	….	….	….	….	….	….	….	….
Salmon GnRH	….	….	….	….	….	….	Trp	Leu	….	….

1.3.2. Pituitary gonadotropins

Pituitary gonadotropin secretion is mainly regulated by GnRH, which is in turn controlled by a number of neurotransmitters and neuropeptides, including kisspeptins (Trudeau et al., 2000; Kah and Dufour, 2011; Zmora et al., 2012; Migaud et al., 2012). Hypothalamic nerve fibers branch throughout the adenohypophysis to regulate the synthesis and release of pituitary GtHs; key processes in both the regulation of sex steroid hormones produced by steroidogenic gonadal cells (Sertoli cells in testes and follicular cells in ovary) and the production of gametes in both male and female teleosts (Zohar et al., 2010; Yaron,and Levavi-Sivan, 2011). There are two main forms of GtHs in teleosts as in most other vertebrates (Redding & Patiño, 1993) known as follicle stimulating hormone (FSH, referred to in the past as GTH I) and luteinising hormone (LH, referred to in the past as GTH II). In addition to FSH and LH, thyroid-stimulating

hormone (TSH) is also important. All three are structurally related proteins and members of the same glycoprotein family. Each consists of a common α subunit whereas, FSH and LH have a hormone specific β-subunit (*fsh*β and *lh*β) which binds and activates specific receptors (FSH receptor (FSHR) and LH receptor (LHR)) localized in the membrane of mainly gonadal target cells (Levavi-Siven et al., 2010).

Studies of seasonal changes in *fshβ* and *lhβ* mRNA expression in the teleost pituitary have revealed different patterns of expression among different species (reviewed in Levavi-Sivan et al., 2010). This is often attributed to the phylogenetic diversity associated with the range of reproductive strategies found in fish (Kah and Dufour 2011; Sambroni et al., 2013). In some batch spawning fish species, *fshβ* and *lhβ* (and their receptors) mRNA expression increase simultaneously while oocytes are developing at different rates to reach a peak at spawning as shown in European sea bass *Dicentrarchus labrax* (Migaud et al., 2012), Atlantic cod *Gadus morhua* (Mittelholzer et al., 2009a and b; Almeida et al., 2011; Cowan et al., 2012) and Atlantic halibut *Hippoglossus hippoglossus* (Weltzien et al., 2003; Kobayashi et al., 2008). Research has shown that both *fshβ* and *lhβ* fluctuate in parallel suggesting that both GtHs are probably actively involved in the regulation of all processes of the reproductive cycle. These species exhibit group-synchronous oocyte development where several generations of oocytes are present simultaneously, each at a different stage of development (Wallace and Selman 1981; Brown-Peterson et al., 2011). In this model synthesis of both FSH β and LH β subunits are most likely to be required for different stages of vitellogenesis as well as for oocyte maturation which occur simultaneously (Yaron et al., 2003). In contrast, in synchronous spawners (whereby all oocytes develop and ovulate simultaneously) like some salmonids, FSH synthetized in the pituitary and

released into the blood stream is generally considered to regulate early phases of gametogenesis, such as vitellogenesis and spermatogenesis, whereas LH would be involved in the regulation of oocyte maturation, ovulation and spermiation (Yaron and Sivan, 2006). Mateos et al. (2003) reported a similar correlation to gonadal development in sea bass concluding that pituitary expression of both *fshβ* and *lhβ* is an accurate representation of pituitary protein abundance. Homologous immunoassays for both FSH and LH have only been developed for a few salmonids (Suzuki et al., 1988; Govoroun et al., 1998), tilapia (Aizen et al., 2007) and more recently sea bass (Molés et al., 2008) therefore, restricting measurements to LH in most other species, a fact that has hampered studies on the functional duality of FSH and LH in fish (Molés et al. 2011).

In summary, gonadotropins are the key factors regulating gonadal maturation therefore determination of pituitary and plasma levels of these hormones is an important tool when investigating fish reproductive physiology. Despite their importance, to the author's knowledge, no research to date has described the sequences or characterized the expression of *fshβ* and *lhβ* in common snook or any other member of the Centropomidae family during their reproductive cycle.

1.3.3. Sex steroids and the control of gametogenesis

The BPG axis ends with the production of sex steroids at the gonadal level (Zohar and Mylonas 2001). There are three main types of steroids: oestrogens, androgens, and progestagens. In addition to their role in gametogenesis and secondary sexual characteristics, sex steroids are of prime importance in the feedback control of reproductive development and provide an indicator to the brain and pituitary as to the

reproductive status of the individual (Diotel et al., 2011). In females 17β-estradiol (E_2) and testosterone (precursor of 17β-estradiol) have important roles in reproduction. As ovarian recrudescence begins E_2 is secreted by the ovarian follicle and stimulates the hepatic synthesis and secretion of vitellogenin which is taken up by the oocyte (Kime, 1993). Androgens including testosterone (T) and 11-ketotestosterone (11KT), produced in the Leydig cells within the testis, are the dominant sex steroids involved in spermatogenesis and spermiogenesis in male teleosts (Schulz et al., 2010). Both the brain and pituitary have been found to contain high densities of estrogen and androgen receptors (Blázquez and Piferrer, 2005). Whether feedback is positive or negative depends on the physiological status of the individual and the species itself (Zohar et al., 2010). Progestagens have also been shown to play a major role during advanced stages of gametogenesis in both male and female teleosts. In females, progestagens such as 17α, 20β-dihydroxy-4-pregnen-3-one (also called DHP) are important during oocyte maturation, namely germinal vesicle breakdown (GVBD) (Nagahama et al., 1987) which is an essential process prior to ovulation. In male rainbow trout, high plasma levels of DHP have been found during spermiation (Ueda et al., 1984) and it is thought to be involved in the acquisition of sperm motility (essential for fertilisation) by increasing the pH of the sperm duct thus increasing cAMP (cyclic adenosine monophosphate) in sperm and facilitating motility (Miura et al., 1991).

1.4. Environmental regulation of reproductive activity in fishes

The synchronization of recruitment, development and release of gametes is one of the important reproductive events in vertebrates. There are a number of reproductive mechanisms found in fish which are attributed to the species-specific regulation of the endocrine system that allows gametogenesis to be initiated and completed at the appropriate timing (Yamahira, 2004). In recent years, there has been an increasing scientific effort to understand circadian and seasonal environmental cues and their molecular basis in a variety of marine organisms (Naylor, 2010); however, the events which entrain the initiation and synchronization of reproduction in fishes still remain largely unknown. Of particular importance in fishes are the role of the environment in sexual differentiation and the diversity of reproductive strategies. Research has shown that in temperate fish species, the brain pituitary gonad (BPG) axis is entrained by external cues such as seasonal variation in environmental conditions including mainly photoperiod and temperature (Bromage et al., 2001; Migaud et al., 2010; Migaud et al., 2013). There are however, a number of additional important environmental factors showing daily and annual variations like temperature, rainfall, lunar phase and food supplies that alone or in combination may entrain the biological rhythms of living organisms (Yamahira, 2004; Naylor, 2010). In the first studies describing successful spawning in captive common and fat snook, broodstock were held under natural temperature and photoperiod conditions (Ibarra-Castro et al., 2011; Cerqueira and Tsuzuki 2009). To date, limited information is available on the use of artificial photothermal regimes for the control of reproduction in many Centropomidae fishes.

1.4.1. Photoperiod

Of all the suggested environmental factors, photoperiod, described as the daily changes in light intensity alongside seasonal changes in day length, seems to provide the most constant signal in most environments (Bromage et al., 2001; Migaud et al., 2010). These inputs are translated into neurological signals that transmit information to target tissues eliciting specific physiological responses (Zohar and Mylonas, 2001). Photoperiod manipulation, through the application of artificial lighting, can therefore be used to control the timing of seasonal reproductive patterns in fish and is now widely adopted in aquaculture to alter spawning season, delay or inhibit maturation during ongrowing and stimulate growth (Pankhurst and Porter, 2003; Rad et al., 2006; Taranger et al., 2010).

Early puberty is a major problem in many farmed fish species due to negative effects on growth performance, flesh composition, external appearance, behaviour, health, welfare and survival, as well as possible genetic impact on wild populations. Late puberty can also be a problem for broodstock management in some species, while some species completely fail to enter puberty under farming conditions. For example, the use of artificial lighting has been used routinely to delay or inhibit maturation throughout the salmon farming industry (Taranger et al., 1999, 2010). In some species, a delay, or complete failure of puberty, rather than precocious puberty, causes problems under farming conditions preventing reproduction and closure of the life-cycle, such as in the European eel (Dufour et al., 2003; van Ginneken et al., 2007). Additionally, groupers and tunas can take many years until puberty starts, increasing costs and risks since potential broodstock has to be maintained for prolonged periods of time until

maturation occurs. In such cases, an advancement of reproduction, will improve the costs-efficiency of the fish farms.

1.4.2 Temperature

In addition to photoperiod, temperature is considered to be an important environmental regulator of a variety of process in fishes. In species which spawn seasonally, the later stages of gonadogenesis (external vitellogenesis onward) were shown to be particularly sensitive to thermal stress (King and Pankhurst, 2003). For example, physiological processes related to both somatic and gonadal development would act as a permissive factor of both male and female gametogenesis as shown by a higher rate of oocyte growth and plasma sex steroid level in males under increasing (higher) water temperature (Pankhurst and King, 2010). In salmonids, water temperature has been shown to be an ultimate factor determining the optimal time for reproduction (Taranger et al., 2010). Although the role of temperature may be less critical in tropical species due to nominal seasonal photo-thermal changes in the natural habitat, studies have confirmed these exogenous cues can still impact reproductive timing and success as show in spiny damsel fish (Hilder and Pankhurst 2003).

1.4.3. Lunar periodicity

The lunar or semilunar-synchronized reproductive cycle has been seen in a wide variety of organisms, particularly those living in shallow waters and reef areas. While most research has focused on photo-thermal requirements of temperate fish species,

other environmental signals like lunar and tidal cycle are involved in the entrainment of reproduction in tropical and sub-tropical fish species (Yamahira 2004). This is due to the relatively small seasonal changes in temperature and photoperiod which occur in the tropics (Naylor, 2010). The importance of lunar cycle, in particular, on fish reproduction has been reported in a range of fish species (Takemura et al., 2004; Ando et al., 2013). In rabbitfishes, from the family Siganidae, studies showed oocytes develop synchronously in the ovaries with mature gametes being released with species-specific lunar periodicity during the reproductive season (Rahman et al., 2003; Takemura 2004; Park et al., 2006). A similar trend was observed where seasonal reproductive and lunar-related spawning cycles were documented in the honeycomb grouper *Epinephelus merra* (Lee et al., 2002). Grass puffer *Takifugu niphobles* were found to spawn in semilunar cycles during the spring tide and exhibit similar spawning behavior to snook where fish have been observed aggregating to certain near shore locations several hours before high tide with spawning occurring 1-2 h during the rising tidal phase (Ando et al., 2013). In the threespot wrasse *Halichoeres trimaculatusi,* the significant role of tidal cycle in gonadal development and spawning was demonstrated where daily spawning reportedly peaked around daytime high tides (Takemura et al., 2008). The importance of the lunar cycle has also been demonstrated in temperate fishes. Studies investigating the effect of the moon light cycle on plasma melatonin rhythms in Senegalese sole (*Solea senegalensis*) showed they could be using melatonin signaling to synchronize their reproduction rhythms to the lunar cycle (Oliveira et al., 2010). In the same study, the lunar cycle was found to influence sex steroids levels in males and females, which remained elevated during a fullmoon and significantly lower during a newmoon (Oliviera et al., 2010).

1.5. Hormonal manipulation of oocyte maturation and spermiation

The control and synchronization of reproduction in captive fishes constitutes the first prerequisite to attain sustainable commercial aquaculture production, and in many species this can be achieved by manipulating photoperiod and water temperature (Migaud et al., 2013). However, the biology of some fish requires external cues that may be impractical or even impossible to simulate under captive culture conditions (i.e. spawning migration, depth, tidal fluctuation, etc.). In these species, the absence of adequate environmental cues and the stressors imposed by confinement can directly impact the neuroendocrine regulation of gametogenesis and therefore, the application of exogenous hormonal treatments are routinely used as an effective method to induce spawning (Mylonas et al., 2010).

Almost all fish reared in captivity (either wild or cultured) exhibit some form of reproductive dysfunction (Hunter and Donaldson 1983; Mañanós et al., 2008). These range from the complete absence of reproductive development as observed in freshwater eel (Kagawa et al., 2005), to the absence of only gamete release. Most commonly, females fail to undergo oocyte maturation and thus, ovulation and spawning, while males produce reduced volumes of milt, or milt of low quality (Bobe and Labbé, 2010). In aquaculture, the most common strategy to stimulate gonad maturation, ovulation and spermiation in captive fish has typically involved treatment with either exogenous luteinizing hormone (LH) preparations, human chorionic gonadotropin (HCG), or gonadotropin releasing hormone agonists (GnRHa), which deliver the hormone over a period of days or weeks (Mylonas and Zohar, 2001; Zohar and Mylonas, 2001). Although a delivery system for LH has been reported (Sato et al., 1995), the ones utilized most commonly in aquaculture contain almost exclusively

GnRHa and are prepared in the form of either injectable biodegradable microspheres (Mylonas and Zohar, 2001) or implantable cylindrical pellets of cholesterol (Weil and Crim, 1983) or ethylene-vinyl acetate (EVAc) (Mylonas et al., 2007). The most classical administration method for GnRH is by injection. Controlled-release delivery systems for reproductive hormones (mainly GnRHa) produce a long-term elevation in plasma gonadotropins, thus providing a better stimulation of oocyte maturation and spermiation, resulting in gametes of better quality, especially in fish with asynchronous ovarian development.

In aquaculture, treatment with GnRHa-delivery enhanced gamete production in a number of commercially important species including European sea bass *Dicentrarchus labrax* L. (Forniés et al., 2001; Prat et al., 2001), gilthead seabream *Sparus aurata* L. (Barbaro et al., 1997), turbot *Scolphtalmus maximus* (Muginer et al., 2000) and newer aquaculture species such Senegalese sole *Solea senegalensis* (Guzmán et al., 2009). Studies have also shown hormonal induction with GnRHa increased volume and quality of male milt in a variety of freshwater and marine species including white bass *Morone chrysops* (Mylonas et al., 1997); Atlantic cod *Gadus morhua* (Garber et al., 2009); greenback flounder *Pleuronectes ferrugineus* (Lim et al., 2004) and Senegalese sole *Solea senegalensis* (Guzmán et al., 2011).

Despite the success of GnRHa treatments among a variety of fish species, these have sometimes been shown to be ineffective when used alone. This is due to a strong dopaminergic inhibition of gonadotropin secretion leading to incomplete or arrested gametogenesis at the later steps of oocyte maturation in females and spermation for males (Dufour et al., 2010). In some, but not in all teleosts species, dopamine exerts

inhibitory actions on both the brain and pituitary through reduction of GnRH synthesis and release, down-regulation of GnRH receptors and interference with the GnRH signal transduction pathways, thus affecting secretion from the pituitary (Chang et al., 1984). In the European eel *Anguilla anguilla* L., which remains at a prepubertal stage as long as the oceanic reproductive migration does not occur (Dufour *et al*., 2003), removal of dopamine inhibition was shown to be required for triggering gonadotropin release and ovarian development. Further, the inhibitory effects of dopamine have been demonstrated mainly in freshwater fish such as Nile tilapia *Oreochromis niloticus* (Levavi-Sivan et al., 2006) and goldfish *Carassius auratus* (Trudeau, 1997) but only recently documented in marine fishes (Guzmán et al., 2011). The most effective strategy for spawning induction in cyprinids and silurids required using a combination of GnRH agonist and dopamine antagonists such as pimozide or domperidone to remove or reduce the inhibition on gonadotroph cells and stimulate release of the GtHs e.g. follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Zohar and Mylonas, 2001; Zohar et al., 2010). An overview of the application of hormonal manipulations with GnRHa for the induction of maturation and spawning in various fish species are presented in Table 1.3.

Table 1.3 Application of hormonal manipulations with agonists of gonadotropin releasing hormone (GnRHa) for the induction of maturation and spawning in female (F) and male (M) broodstocks (Taken from Zohar and Mylonas, 2001).

Species	Common name	Sex	GnRHa[a]	Other Treatment[b]
Anoplopoma fimbria	sablefish	F	A	
Aristichthys nobilis	bighead carp	F	A	
Carassius auratus	goldfish	F	A,T	Pim
Chanos chanos	milkfish	F	A,R	
Clarias batrachus	Asian catfish	F	L	Dom
Clarias macrocephalus	catfish	F	A	Pim
Clupea harengus	Pacific herring	F	A	
Cynoscion nebulosus	spotted seatrout	F	A	
Cyprinus carpio	common carp	F	R	Dom, Met
		M	A	
Dicentrarchus labrax	European seabass	F	A	
Epinephelus striatus	Nassau grouper	F	A	
Lates calcarifer	barramundi	F	A	
Mugil cephalus	grey mullet	F	A	PiT
Mylopharyngodon piceus	black carp	F	A	Res, Pim
Heteropneustes fossilis	catfish	F	L	
Hypophthalmichthys molitrix	silver carp	F	A	Pim
Oncorhynchus kisutch	coho salmon	F	A	
O. mykiss	rainbow trout	F	T	
O. nerka	sockeye salmon	M /F	A	
Oreochromis spp.	tilapia hybrid	F	T	Dom, Met
Parabramis pekinensis	bream	F	A	Pim
Paramisgurnus dabryanus	loach	F	A	Dom, Pim
Perca flavescens	yellow perch	M / F	A	
Salmo salar	Atlantic salmon	M	A	
S. trutta	brown trout	F	A	
Salvelinus fontinalis	Artic charr	F	R	Pim
Sciaenops ocellatus	red drum	F	A	
Solea solea	common sole	F	A	
Sparus aurata	gilthead seabream	F	A	
Stizostedion vitreum	walleye	F	A	

[a]A = $_{D}$-Ala^{6} Pro^{9} NEt-mGnRH; L = $_{D}$-Lys^{6}-sGnRH; R = $_{D}$-Arg^{6} Pro^{9} Net-sGnRH; T = $_{D}$-Trp^{6}-mGnRH.

[b]Combination treatment with a dopamine antagonist (Pim = pimozide, Dom = domperidone, Met = metoclopramide, Res = reserpine) or a pituitary extract (PiT.)

1.6. Experimental Aims

Advances in aquaculture technologies are being investigated to support the replenishment of local fisheries, develop marine food fish farming opportunities and to increase seafood production globally. In order to promote the expansion and development of aquaculture technologies required to raise new finfish species, a number of key bottlenecks restricting commercial-scale culture need to be addressed including the ability to control fish reproduction in captivity and to produce high quality seeds. One candidate species for large-scale production and the focus of this work is common snook. As one of the three most popular inshore sport fish in Florida, common snook have long since been an economically important part of Florida's saltwater recreational fishing industry. Their popularity, however, has a downside: fishing pressures have placed snook on the state's list of "species of special concern" and resulted in the need for fishing restrictions and careful monitoring. Bag limits and limitations on size and seasons for snook harvest have been implemented and a sizeable investment has been apportioned for fisheries managers to develop an effective marine fish stocking technology for rapid restoration of depleted stocks. Despite recent advances in captive spawning, a number of reproductive bottlenecks still need to be addressed including the failure of females to ovulate without hormonal manipulation; reduced milt production in males; inconsistent supply of high quality eggs; high incidence of larval mortality and limited knowledge of nutritional requirements.

In summary, more detailed scientific knowledge is required regarding the photo-neuroendocrine control of reproduction in temperate tropical fish, like common snook, to better enable reliable control of reproduction under captive conditions and to

maximize the production of fingerlings either for fisheries enhancement or commercial-scale food fish production.

The specific objectives of this thesis were as follows:

1. To describe oocyte development in common snook and validate a noninvasive method for assessing reproductive condition in wild and captive stocks (**Chapter 2**).

2. To study the effects of the dopamine antagonist pimozide and different sustained release GnRHa implants on milt characteristics and plasma levels of gonadal steroids in male common snook *Centropomus undecimalis* broodstock (**Chapter 3**).

3. To determine parental contribution to single (daily) batches of fry produced by a mass spawning broodstock tank (**Chapter 4**).

4. To confirm the role of tidal pattern on the synchronization of oocyte maturation and spawning in common snook and to determine the chain of endocrine events that support it (**Chapter 5)**.

5. To utilize a combination of digital photography and histological techniques to document embryonic and early larval development in common snook to gain a better understanding of their early ontogeny and refine hatchery production techniques accordingly (**Chapter 6**).

CHAPTER 2

RESEARCH ARTICLE

ASSESSING REPRODUCTIVE CONDITION IN CAPTIVE AND WILD COMMON SNOOK STOCKS: A COMPARISON BETWEEN THE WET MOUNT TECHNIQUE AND HISTOLOGICAL PREPARATIONS

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Keywords: *Centropomus undecimalis*; oocyte maturation; spawning; broodstock

Abstract

We describe oocyte development in common snook *Centropomus undecimalis* and, secondarily, present results from a comparison of the wet mount technique with histological preparations of ovarian biopsies. Potential differences in ovarian development between wild and captive broodstock were investigated. Results showed that mean oocyte diameter (µm) was not statistically different between the two groups or within each defined stage and step of reproductive condition. Histological preparations were used to validate the wet mount technique as a rapid, yet accurate, low-cost alternative for assessing reproductive condition in common snook. When compared with histology, the wet mount provided a precise method for determining whether female broodstock were candidates for hormone induction in aquaculture applications. However, due to the loss of fine resolution, it was not possible to identify cortical alveoli, oocyte atresia, and postovulatory follicle complexes by using the wet mount technique. Despite these limitations, findings from this study indicate that the wet mount technique may have applications in fishery biology as a noninvasive method for assessing reproductive condition in wild fish stocks.

1. Introduction

A critical component for many studies of fish reproductive biology is an accurate assessment of the reproductive condition of individual fish. There are numerous macroscopic and microscopic methods for evaluating the gonadal condition of fish, particularly with regard to ovarian development. West (1990) reviewed some of these in detail and discussed the level of accuracy and usefulness for each. The methods in use range from histology, which can be very accurate yet time consuming, to more rapid but less certain visual evaluation based on whole gonad appearance (Hunter and Macewicz, 1985). Other methods include assessments based on the measurement of oocyte size and gonad indices (Honji et al., 2006). One additional method, the wet mount technique, assigns reproductive stage based on microscopic visual inspection of whole oocytes obtained from ovarian biopsies (Neidig et al., 2000; Grier, 2009). In the fields of aquaculture and fisheries, a variety of these methods can be used to answer important questions about when fish have spawned or are preparing to spawn.

Fishery biologists typically gather information on fish reproductive status by sacrificing fish to score whole ovaries (García-Díaz et al., 1997). During ecological assessments, a combination of histology and gross morphological evaluations of whole gonads is used to answer questions about fecundity, size at maturity, spawning frequency, and overall reproductive health of fish stocks (Hunter and Macewicz, 1985; West 1990). An understanding of fish reproductive success and population reproductive potential is critical for designing and implementing effective fisheries management strategies (Lowerre-Barbieri, 2009). In fields such as aquaculture, determining the reproductive condition of captive broodstock is important for

administering hormonal therapies and inducing ovulation and spawning (Mylonas et al., 2010). Determinations of oocyte development are often based on macroscopic visual inspection, which involves documenting the color and general appearance of oocytes collected from ovarian biopsies (Honji et al., 2006). In addition, microscopic methods like the wet mount technique have been utilized for quick, low-cost oocyte staging (Neidig et al., 2000; Grier and Neidig, 2011). Unlike in fisheries applications, aquaculture assessments of gonadal condition must be performed at the time of the ovarian biopsy and cannot be hampered by the delay of histological processing. The fields of aquaculture and fisheries require the same information but on different temporal scales and with different levels of precision.

Although histology is recognized as the most accurate method for examining reproductive status in fish populations, it often requires sacrificing individuals for evaluation (West, 1990). This is not optimal in cases where (1) fish stocks are under conservation management, with limited numbers available for sampling; or (2) fish have a high commercial value, such as broodstock that are used for spawning in aquaculture facilities. The common snook *Centropomus undecimalis* is an example of a species for which sacrificing fish to assess reproductive condition is not ideal. The common snook is a high-priority species for conservation as it is both ecologically and economically important in select regions of North America and South America (Alvarez-Lajonchère and Tsuzuki, 2008; Winner et al., 2010). Along the Gulf coast of the United States, common snook are part of a popular recreational fishery. However, factors such as environmental change (cold kills), habitat destruction and overfishing have left the stocks vulnerable to decline. Recent findings highlighting changes in common snook stock abundance and recruitment have prompted the Florida Fish and

Wildlife Conservation Commission to regularly assess the condition of wild stocks (Muller and Taylor, 2012). From a fisheries management perspective, noninvasive techniques for assessment of fish reproductive condition and spawning potential would be beneficial for common snook as well as for other species. One such technique, the wet mount, has been used for a number of years by fisheries biologists and aquaculturists as an accurate, low-cost alternative to histology. There is a variety of forms of the wet mount technique and staging, but not all forms have been properly validated for accuracy (Patiño and Sullivan, 2002; Kjesbu, 2009). Therefore, the aims of our study were to describe oocyte development in common snook and, secondarily, to evaluate the wet mount technique of staging ovarian development in comparison with histological techniques.

2. Materials and Methods

2.1 Broodstock systems

In 2009, wild adult common snook were caught and transported to Mote Aquaculture Research Park, Sarasota, Florida. Collected broodstock were divided between two separate, indoor, photoperiod- and temperature- controlled recirculating tank systems (tanks A and B). Tank A contained 22 males and 11 females, whereas tank B contained 18 males and 15 females. Each system consisted of a 4.57 m diameter fiberglass tank with a total system volume of 28 m^3. Temperature was controlled ($\pm 1^{\circ}$ C) in each tank by cycling water through an individual heater/chiller unit. The filtration system included a 0.085 m^3 drop filter (Aquaculture Systems Technologies), a 900-L moving bed for biofiltration, a protein skimmer, and an ultraviolet light sterilization unit. Salinities were maintained at 35‰.

2.1 Broodstock sampling

To sample the broodstock, the tank water level was lowered and, by using two dividers made from plastic mesh stretched across a polyvinyl chloride pipe frame, the fish were gently corralled into a section of the tank. From this restricted section, individual fish were netted into a 500-L tank containing 200-L of water and were anesthetized with tricaine methanesulfonate (MS-222) at a concentration of 300 mg/L. Male and female common snook were then weighed (kg) and measured (FL and TL).

Sixteen broodstock sampling events took place between 2010 and 2012. During each event, a cannulation biopsy was taken from every female (n = 26 fish) by inserting a soft tubing catheter (1.0 mm inside diameter) into the gonoduct and applying a gentle suction (using a 3-mL syringe) to collect an ovarian biopsy. A small portion of the biopsy was prepared for observation as a wet mount by placing the biopsy on a glass slide and covering it with a 22 × 22 mm, number-1 glass coverslip. Oocytes from the biopsy (n = 20 oocytes/biopsy) were immediately staged and photographed. The remaining portion of the biopsy was then placed in Trump's fixative (McDowell and Trump, 1976). For light microscopy, the fixed portion was subsequently embedded in glycol methacrylate and was sectioned at 6 µm on an LKB Bromma 2218 Historange Microtome (LKB Bromma, Sweden). Tissue sections were stained with periodic acid Schiff, metanil yellow, and Weigert's hematoxylin and eosin (Quintero-Hunter et al., 1991). Both wet mounts and histological tissue sections that were collected from individual females at each sampling event were photographed by using an Olympus BX53 microscope fitted with a DP-72 digital camera. Oocytes were measured using Olympus cellSens version 1.3 imaging software.

2.2 Capture and sampling of wild stocks

Wild female common snook ($n = 152$) were captured from April to September over the course of 3 years (2010–2012). All fish were weighed (kg) and measured (TL and FL) at the time of collection. Ovarian biopsies obtained from individual females were prepared as wet mounts and for histology by following the same procedures as described above for captive broodstock. Oocytes were placed on ice in the field and were later staged, measured (n = 20 oocytes/biopsy), and photographed at Mote Aquaculture Research Park within 3–5 h after collection.

2.3 Oocyte staging

For each wild and captive fish, wet mounts of ovarian biopsies were compared with histological preparations of the same biopsy. The wet mount technique and oocyte staging terminology from Grier et al. (2009) were used to identify the reproductive condition (stage and step) of each female and to determine which individuals were suitable for hormonal implantation. The same oocyte staging method was applied to classify the reproductive status of wild females. Maturation and spawning of common snook for aquaculture purposes have only recently been achieved. Consequently, it was important to confirm that oocyte development in fish held under captive conditions was similar to oocyte development in wild fish. Therefore, oocyte development in wild common snook was examined and compared with that of captive individuals. The terminology used for staging oocytes and describing oocyte development in common snook was adapted to the wet mount technique, wherein letters rather than numbers are used to describe oocyte development (Grier et al., 2009; Grier, 2012). In the abbreviations used here, stages are indicated by uppercase letters, whereas their

subdivisions (called “steps”) are indicated by lowercase letters (for example, the preovulatory step within the oocyte maturation stage is referred to as “OMpov”).

2.4 Statistical analysis.

To examine potential differences between wild and captive broodstock, a statistical analysis was performed using SPSS version 12.0 (SPSS, Inc., Chicago, Illinois). Independent sample t-tests were used to detect within stage/step differences in mean oocyte diameter for captive and wild fish; an α of 0.05 was used as the statistical significance criterion.

3. Results

3.1 Oocyte diameter

Wet mount stage and step descriptions and the corresponding mean oocyte diameters are presented in Table 1. In total, 3,040 and 4,780 oocyte diameters (n = 20 oocytes/biopsy) were recorded for wild and captive fish, respectively. Mean oocyte diameter (μm) within each defined stage and step of reproductive condition was not statistically different between wild and captive fish ($P > 0.05$).

3.2 Validation of the wet mount technique

3.2.1 Primary growth

Primary growth (PG) oocytes, which were transparent in wet mounts, were the only oocytes present in biopsies from regressed ovaries of common snook (Figure 1). The PG oocyte stage includes four steps: one nucleolus (PGon; Figure 2a, b); multiple nucleoli (PGmn; Figure 2c, d); perinucleolar (PGpn; Figure 2e, f); and oil droplets

(PGod; Figure 2g, h). The best way to distinguish among the steps of PG in wet mounts and histological preparations is by looking closely at the nucleoli within the germinal vesicle (Figure 2). Another way to clearly identify the steps of PG is by determining the oocyte diameter (Table 1). As PG progressed, the gross oocyte characteristics did not change much, despite an increase in oocyte diameter (Figure 2; Table 1). In captive broodstock, PGon oocytes were the smallest, with a mean diameter of 66.9 µm (SE = 1.2), whereas PGod oocytes were the largest at 159.2 µm (SE = 1.5; Table 1).

Table 1. Stages and steps of common snook oocyte development as they appear when the wet mount technique is used; mean ± SE oocyte diameter (µm) in implanted captive and wild common snook (n = 20 oocytes measured per female) and number of females sampled are also shown.

Stage	Step	Abbreviation	Wet mount stage and step descriptions	Oocyte diameter (captive females)	Oocyte diameter (wild females)	No. females sampled (captive/wild)
PRIMARY GROWTH (PG)	one nucleolus	PGon	Germinal vesicle (gv) has a single nucleolus, ooplasm transparent	66.9 ± 1.2	63.2 ± 1.1	11 / 10
	multiple nucleoli	PGmn	Spherical gv with two or more nucleoli not at the periphery, ooplasm transparent	73.4 ± 0.51	75.6 ± 0.47	43 / 22
	perinuclear	PGpn	Gv nucleoli are peripheral and gv may have undulating outline, ooplasm transparent	87.6 ± 0.61	82.3 ± 0.69	56 / 23
	oil droplets	PGod	Oil droplets located around periphery of gv resolved as a black ring (ring oocyte), ooplasm transparent	159.2 ± 1.5	147.2 ± 1.2	10 / 21
SECONDARY GROWTH (SG)	early	SGe	Some scattered, small yolk globules appearing as clear spheres, still called ring oocytes	231.3 ± 2.9	239.8 ± 2.2	10 / 13
	late	SGl	Oil droplets masked by density of yolk globules, gv a diffuse, clearer area in center of oocyte. Ooplasm dark and granular	356 ± 1.7	335.7 ± 1.6	19 / 10
	full-grown	SGfg	Contains dark granular ooplasm; distinguishable from SGl by oocyte diameter, oil droplets are masked by yolk globles	399.5 ± 1.0	388.2 ± 0.98	60 / 15
OOCYTE MATURATION (OM)	eccentric germinal vesicle	OMegv	Oil globules, with black borders, coalesce until a single, central oil globule is formed displacing the germinal vesicle to an eccentric position	429.0 ± 1.9	422.6 ± 1.7	10 / 10
	germinal vesicle migration	Omgvm	Oocyte hydrates; single oil droplet is generally present, but multiple droplets can occur; germinal vesicle not visible	469.1 ± 4.3	488.3 ± 3.9	10 / 10
	preovulatory	OMpov	Ooplasm cleared (hydrated) with extensions of peripheral ooplasm extending into yolk. Gv has broken down (not visible)	501.8 ± 2.9	522.4 ± 2.4	10 / 18

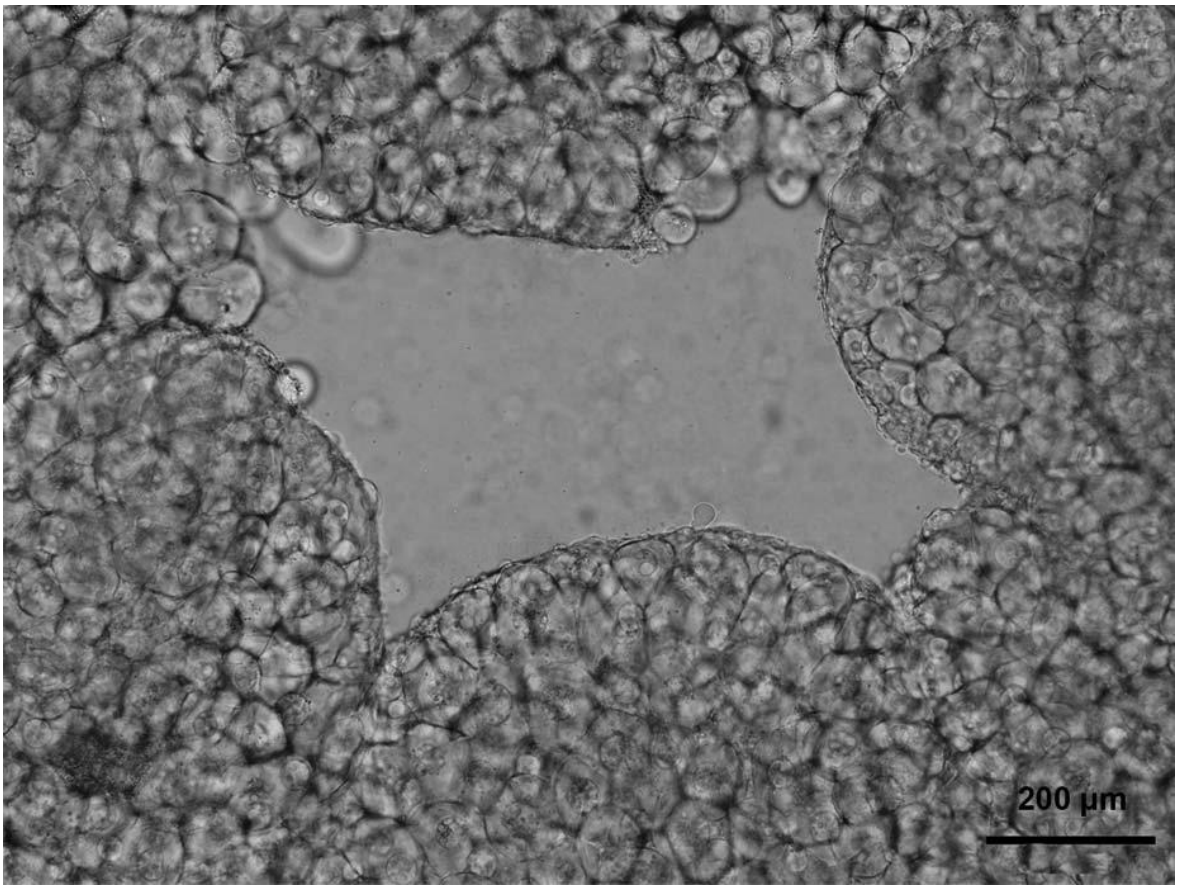

Figure 1. Wet mount technique applied to a regressed ovary of a common snook. The edges of ovarian lamellae are depicted. Numerous transparent, primary growth oocytes in different steps are present.

A number of differences were seen when photographs of PG oocyte wet mounts and histology were compared. In wet mounts, the ooplasm remained clear in PGon, PGmn, and PGpn oocytes (Figure 2a, c, and e). In histological preparations, the ooplasm was stained blue because it is basophilic (Figure 2b, d, and f). Furthermore, PGpn oocytes in the wet mounts (Figure 2e) appeared to have nucleoli randomly scattered within the germinal vesicle, whereas histologically the nucleoli were located around the periphery of the germinal vesicle (Figure 2f).

As oocyte growth progressed from PGpn to PGod, additional details reflecting the lower resolution of wet mounts relative to histological preparations became

apparent, as in the “ring oocyte.” The black ring is composed of numerous small oil droplets that are not individually resolved due to the limited resolution of the wet mount technique (Figure 2g). Using histology, the oil droplets are seen as clear vesicles encircling the germinal vesicle (Figure 2h). Ring oocytes are found at the initiation of oocyte development and are usually scattered among transparent PG oocytes in an earlier step of development. They are indicative of the transition from PG to secondary growth (SG) and are present in both stages.

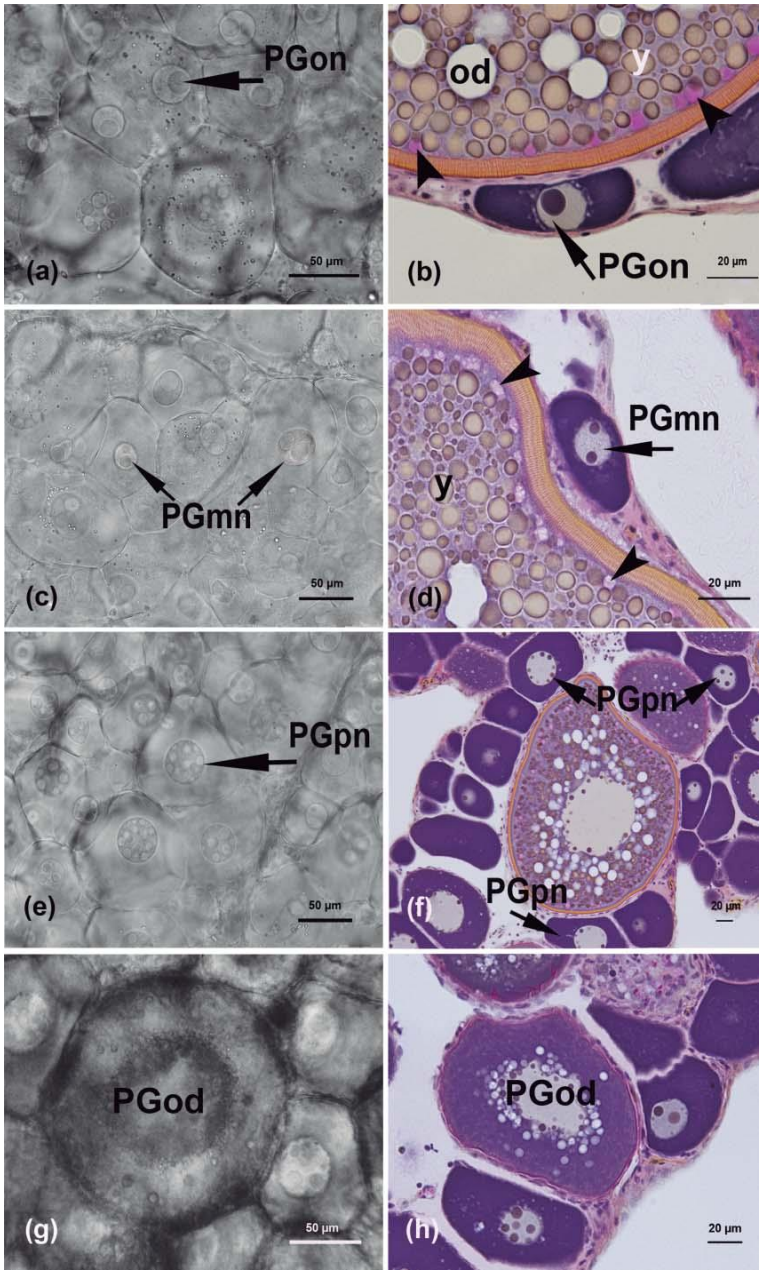

Figure 2. Primary growth (PG) oocytes in ovarian biopsies collected from common snook. Micrographs of wet mounts (a, c, e, and g) and their corresponding histological preparations (b, d, f, and h) are presented: (a) PG stage, one-nucleolus step (PGon) oocytes; (b) PGon oocyte below a vitellogenic oocyte containing oil droplets (od), yolk (y), and periodic acid Schiff positive cortical alveoli (indicated by arrowheads); (c) PG stage, multiple nucleolus step (PGmn) oocytes; (d) PGmn oocyte and a vitellogenic oocyte with yolk (y) and cortical alveoli (arrowheads); (e) PG stage, perinucleolar step (PGpn) oocytes; (f) PGpn oocytes, with a larger, early secondary growth stage oocyte visible in the central part of the micrograph; (g) PG stage, oil droplet step (PGod) oocyte (i.e., "ring oocyte"); and (h) PGod oocyte.

3.2.2 Secondary growth

Secondary growth includes three steps: early (SGe; Figure 3a–c), late (SGl; Figure 3d, e), and full grown (SGfg; Figure 3f, g). Within the SG stage, SGe, SGl, and SGfg can be distinguished from each other by determining the diameter of the oocyte (Table 1). In a spawning-capable female common snook, SGfg oocytes had a mean diameter of approximately 400 µm (Table 1).

As in PG, greater details in SG oocytes were revealed by histology than by wet mounts. Secondary growth commences upon the appearance of yolk in the form of globules scattered throughout the ooplasm (Figure 3b, c); the yolk appears as fine, clear vesicles in the wet mount ring oocytes (Figure 3b). In histological preparations, the yolk is visible as fine, distinctively stained globules in SGe, SGl, and SGfg oocytes (Figure 3c, e, and g). As oocyte growth continues, the accumulating yolk globules cause the ooplasm to become opaque in wet mounts, but they always remain as clear yolk globules over the germinal vesicle (Figure 3f). The SGe and SGl oocytes typically have zonation in the ooplasm, with oil droplets surrounding the germinal vesicle and intermixing with yolk globules (Figure 3c, e, and g). Furthermore, there is a zone of primarily yolk globules that mix with cortical alveoli at the oocyte periphery (Figure 3c, e).

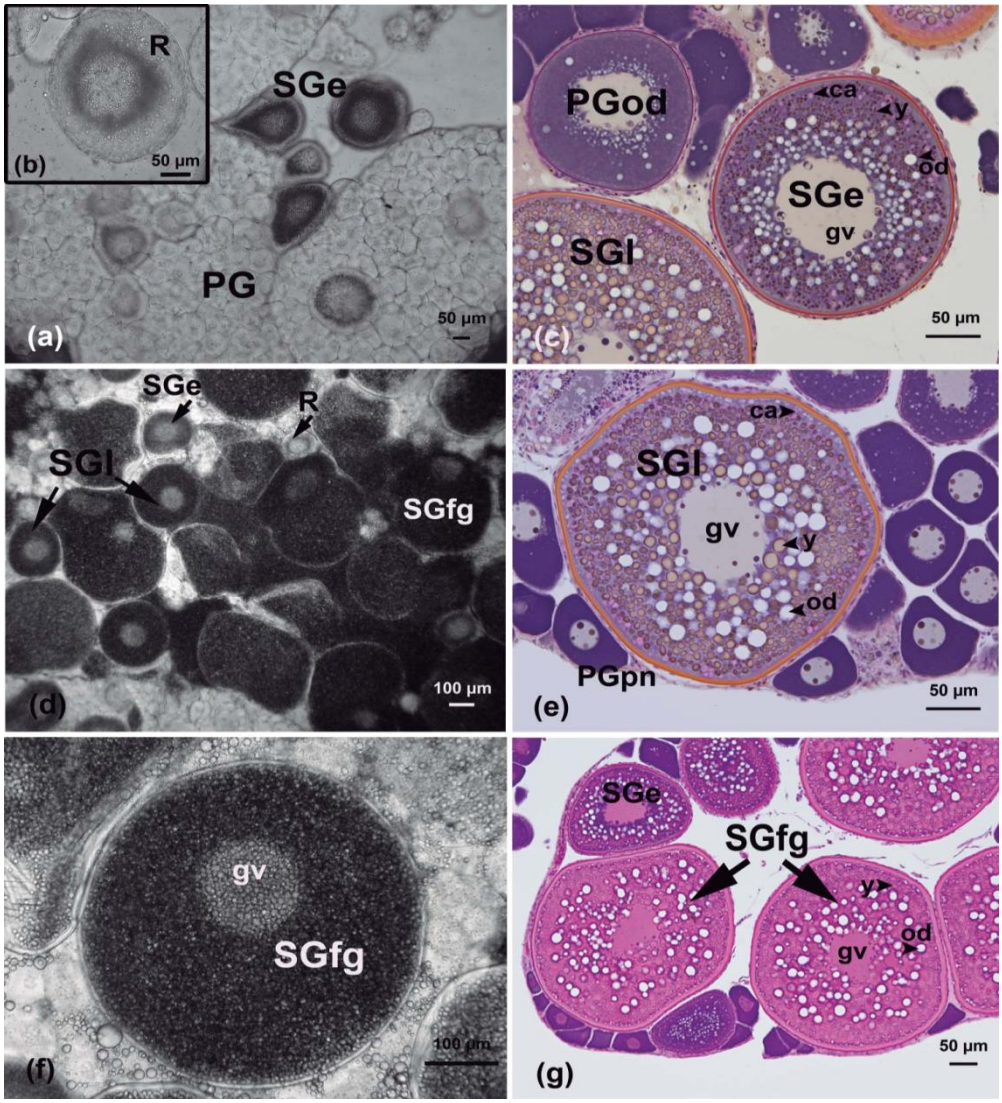

Figure 3. Micrographs of common snook secondary growth (SG) oocytes from wet mount preparations (a, b, d, and f) and corresponding micrographs of histological preparations (c, e, and g) are shown: **(a)** primary growth (PG) oocytes and SG stage, early step (SGe) oocytes, appearing as ring oocytes; **(b)** ring (R) oocyte with clear yolk globules in the ooplasm and over the germinal vesicle; **(c)** a PG stage, oil droplet step (PGod) oocyte, an SGe oocyte (with a well-defined germinal vesicle [gv], cortical alveoli [ca], yolk [y], and oil droplets [od]), and an SG stage, late step (SGl) oocyte (visible in the left corner); **(d)** SG stage, full-grown step (SGfg) oocyte, with R, SGe, and SGl oocytes (nonspherical shape of SGfg oocytes is due to the pressure applied to the coverslip); **(e)** PG stage, perinucleolar step (PGpn) oocytes surround an SGl oocyte with clear oil droplets (od), yolk (y), and peripheral cortical alveoli (ca); **(f)** SGfg oocyte, with the germinal vesicle (gv) present as a clearer area; and **(g)** SGfg oocytes, with central germinal vesicles (gv) and ooplasm containing clear oil droplets (od) intermixed with yolk globules (y), and an SGe oocyte also present.

3.2.3 Oocyte maturation

Oocyte maturation (OM) includes ooplasmic and germinal vesicle changes and the breaking of meiotic arrest prior to ovulation. Oocyte maturation includes three steps: eccentric germinal vesicle (OMegv), germinal vesicle migration (OMgvm), and preovulatory (OMpov; Figure 4). When using the wet mount technique, the first sign of OM (i.e., OMegv) is the coalescence of oil droplets around the central germinal vesicle (Figure 4a). When the oil droplets begin to coalesce at the beginning of OMegv, they are thereafter called "oil globules," a terminology change that separates oocyte growth from OM (Grier et al., 2009). The dark borders of oil globules in wet mounts (Figure 4a) distinguish them from the diffuse border of the germinal vesicle that is located behind numerous spherical yolk globules, as during SG (Figure 4f). The germinal vesicle becomes displaced to an eccentric position during OMegv as the oil globules coalesce centrally. In the wet mount preparation, an eccentric germinal vesicle is the first observed polarity of the oocyte in the developing common snook egg (Figure 4b). Histological preparations also reveal the appearance of fluid yolk at the vegetal pole during OMegv (Figure 4c). By the completion of OMegv, there is a single, central oil globule and the germinal vesicle has become eccentric, being displaced toward the animal pole of the oocyte.

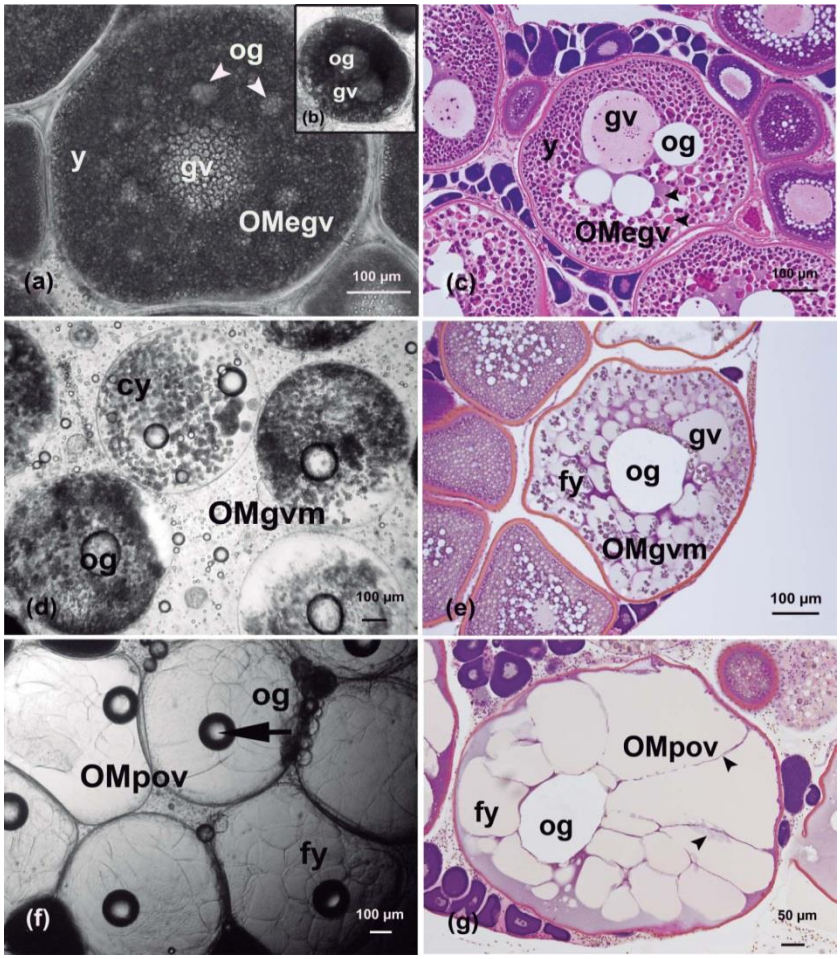

Figure 4. Oocyte maturation (OM) in common snook (a, b, d, and f are wet mounts; c, e, and g are histological preparations): (a) early in the OM stage, eccentric germinal vesicle step (OMegv) of oocyte development, the germinal vesicle (gv) is central and the oocyte is full of yolk globules (y), rendering it opaque, and oil globules with dark borders (og) encircle the central germinal vesicle; (b) an oocyte at the end of the OMegv step has a central oil globule (og) that has displaced the germinal vesicle (gv) to an eccentric position; (c) OMegv oocyte (more advanced than the OMegv oocyte in panel a), with multiple oil globules (og) coalescing at the center of the oocyte, displacing the germinal vesicle (gv) to an eccentric position toward the animal pole, and with yolk globules (y) that are smaller at the animal pole but are coalescing (arrowheads) at the vegetal pole; oocyte polarity is clearly established; (d) OM stage, germinal vesicle migration step (OMgvm) oocyte, showing black patchiness in the ooplasm (clearing yolk [cy]) and oil globules (og) with a dark border; (e) OMgvm oocyte at the beginning of migration, signified by ooplasm between the oil globule (og) and the germinal vesicle (gv), and fluid yolk (fy) surrounds the germinal vesicle, still having yolk globules within; (f) OM stage, preovulatory step (OMpov) oocytes, exhibiting numerous "lines" through the fluid yolk (fy) and generally a single oil globule (og); and (g) OMpov oocyte with fluid yolk (fy) and a single oil globule (og), and arrowheads indicate the extension of ooplasm through the fluid yolk.

The germinal vesicle is no longer observed in wet mounts of OMgvm oocytes, when the yolk is hydrating or clearing, and one or more oil globules are observed (Figure 4d). However, in histological preparations of OMgvm oocytes, germinal vesicle migration is observed to begin when there is ooplasm between the single oil globule and the germinal vesicle (Figure 4e). Although germinal vesicle migration is not observed in wet mounts during OMgvm, oil globules and clearing yolk are observed and serve to distinguish this OM step. Germinal vesicle breakdown and the resumption of meiosis are also not observed in either the wet mounts (Figure 4d) or the histological preparations (Figure 4e, g).

In the OMpov step, the germinal vesicle has broken down; meiosis has resumed, and meiotic arrest has occurred in metaphase of the second division of meiosis. A single oil globule is present, and the ooplasm extends from the oocyte periphery into the fluid yolk, subdividing the yolk into large, fluid yolk globules (Figure 4f, g). The yolk globules are distinct in wet mounts (Figure 4f) and are outlined by basophilic ooplasm in histological preparations (Figure 4g).

3.2.4 Oocyte atresia

Atretic oocytes were observed (Figure 5) in ovaries of captive and wild common snook. Oocyte atresia is not visible in wet mounts because oocytes in early atresia appear as normal SG oocytes; however, early atretic oocytes are clearly revealed in histological preparations. The germinal vesicle was never observed in atretic oocytes by using either wet mounts or histology, as it had broken down. Histologically, the salient features of early atresia in captive common snook include the fragmented zona pellucida, which primarily becomes clumped toward the center of

the oocyte in the generally disorganized ooplasm. The ooplasm is mostly composed of scattered oil droplets and yolk globules; the latter are seen to be breaking down toward the oocyte periphery. Numerous oil droplets are scattered throughout the ooplasm.

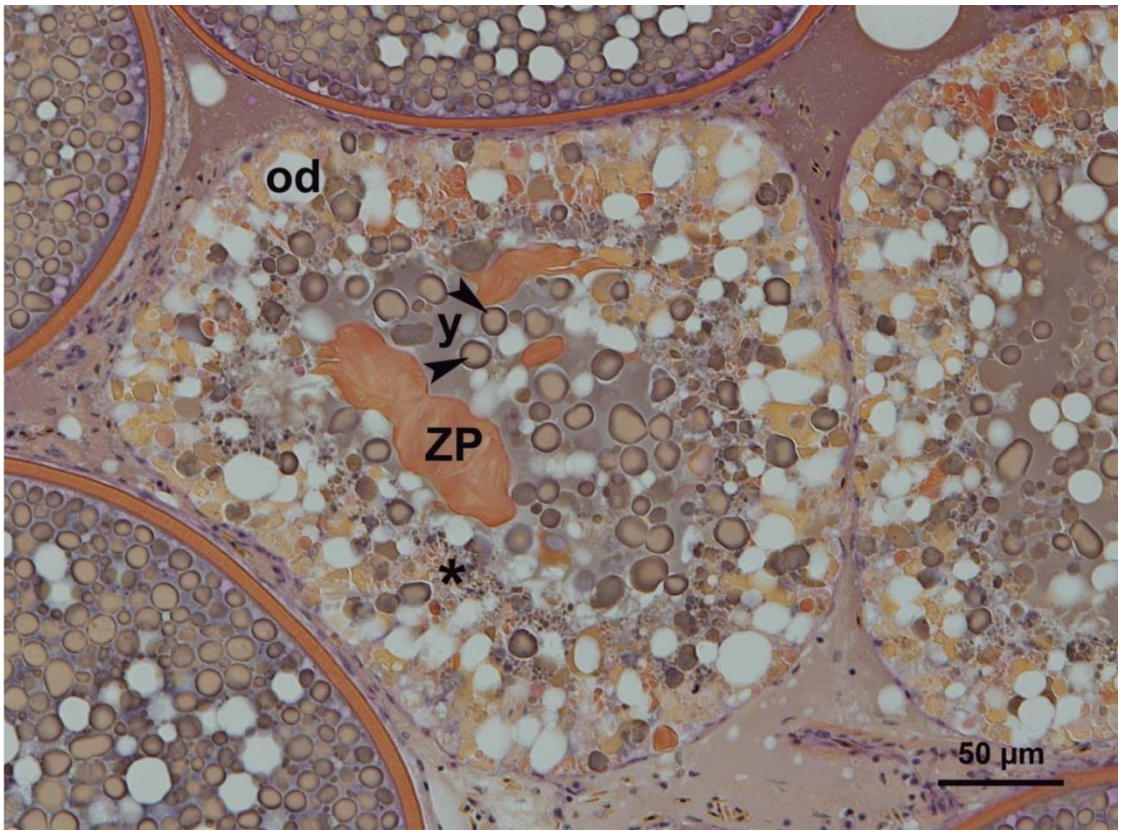

Figure 5. An atretic oocyte in a common snook. The zona pellucida (ZP) is fragmented and is clumped in the atretic oocyte's center. Oil droplets (od) and yolk globules (y) have a disorganized arrangement. Smaller yolk globules (*) are fragmented toward the oocyte periphery.

4. Discussion

Accurate assessment of fish reproductive condition and spawning potential is critical in aquaculture and fisheries. In the study of fish reproductive biology, numerous techniques and corresponding staging methods are available for documenting and interpreting gonadal development. However, not all of these techniques have been validated with very precise methods like histology, and not all of the staging descriptions use the same terminology. Some of the staging schemes use letters (Grier et al., 2009), while others use numbers (Bromley, 2003) to describe and define the development of fish oocytes. The problems encountered using different numbered staging schemes to describe oocyte growth and maturation events in fish are well documented (Mayer et al., 1990; West, 1990; Patiño and Sullivan, 2002; Brown-Peterson et al., 2011). The lack of generally accepted, consistent ovarian terminology has limited communication among scientists within and across research fields by making it difficult to conduct data comparisons (Grier et al., 2009; Brown-Peterson et al., 2011). Bromley (2003) highlighted the problem with the terminology used in fisheries literature by citing the incongruent staging (i.e., based on different numbered stages) used by fisheries biologists for the plaice *Pleuronectes platessa*. The resulting data for plaice could not be compared within the same fishery, thus confirming the need for validated sampling methods as well as clear and consistent definitions of oocyte growth and maturity (Blazer, 2002; Parenti and Grier, 2004).

Although the timing of events during gamete development may vary among species, each reproductive phase that occurs during annual reproductive cycles has specific physiological and histological markers that are conceptually universal. Variability in oocyte development may occur due to reproductive diversity among

fishes (Lubzens et al., 2010). For example, in gulf killifish *Fundulus grandis*, cortical alveoli are large and well developed prior to the appearance of oil droplets but become smaller later in development (Grier et al., 2009). In common snook, cortical alveoli appear shortly after the PGod step. The appearance of oil droplets and the appearance of cortical alveoli in PG have been designated as transitory steps to SG (Grier, 2012). Cortical alveoli cannot be observed in wet mounts due to the limited resolution of the technique. For fish that produce pelagic eggs with an oil globule, a ring of blackened oil droplets surrounds the germinal vesicle in wet mount preparations. The appearance of oil droplets around the germinal vesicle and the appearance of cortical alveoli at the oocyte periphery are events that occur nearly simultaneously during oocyte growth, as indicated histologically in common snook.

Within a population, fish are not spawning capable unless full-grown oocytes are present in the ovary (Brown-Peterson et al., 2011). In fish that produce pelagic eggs, the appearance of ring oocytes in wet mounts should suffice to determine reproductive condition for purposes of aquaculture and fisheries applications when histology is not used. Again, the designation “ring oocyte” is not a stage of oocyte development. Ring oocytes may encompass two stages and multiple steps of oocyte development (e.g., PGod and SGe) when yolk begins to form. The dark appearance of oil globules around the germinal vesicle is an important diagnostic criterion found during hatchery evaluations of wet mounts. The same criterion could be applied in fisheries for pelagic egg development where an oil globule is present in the egg. Oil droplets in PG and SG oocytes become oil globules during OM. By definition, this is meant to distinguish oocyte growth from OM (Grier et al., 2009). However, ring oocytes are not full-grown oocytes (i.e., SGfg). Until SGfg oocytes are present in the

ovary, a fish cannot be induced to undergo maturation and ovulation by using hormones. The SGfg oocytes are easily detected using either the wet mount technique or histological processing, and oocyte diameter is a good indicator.

The wet mount technique for ovarian biopsies has been applied in aquaculture spawning procedures, and the technique has been validated by comparison with histological preparations. In an aquaculture setting where a simple and quick method is needed to determine whether a female common snook is a potential candidate for hormone induction of ovulation, the microscopic evaluation of whole oocytes by using the wet mount technique will be accurate and informative. Use of the wet mount technique need not be restricted to aquaculture applications; it may also have applications in fishery biology as a tool for immediate, low-cost determination of a population's reproductive condition. Gross ovary evaluations are commonplace in fisheries science, especially when histology is not available, because gross evaluations constitute an inexpensive way to routinely monitor the reproductive state of the catch. Depending on the needs of the study, the wet mount technique can be advantageous by providing a noninvasive method of observation in situations where sacrificing the fish to determine their reproductive condition is not optimal. Although the wet mount technique has potential as a tool that can be used across research fields, it does have some limitations. In common snook, oocytes in early atresia appeared to be normal oocytes when viewed in wet mounts but were easily identified as atretic when using histology. Additionally, due to the loss of fine resolution, it was not possible to accurately identify cortical alveoli, oocyte atresia, and postovulatory follicle complexes by use of the wet mount technique. This limitation negates the technique's utility for

evaluating spawning frequency, which is an important part of many fisheries management studies.

In conclusion, use of the wet mount technique with a tiered and adaptable staging scheme has been validated for aquacultural and field applications with common snook. Results indicate that use of the side-by-side comparison of the wet mount technique and histological preparations could serve as guide for researchers in aquaculture and fisheries to conduct similar validations for species of interest in addition to common snook.

Acknowledgments

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CHAPTER 3

RESEARCH ARTICLE

SPAWNING PERFORMANCE AND ENDOCRINE PROFILES OF CAPTIVE COMMON SNOOK *CENTROPOMUS UNDECIMALIS* BROODSTOCK TREATED WITH DIFFERENT SUSTAINED RELEASE GNRHA-DELIVERY SYSTEMS.

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Keywords: spermiation; common snook; GnRH; sperm quality; induced spawning

Abstract

The acclimation of wild-caught common snook to captivity and the development of reliable protocols for inducing consistent multiple spawns for the mass production of larvae have not previously been reported. Reproductive bottlenecks among captive common snook broodstock include the failure to complete oocyte maturation and ovulation in females and reduced milt production and quality in males. The aims of this study were to investigate the efficacy of gonadotropin releasing hormone analogue (GnRHa) to induce spontaneous spawning and to study the effects of GnRHa-based hormonal treatment on milt characteristics and plasma steroid levels in captive male common snook broodstock. The annual plasma sex steroid profile of male and female broodstock maintained under ambient natural photo-thermal conditions was also examined. Two experiments were conducted. The first aimed to compare the effects of slow and regular release GnRHa implants, whereas the second aimed to investigate the effects of GnRHa, alone or in combination with the dopamine antagonist, pimozide (PIM). A single treatment of regular release GnRHa, administered at a minimum stage of oogenesis (late secondary growth-SGl), induced and synchronized ovulation and spontaneous spawning in captive females for up to 72 hours post-implantation. Overall, spawning performances were variable in terms of the total number of eggs produced (from 359,190 to 878,630 eggs/spawn), fertilization (from 44.4 to 78.6 %) and hatch rate (from 44.5 to 94.1 %). No effects on the quality or volume of milt produced by male broodstock were observed when treated with different GnRHa delivery systems nor with GnRHa alone or in combination with a dopamine antagonist (pimozide).

1. Introduction

The control of reproduction in captivity constitutes the first prerequisite to achieve sustainable commercial scale production of emerging aquaculture species. However, this requires the development of reliable protocols for inducing consistent spawning from captive broodstock and obtaining large quantities of high quality eggs and larvae for the mass production of juveniles (Migaud et al., 2013). The reproductive dysfunctions often reported in captive marine fish populations of either wild or cultured brood fish represent critical bottlenecks for commercialization (Mylonas et al., 2010). Some of the most common reproductive bottlenecks are the failure to complete oocyte maturation and ovulation in females and reduced milt production and quality in males (Bobe and Labbé, 2010). The absence of adequate environmental cues and the stressors imposed by confinement can directly impact the neuroendocrine regulation of gametogenesis in fish and therefore, the application of exogenous hormonal treatments are routinely used as an effective method to induce spawning in captive fish (Mylonas et al., 2010).

In cultured fish stocks, artificial manipulation of the endocrine system has typically involved treatment with GnRH analogues (Mylonas and Zohar 2001; Zohar and Mylonas 2001). Results showed enhanced gamete production in females such as European sea bass *Dicentrarchus labrax* L. (Forniés et al., 2001; Prat et al., 2001), gilthead seabream *Sparus aurata* L. (Barbaro et al., 1997), turbot *Scolphtalmus maximus* (Mugnier et al., 2000) and newer aquaculture species such Senegalese sole *Solea senegalensis* (Guzmán et al., 2009). Additionally, the use of GnRHa implants increased volume and quality of male milt in a variety of freshwater and marine species including white bass *Morone chrysops* (Mylonas et al., 1997), greenback flounder

Pleuronectes ferrugineus (Lim et al., 2004), Atlantic cod *Gadus morhua* (Garber et al., 2009) and Senegalese sole *Solea senegalensis* (Guzmán et al., 2011a). Low volume of expressible milt is a common problem among captive broodstock and represents a significant bottleneck for those species in which hatchery production is based on artificial fertilization and the acquisition of gametes by manual stripping (Billard, 1986). This has also been problematic for commercial aquaculture operations working with multiple batch spawner species, such as barramundi *Lates calcifer*, where inadequate milt production was shown to significantly limit fertilization success, directly impacting the production of fry (Hilomen-Garcia et al., 2002; Loughnan et al, 2013).

Despite the success of GnRHa treatments among a variety of fish species, these have sometimes been shown to be ineffective when used alone, due to a strong dopaminergic inhibition of gonadotropin secretion, leading to incomplete or arrested gametogenesis at the later steps of oocyte maturation stage in females and spermation for males (Dufour et al., 2010). In some, but not in all teleost species, dopamine exerts inhibitory actions on both the brain and pituitary through reduction of GnRH systhesis and release, down-regulation of GnRH receptors and interference with the GnRH signal transduction pathways, thus affecting secretion from the pituitary (Chang et al., 1984). In the European eel *Anguilla anguilla* L., which remains at a prepubertal stage as long as the oceanic reproductive migration does not occur (Dufour *et al*., 2003), removal of dopamine inhibition was shown to be required for triggering gonadotropin release and ovarian development. The inhibitory effects of dopamine have also been demonstrated mainly in freshwater fish such as tilapia *Oreochromis niloticus* (Levavi-Sivan et al., 2006), grey mullet *Mugil cephalus* (Aizen et al., 2005) and goldfish *Carassius auratus*

(Trudeau 1997). In contrast, dopamine inhibition of gonadotropin release has not been observed in many other teleosts and has only recently been documented in marine fishes (Guzmán et al., 2011b). The most effective strategy for spawning induction is therefore, using a combination of GnRH agonist and dopamine antagonists such as pimozide or domperidone to remove or reduce the inhibition on gonadotroph cells and stimulate release of the gonadotropins e.g. follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Zohar and Mylonas, 2001; Zohar et al., 2010).

One marine finfish of interest for commercial scale aquaculture production is the common snook *Centropomus undecimalis*. Prized as a food fish in several Central and South American countries (Alvarez-Lajonchère et al., 2008) and among the most popular inshore game fish along the Gulf coast of the United States (Winner et al. 2010); common snook have both a high market value and recreational demand. These characteristics have led to research directed towards the development of induced spawning protocols however, to date; there is still no established large scale production of juveniles for on-growing or the enhancement of wild stocks. The failure of captive female broodstock to ovulate in the absence of hormonal manipulation (Ibarra-Castro et al., 2011; Rhody et al., 2013, Chapter 2) and the inconsistent production of high quality eggs and larvae remain culture constraints. In addition, it has been hypothesized that sperm production among captive males may be reduced when compared to that of wild males, a possible explanation for the variation in fertilization success rates observed during mass spawning events in captive broodstock (Rhody et al., 2014, Chapter 4).

In the present work two experiments were conducted. The first aimed to compare the effects of slow and regular release GnRHa implants, whereas the second aimed to investigate the effects of GnRHa, alone or in combination with the dopamine

antagonist, pimozide (PIM), on milt characteristics and plasma steroid levels in captive male common snook broodstock. In both studies, treatment effects were assessed by measuring plasma levels of the sex steroids testosterone (T) and 11-ketotestosterone (11-KT) where quality of milt was evaluated in terms of sperm concentration, milt volume, sperm motility and density. In an effort to better enable reliable control of reproduction under captive conditions, the annual plasma sex steroid profile of captive male and female broodstock maintained under ambient natural photo-thermal conditions was also examined.

2. Materials and methods

2.1 Broodstock acquisition and maintenance

In the fall of 2009, using a 92-m seine net deployed from a research vessel, wild adult male (mean body weight: 2.0 ± 0.1 kg, fork length: 56.6 ± 0.9 cm) and female (mean body weight: 2.7 ± 0.3 kg, fork length: 61.7 ± 1.7 cm) common snook were collected from three locations in Sarasota County (Florida, USA). At the time of collection, gonadal biopsies were used to determine gender. Additionally, each fish was implanted subcutaneously with a passive intergrated transponder (PIT tags, Oregon RFID, OR, USA) for future identification. Captured fish were transported in oxygenated tubs by vehicle to holding tanks at Mote Aquaculture Research Park (Sarasota, Florida, USA). Following a 40 day quarantine period, collected broodstock were divided among two separate, indoor, photoperiod (10-15H light) and temperature (20 - 30°C) controlled recirculating tank systems (A and B). A male: female sex ratio of 2 : 1 was established in each tank with a target stocking density of 2.0 to 2.5 kg m^3

(Table 1). Tanks A and B each contained 24 males and 12 females (Table 1). Over the study period (from January 2010 to September 2011, 20 months), broodstock in Tanks A and B were subjected to a simulated natural photoperiod and temperature cycle (Figure 1). Broodstock were given a fresh frozen diet consisting of 50 % shrimp and 50 % herring fed at 2.5 % of the total tank biomass five times per week.

Systems A and B each consisted of one 28 m^3 circular, green, fiberglass tank equipped with a 300 L side-mounted egg collector containing a 300 µm collection bag (Figure 2). Temperature was controlled in each tank system by cycling water through an individual heater/chiller unit (AquaCal, St. Petersburgh, FL, USA). Filtration included a drop filter (Aquaculture Systems Technologies, New Orleans, LA, USA) for the collection of fine solids, 900 L moving bed for bio-filtration, protein skimmer and an ultraviolet light (UV) sterilization unit. Water quality parameters were monitored throughout the study period. Salinity and dissolved oxygen were recorded daily and maintained at 33 - 35 ‰ and 7.0 ± 0.5 mg/L, respectively. Ammonia, Nitrite and Nitrate were checked weekly and averaged 0.21mg/L NO_2-N, 0.13 mg/L NO_3-N and 45.4 mg/L NH_3-N, respectively.

All fish were collected under a Florida Fish and Wildlife Conservation Commission Special Activity License (Contract No.10087, Permit # SAL 09-522-SR). Handling of the fish for routine management and experimentation was done in accordance with United States legislation concerning the protection of animals used for experimentation. All methods were conducted in accordance with Mote Marine Laboratory's Institutional Animal Care and Use Committee approved protocols (IACUC Approval No. 12-03- KM1).

Table 1. Description of captive common snook broodstock populations held at Mote Aquaculture Research Park. Weight and fork length (FL) ± SEM. Means with different letters in the same column are significantly different ($P < 0.05$).

Broodstock Tank	Total No. Fish/Tank	Male : Female Sex Ratio	Female Mean Weight (kg)	Female Mean Length (cm)	Male Mean Weight (kg)	Male Mean Length (cm)	Tank Biomass (kg/m^3)
A	36	2 : 1	3.2 ± 0.4^a	63.9 ± 2.6^a	1.8 ± 0.1^a	59.4 ± 1.1^a	2.4
B	36	2 : 1	3.0 ± 0.3^a	58.8 ± 2.2^b	2.0 ± 0.2^a	56.9 ± 1.5^a	2.1

Means with different letters in the same column are significantly different ($P < 0.05$).

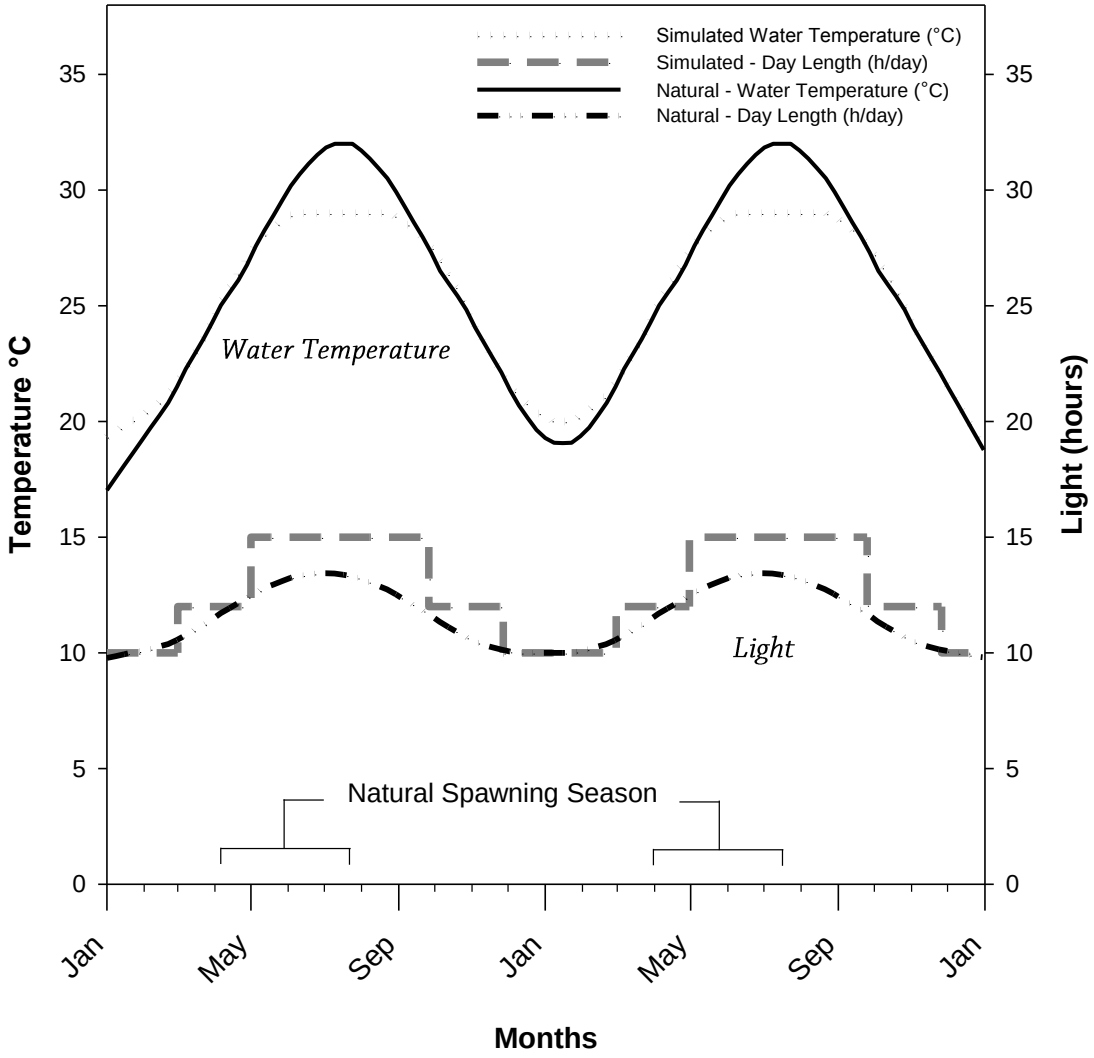

Figure 1. Schematic representation of ambient natural and artificial (simulated) environmental conditions associated with the annual reproductive cycle of wild common snook located on the Gulf coast of Florida and captive common snook broodstock held at Mote Aquaculture Research Park, Sarasota, FL. Natural ambient cycle of day length (light h/day) (**— —**) and water temperature (°C) (**—**) in Tampa Bay, FL. Imposed photo-thermal cycle used to mature and spawn captive broodstock including day length (light h/day) (— ·· —) and water temperature (°C) (**▪▪▪**).

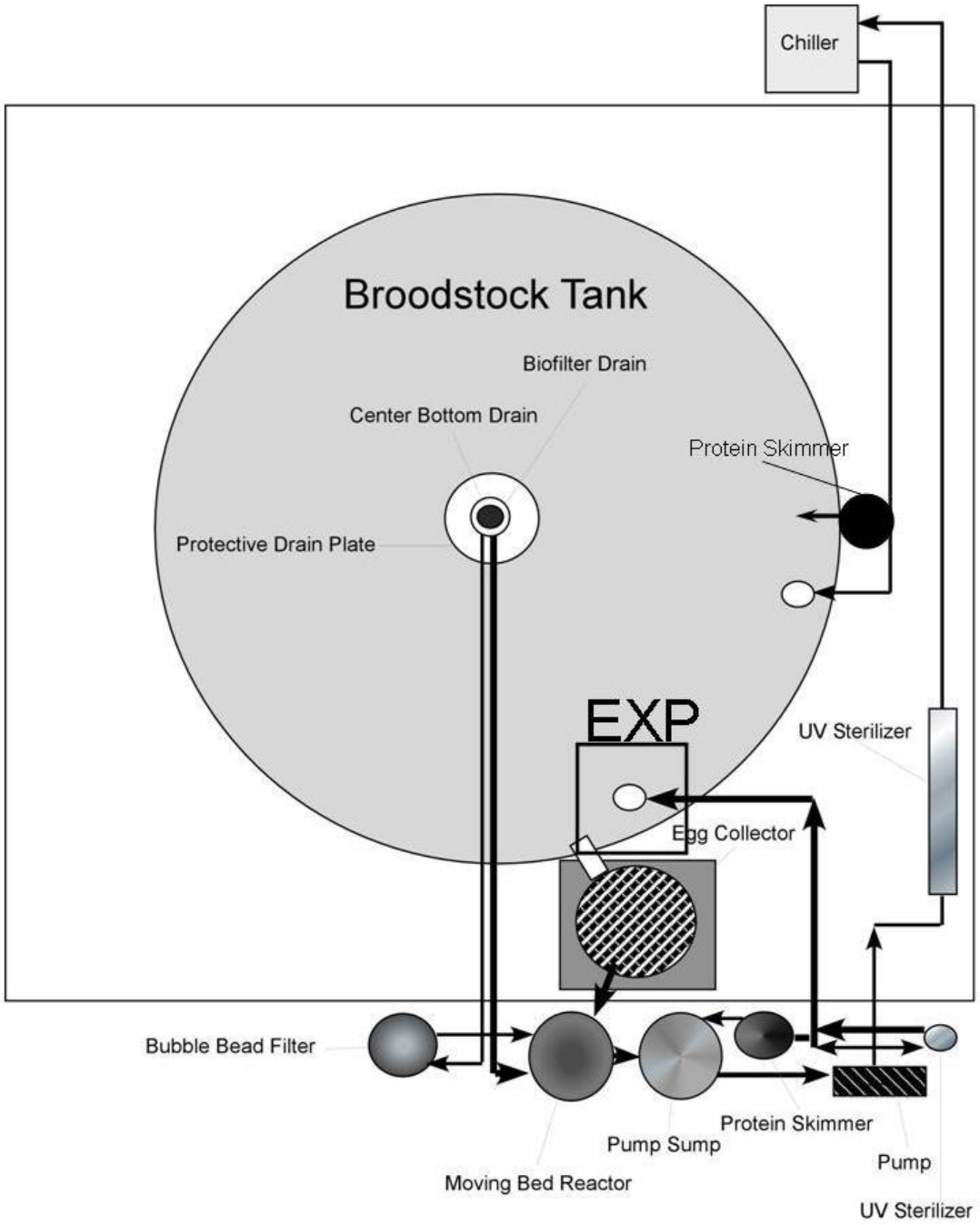

Figure 2. Broodstock tank and filtration design at Mote Aquaculture Research Park, Sarasota, FL, USA. Total tank volume is 28m^3.

2.2 Broodstock sampling procedures

Experiments were carried out between June and August, part of the natural spawning season of common snook, for two consecutive years (2010 and 2011). During that time, trials were carried out which required common snook broodstock in Tanks A and B to be routinely evaluated. At each sampling event the tank water level was lowered and two dividers, made from plastic mesh stretched across a polyvinyl chloride (PVC) pipe frame, were used to corral the fish into a section of the tank. From this restricted section, individual fish were netted into a 500 L tank containing 200 L of water and anesthetized with tricaine methanesulfonate (MS-222) at a concentration of 300 ppm for 1-2 minutes. Following sedation, both male and female common snook were weighed (to the nearest gram), measured (fork length, FL, mm) and the reproductive status of each individual was determined from wet mount preparations of ovarian biopsies (Rhody et al., 2013, Chapter 2). In addition to being anesthetized during activities associated with the experimental trials, males and females in Tank B were sedated approximately every four weeks between March 2010 and August 2011 (17 months) in order to collect a blood sample from each individual. This work was conducted as part of a two year effort to monitor the seasonal reproductive cycle of captive broodstock through sex steroid profiling. Blood (3 ml) was taken from the caudal vein using a 23-gauge needle attached to pre-heparinized syringe and placed into a vacutainer tube coated with lithium heparin. Immediately after collection, plasma was obtained by centrifugation (1,500 *g*, 10 min, 4 °C) and stored at -70 °C for sex steroid analysis. From the two groups of replicated experimental tanks, only fish in Tank B were sampled monthly for blood sampling. The other treatment group (Tank A) was

only manipulated for hormone administration and blood sampling associated with experimental trials conducted during the study period.

2.3 Steroid analyses

Plasma testosterone (T) and 17β-estradiol (E_2) levels were measured by radioimmunoassay (RIA) according to methods described by Duston and Bromage (1987). Tritiated T (GE Healthcare, UK) and E_2 (PerkinElmer, Boston, MA, USA) radiolabels along with anti-testosterone and anti-estradiol antisera (CER group, Marloie, Belgium) were used. Plasma levels of 11-ketotestosterone (11-KT) were also measured by RIA according to Fostier et al. (1982). A parallelism test was performed for all three hormonal assays to confirm that the serial dilutions of common snook plasma extracts and hormone standards were immunologically comparable. Radiolabel and antibody were provided by Dr. Alexis Fostier (Department of Animal Physiology and Livestock Systems, Rennes Research Centre, France). Intra- and inter-assay coefficients of variation were; 4.2 and 9.1 %; 6.4 and 8.9 %; 5.4 and 7.1% for T, E_2, and 11-KT respectively (n = 10 to 15 assays for each hormone). All standards and samples were assayed in duplicate.

2.4 Ovarian biopsies and hormonal induction of females

The reproductive status of each female was confirmed by ovarian biopsy at the beginning and end of each experiment. Ovarian biopsies were obtained by inserting a soft tubing catheter (5Fr; 1.0-mm inside diameter) into the oviduct and applying a gentle suction (using a 3-mL syringe) to collect a small sample of oocytes. A portion of

the oocytes were immediately prepared as wet mounts and examined microscopically to determine oocyte diameter and morphology (Rhody et al., 2013, Chapter 2). The remaining portion of the biopsy was then placed in Trump's fixative (McDowell and Trump 1976) for later comparison and confirmation of wet mount staging. For light microscopy, the fixed portion was subsequently embedded in glycol methacrylate and was sectioned at 6 μmon an LKB Bromma 2218 Historange Microtome (LKB Bromma, Sweden). Tissue sections were stained with periodic acid–Schiff, metanil yellow, and Weigert's hematoxylin and eosin (Quintero-Hunter et al., 1991). The phase of reproductive development was assessed using a classification proposed by Grier et al. (2009) and adapted to common snook according to Rhody et al. (2013). Oocytes from the wet mount biopsy were photographed (n = 50 oocytes/individual) within 30 minutes post collection using an Olympus BX53 microscope fitted with a DP-72 digital camera and Olympus cellSens (version 1.3) imaging software (Olympus, USA) and measured.

The absence or presence of mature oocytes was determined by ovarian biopsy. Based on previous studies with common snook (Rhody et al., 2014, Chapter 2), only females determined to have a minimum oocyte developmental stage of late Secondary Growth (SGl) were treated with a non-degradable implant containing regular release GnRHa ([$_{D}$-Ala^{6} Pro^{9} NEt]-GnRH) at a dosage of 50 μg kg^{-1} body weight. All implants were composed of an ethylene and vinyl acetate (EVAc) delivery system (Zohar and Mylonas 2001) and were fabricated at the Institute of Marine and Environmental Technology (IMET), Department of Marine Biotechnology, University of Maryland Baltimore County. The EVAc implants release GnRHa for a duration of approximately

5 weeks for the ‘slow release’ implant or 2-3 weeks for the ‘regular release’ implant (John Stubblefield, personal communication).

2.5 Experimental design and hormonal treatment of captive male broodstock

2.5.1 Experiment 1: *Effect of slow and regular release GnRHa*

The first experiment took place in June 2010 and aimed to determine the effects of slow release (SR) and regular release (RR) GnRHa implants on milt characteristics and plasma levels of gonadal steroids in male broodstock. A ten day trial was conducted during which time three broodstock sampling events occurred in Tanks A (1, 6 and 11 June 2010) and B (2, 7 and 12 June 2010). Males were randomly divided into three experimental groups (n = 16) and treated 10 days prior to females with a single intramuscular implant (100 μg kg^{-1} bw) of either slow or regular release GnRHa or with a ‘zero load’ implant serving as the control (Sham). The total amount of expressible milt was collected (stripped) from males by applying gentle abdominal pressure immediately prior to hormonal stimulation (day 0) and at 10 days afterward. Each individual milt sample was held in a volumetric vial and promptly placed on ice until total milt volume, motility and sperm density could be assessed. A blood sample (3 ml) was also obtained from each male at the start of the study (day 0) and at days 5 and 10. Blood samples were collected according to methods previously described in section 2.2 and processed for sex steroid analysis (T and 11-KT) as detailed in section 2.3.

2.5.2 Experiment 2: *Effect of GnRHa and dopamine antagonist*

The second experiment was 21 days in length and took place in 2011 (4, 9, 15 and 24 August 2011). The primary objective was to investigate the effects of regular release GnRHa (100 μg kg^{-1} bw), alone or in combination with the dopamine antagonist, pimozide (1,000 μg kg^{-1} bw) (both administered as implants). A 'zero load' implant served as the control (Sham). As in the previous experiment, each of the three experimental groups consisted of 16 males split in two tank replicates (Tank A and B). Implants were administered to the fish at three time points during the study; at the start (day 0) and again 5 and 10 afterwards. Blood samples were taken prior to the initial implantation (day 0) and again at days 5, 10 and 20 thereafter. Blood plasma was obtained and later processed for sex steroid analysis (T and 11-KT) as detailed in section 2.3. Milt quality evaluations were conducted twice; at the start of the study (day 0) and again at the end of the trial (20 days after the initial implants were administered to each male). Ovarian biopsies revealed all females were regressed at the time of sampling and therefore, no females were implanted during this trial.

2.6 Milt evaluation

Sperm volume, sperm motility (total duration of movement and percentage of motile sperm cells), spermatocrit, and sperm density were documented for individual males using methods reviewed by Rurangwa et al. (2004). The total volume of expressible milt was measured directly after its collection using sterile syringes calibrated in 0.01 ml increments. Duration of sperm motility was measured in seconds determined by diluting 10 μl of milt in 1 ml non-activating solution (0.9 % NaCl), and

then 1 µl of this diluted solution was pipetted onto a 1% (w/v) BSA-coated microscope slide. The following scale was used to assess the percentage of motile spermatozoa: 0, when no movement was observed; 1, when up to 25% cells were moving; 2, when up to 50% of cells were moving; 3, when up to 75% of cells were moving and 4, when more than 75% of cells were moving (Viveiros et al., 2003). Activation was achieved by adding 25 µl of a standardized (33 ‰) saltwater solution prepared by adding Instant Ocean™ salt to seawater. A total of four replicates were evaluated per sample by the same observer to reduce the degree of variation in the assessments. Once diluted, milt was held on ice in a cooler between preparations of replicate slides. The non-activating and seawater solutions were held at 4 °C until they were needed.

Spermatocrit was determined using milt collected in micro hematocrit capillary tubes (75 mm length, 0.5–0.6 mm inner diameter) and centrifuged at 12,500 x *g* for 20 minutes within 1 hour post collection. Spermatocrit for each milt sample was estimated based on the mean value of 3 microhaematocrit capillary tubes. To measure spermatozoan density 10 µl of milt were diluted (1:100) into 1 ml of a non-activating solution (0.9% NaCl) solution and the number of spermatozoa was counted from triplicate samples using a Neubauer hemocytometer under a 400x magnification.

2.7 Spawning

When spawning occurs in the common snook, eggs are spontaneously released. Consequently, in this study, egg collectors were checked every 4 to 6 hours for the presence of eggs and the spawning event or ‘spawn’ was defined as the sum of eggs or offspring produced during that time frame. Following each spawning event, eggs were

transferred from the broodstock tank to 100 L hatching tanks where volumetric counts (*n*=3) were used to determine the total number of eggs collected. At 4 - 6 h post fertilization (blastula stage), aeration was removed and non-viable (sinking) eggs were enumerated and discarded. At this time approximately 250 viable (non-sinking) eggs were stocked into individual microcosms. Accuracy of initial stocking ranged from 226 to 272 larvae per microcosm. The microcosms (n = 5 for each spawn), made of PVC (2.6 cm diameter), were held in a shallow rectangular recirculating raceway tank system equipped with UV sterilization. Each microcosm had an operational volume of 330 ml. The base of each microcosm was covered with a 200 µm nylon mesh to prevent the escape of larvae while allowing circulation of the water. Temperature in the raceway tank was maintained at 28 ± 1 °C. At approximately 17 h post fertilization eggs began to hatch; between 21-23 h post fertilization hatch rate for each microcosm was assessed.

2.8 Statistical analysis

All statistical analyses were carried out with Statistica 8.0 (StatSoft, Inc., Tulsa, OK, USA). Data were ln-transformed prior to analyses. Differences in seasonal plasma levels of steroids (E_2, T and 11-KT) were assessed using a General Linear Model (GLM) one-way analyses of variance (ANOVA) followed by Bonferroni post-hoc tests with Bonferroni-adjusted p-level for multiple comparisons. GLM repeated measures ANOVA (RM-ANOVA) was used to (i) detect significant differences between experimental and control groups in response to GnRHa treatment and (ii) assess changes in total expressible milt, sperm density, sperm motility (total duration of movement) and plasma sex steroids levels data from individual fish sampled

repetitively. Although percentage of motile sperm was also recorded, statistical analysis could not be performed on this parameter since an arbitrary, nonlinear scale was used to conduct the assessment. Results are presented as means ± SEM.

For the first experiment, general models for (i) were developed with 'treatment' [three levels: slow release GnRHa, regular release GnRHa and Control (SHAM)] as the between-subjects factor and time-post-implantation [three repeated measure variables: day 0, 5 and 10] as within-subjects factors. General models for (ii) were developed with 'treatment' [three levels: slow release GnRHa, regular release GnRHa and Control (SHAM)] as the between-subjects factor and time [two repeated measure variables: day 0, day 10] as within-subjects factors.

For the second experiment, general models for (i) were developed with 'treatment' [three levels: GnRHa, GnRHa + Pimozide and Control (SHAM)] as the between-subjects factor and time post-implantation [four repeated measure variables: day 0, 5, 10 and 20] as within-subjects factors. General models for (ii) were developed with 'treatment' [three levels: GnRHa, GnRHa + Pimozide and Control (SHAM)] as the between-subjects factor and time [two repeated measure variables: day 0, day 20] as within-subjects factors.

3. Results

3.1 Spawning

Based on ovarian biopsy evaluations, 75 % of the females in Tank A and 40% of females in Tank B were implanted with regular release GnRHa in the 2010 experiment (Table 2). Among females which received implants, 8 of 12 females were

found to have oocytes in the late stages of vitellogenesis (Secondary Growth, full-grown) having a mean oocyte diameter of 400 µm. Four mass spawning events were documented during the study period, all of which occurred during the first experiment in June 2010; approximately 24 to 72 hours post implantation, between 1500 - 2200 h (Table 2). Spawns were collected from Tank A over three consecutive days (12, 13 and 14 June 2010), whereas only one spawn was documented in Tank B (13 June 2010). In 2011, no females were implanted during the second experiment. Despite broodstock populations in both tanks being subjected to the same environmental conditioning regime throughout the study period; biopsies obtained in August 2011 revealed that female oocyte development had not advanced beyond the Primary Growth Stage having a mean oocyte diameter of 65 µm. Histological analysis revealed there were a number of oocytes which were atretic however, the proportion of these oocytes was not quantified. Approximately 2,450,000 eggs were collected during the first trial in June 2010. Overall, spawning performances between the two tank groups were variable in terms of the total number of eggs produced (from 359,190 to 878,630 eggs/spawn), fertilization (from 44.4 to 78.6 %) and hatch rate (from 44.5 to 94.1 %) (Table 2). The mean number of eggs produced per spawn in tank A was 563,940 ± 159,708 (SEM), whereas 562,160 ± 28,900 were obtained from the single spawn which occurred in tank B.

Table 2. Data presented represents time of spawning, total number of eggs collected and percentage hatch of fertilized eggs collected over the course of the three common snook spawning trials conducted in 2010 and 2011.

Tank (s)	Total Females / Tank (n)	Date of Female Implatation (mm/dd/yy)	Number Female Implanted (n)	Percentage of Females Implanted (%)	Spawning Event (mm/dd/yyyy)	Time of Spawning (h)	Number of Eggs Collected (*n*)	Fertilization Rate (%)	Hatch Rate (%)
A	12	Jun 11, 2010	9	75	Jun 12, 2010	1800-2200	878,630 ± 10,500	78.6 ± 2.6	44.5 ± 3.3
					Jun 13, 2010	1500-1900	454,000 ± 11,650	74.8 ± 2.9	79.9 ± 4.6
					Jun 14, 2010	1600-2100	359,190 ± 16,200	46.1 ± 3.0	95.1 ± 2.9
B	12	Jun 12, 2010	3	40	Jun 13, 2010	1900-2300	562,160 ± 28,900	44.4 ± 4.8	54.3 ± 7.2
A / B	12	Not implanted	0	0	……	No Spawns Occurred		……	……
A / B	12	Not implanted	0	0	……	No Spawns Occurred		……	……

3.2 Annual plasma sex steroids levels for captive male and female snook broodstock

Circulating levels of T and E_2 measured in captive female common snook broodstock during the two consecutive years are shown in Figure 3A and B. In year 1, testosterone gradually increased throughout the summer months reaching peak levels in September (T, 0.7 ± 0.02 ng/ml) then falling sharply in October to basal winter levels (T, 0.2 ± 0.03 ng/ml). In contrast, plasma E_2 peaked in April (E_2, 0.3 ± 0.02 ng/ml) and remained at a similar level throughout the spring (May) and early summer (June). In year 2, mean T and E_2 levels appeared to be more synchronized, increasing together gradually in the spring but overall plasma levels were significantly lower (40%) than in year 1.

Changes in mean circulating levels of T and 11-KT in males are shown in Figure 3C and D. Levels of T were at low levels in March (year 1) and rose significantly in April (0.8 ± 0.04 ng/ml). They remained high in the spring reaching a peak in July (1.0 ± 0.02 ng/ml) but then declining sharply in August (0.4 ± 0.02 ng/ml) where they remained at basal winter levels until the following spring. In the following year, maximum levels of T were significantly lower than in year 1, reaching a peak in May (0.5 ± 0.02 ng/ml) and sharply declining in June. Mean circulating levels of 11-KT doubled (3.0 ± 0.03 to 6.0 ± 0.02 ng/ml) between May and August only to fall sharply again in September (≤ 2.0 ng/ml) in year 1. In summary, gonadal steroid levels were on average higher in female and male broodstock in year 1 than in year 2 during the months of June, July and August (Figure 3A, B).

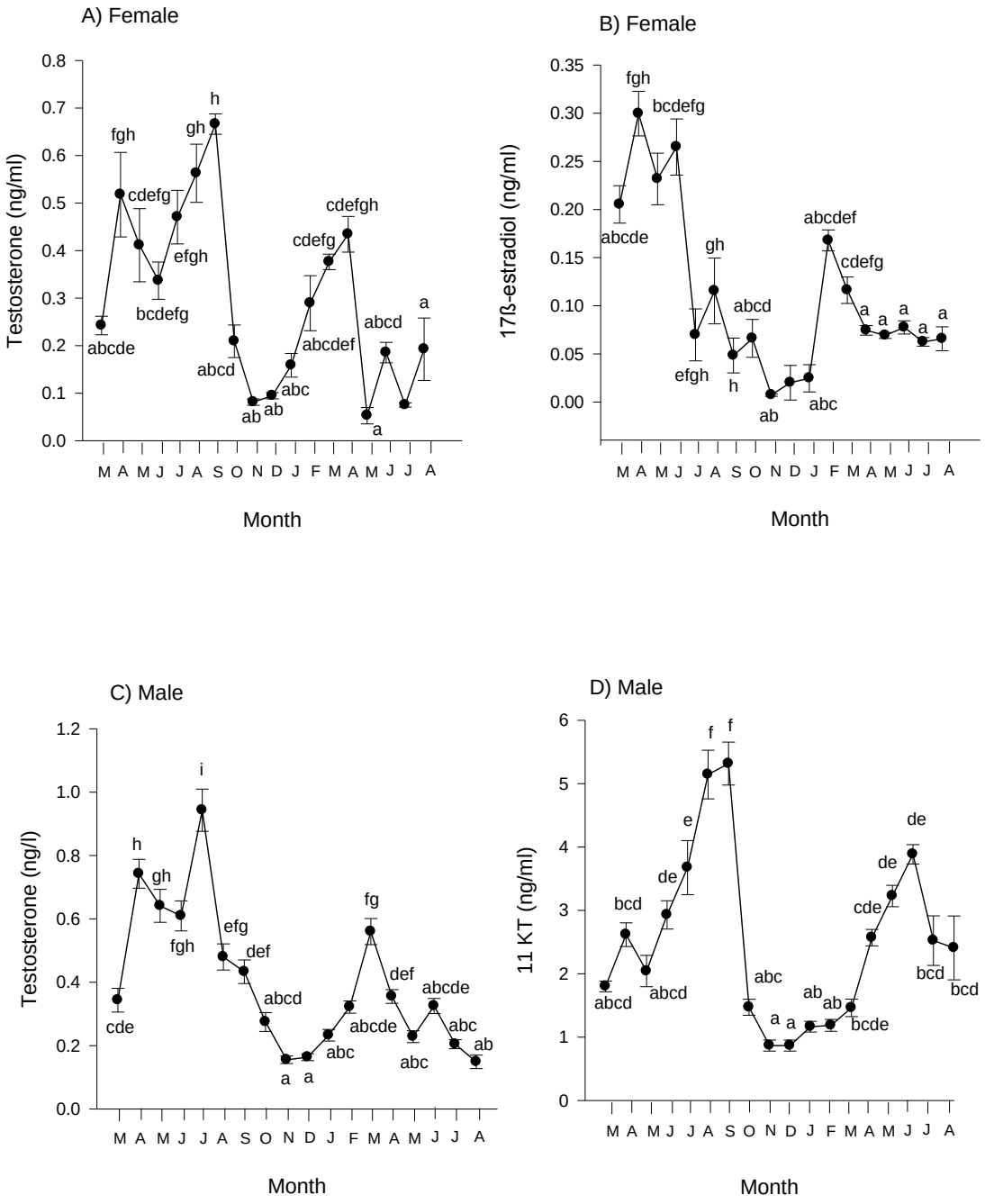

Figure 3. Changes in plasma testosterone (A) and 17β-estradiol (B) in captive females (n=12) and testosterone (C) and 11-ketotestosterone (D) in captive males (n=24) maintained under natural photothermal conditions from 2010 to 2011. Lower-case letters indicate significant differences given sampling points (Repeated measures ANOVA). Data are presented as mean ± SEM.

3.3 Response of male broodstock to hormonal treatment with slow and regular release GnRHa

Levels of sex steroids were analyzed prior to implantation (day 0) and at 5 and 10 days after initiation of treatments (Figure 4). Prior to the initial hormonal implantation, no significant difference in plasma T or 11-KT were observed among treatment groups. Plasma steroid levels over time declined in all three treatment groups from the initial levels measured at the start of the study (day 0). There was no effect of treatment with either slow or regular release GnRHa on levels of male testosterone (T). Levels of male 11-KT were significantly lower [$F_{(2, 23)} = 3.7368$, $p = 0.04$] in the control and regular release GnRHa groups than in the group treated with slow release GnRHa at day 5.

A series of milt quality characteristics were assessed and analyzed prior to implantation (day 0) and 10 days after initiation of treatments (Table 3). There was no effect of treatment with slow or regular release GnRHa on motility, spermatocrit or density. Overall, at the start of the trial (day 0), the expressible volume of milt obtained from each individual was low, ranging from approximately 400 to 480 µl. Irrespective of treatment, the total expressible volume of milt decreased significantly (by 50%) ($p = 0.03$) over the study period (Table 3). Sperm density ranged from 9.25 ± 3.8 to 11.3 ± 1.2 x 10^{10} spermatozoa/ mL (± SEM) during the trial period.

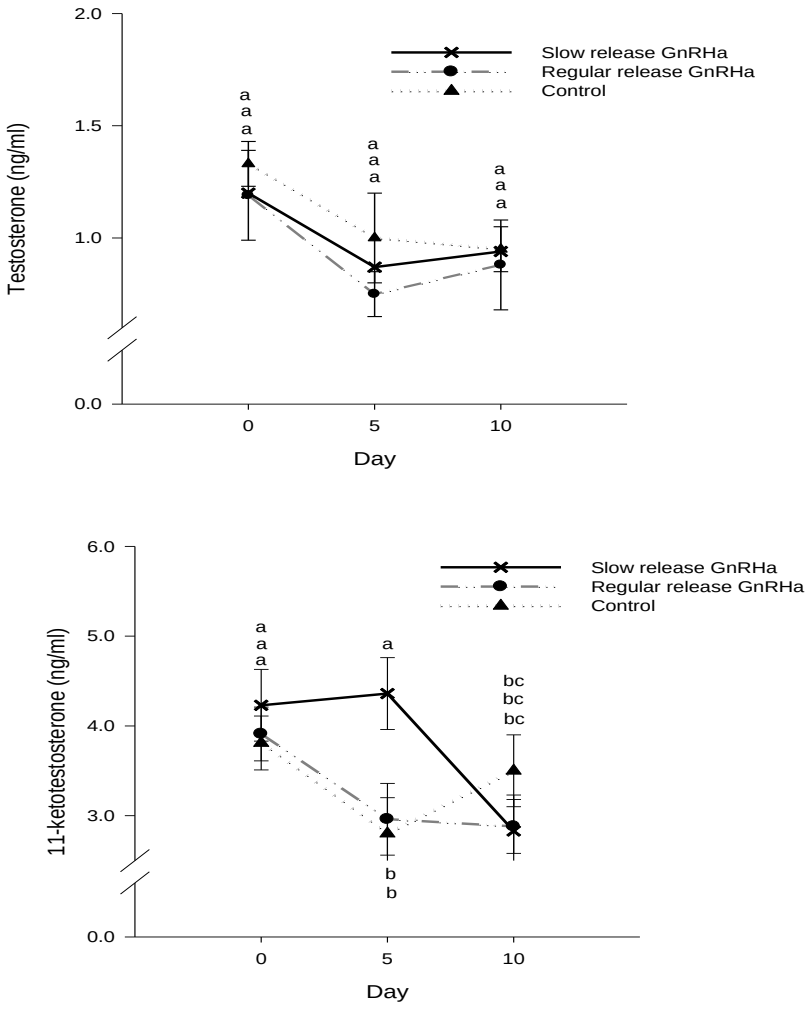

Figure 4. Plasma levels of testosterone (A) and 11-ketotestosterone (B) in male common snook broodstock treated with 'zero load' (Control), slow or regular release GnRHa. Plasma levels were collected on days 0, 5 and 10 from initiation of treatments. Lower-case letters indicate significant differences given sampling points Data are expressed as a mean ± SEM (n = 8).

Table 3. Mean (± SEM) total expressible milt, duration sperm motility, spermatocrit and sperm density of captive male common snook broodstock (n = 8) treated with a single dosage of slow or regular release GnRHa. Treatment effects were measured immediately prior to implantation and 10 days afterward. Different superscripts indicate significant differences between treatments at that sampling time (ANOVA, p = 0.05).

	Treatment Group	Day 0	Day 10
Total milt volume (µl)	Slow Release GnRHa	420 ± 50	220 ± 25
	Regular release GnRHa	455 ± 25	205 ± 35
	Control (Sham)	480 ± 45	250 ± 40
Duration of sperm motility (s)	Slow Release GnRHa	225 ± 5	200 ± 10
	Regular release GnRHa	236 ± 10	222 ± 11
	Control (Sham)	265 ± 15	252 ± 28
Percentage of motile sperm (%)	GnRHa	≥ 75 %	≥ 75 %
	GnRHa + Pimozide	≥ 75 %	≥ 75 %
	Control (Sham)	≥ 75 %	≥ 75 %
Spermatocrit (%)	Slow Release GnRHa	57.2 ± 3.0	49.4 ± 5.5
	Regular release GnRHa	60.3 ± 3.0	56.9 ± 8.1
	Control (Sham)	62.9 ± 4.0	55.3 ± 2.3
Spermatozoa ($x10^{11}/ml^{-1}$)	Slow Release GnRHa	10.06 ± 1.3	9.33 ± 1.27
	Regular release GnRHa	10.03 ± 1.8	9.25 ± 3.84
	Control (Sham)	11.37 ± 1.2	10.14 ± 4.6

3.4 Experiment 2: Response of male broodstock to hormonal treatment with GnRHa and pimozide

Levels of sex steroids were analyzed prior to implantation (day 0) and on 5, 10 and 20 days after initiation of treatments (Figure 5). Prior to the initial hormonal implantation, no significant difference in plasma T or 11-KT were observed among treatment groups. Plasma steroid levels over time declined in all three treatment groups from the initial levels measured at the start of the study (day 0). Levels of male testosterone (T) were significantly higher [$F_{(2, 23)} = 5.1043$, $p = 0.017$] in the treatments than in control at day 5 and significantly lower in fish administered GnRHa [$F_{(2, 23)} = 5.7347$, $p = 0.011$] at day 20. A significant difference [$F_{(2, 23)} = 17.6$, $p \leq 0.001$] in plasma 11-KT levels was observed on day 5 of the experiment with the highest level reported in fish administered GnRHa + Pimozide (4.6 ± 0.9 ng/ml), followed by GnRHa alone (2.0 ± 0.5 ng/ml) and finally the control (1.0 ± 0.2 ng/ml). Levels of 11-KT were significantly higher [$F_{(2, 23)} = 6.6901$, $p = 0.022$] in the treatments than in control at day 10.

A series of milt quality characteristics were assessed and analyzed prior to implantation (day 0) and 20 days after initiation of treatments (Table 4). In summary, there was no effect of treatment with GnRHa alone or in combination with pimozide on sperm volume, motility, spermatocrit, or density. Irrespective of treatment, the expressible volume of milt obtained was low, ranging from approximately 180 to 265 µl per individual male. In the GnRHa + Pimozide and control treatment groups, milt volume increased slightly after the first implants were administered but no significant

changes were observed. Sperm density ranged from 7.4 ± 1.6 to 9.5 ± 3.2 x 10^{10} spermatozoa/ mL (± SEM) during the trial period.

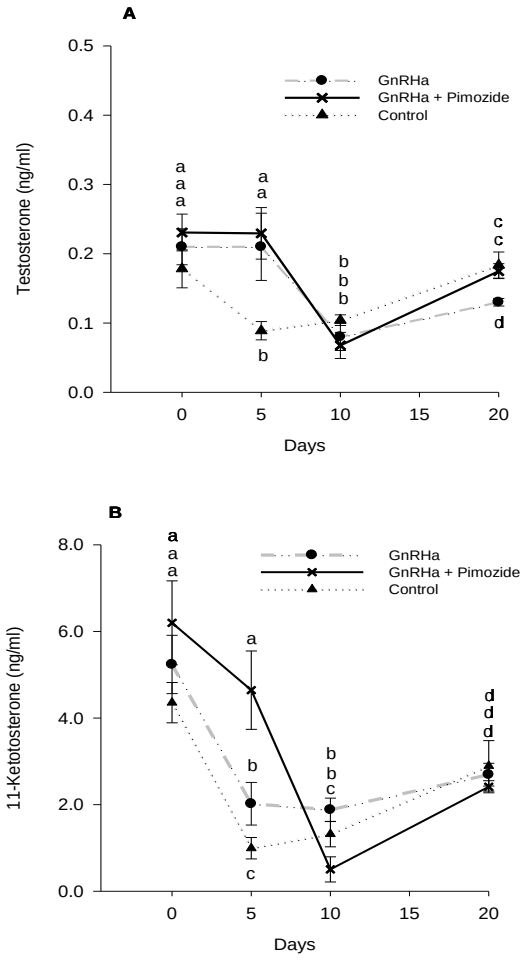

Figure 5. Plasma levels of testosterone (A) and 11-ketotestosterone (B) in male common snook broodstock treated with 'zero load' (Control), GnRHa, and combined Pimozide + GnRHa. Plasma levels were measured on days 0, 5, 10 and 20 from initiation of treatments. Lower-case letters indicate significant differences given sampling points. Data are expressed as a mean ± SEM (n = 8).

Table 4. Mean (± SEM) total expressible milt, duration sperm motility, spermatocrit and sperm density of captive male common snook broodstock (n = 8) treated with GnRHa alone or in combination with the dopamine antagonist, pimozide, prior to hormonal induction (day 0) and 20 days post hormonal implantation. Different superscripts indicate significant differences between treatments at that sampling time (ANOVA, p = 0.05).

	Treatment group	Day 0	Day 20
Total milt volume (µl)	GnRHa	225 ± 30	211 ± 10
	GnRHa + Pimozide	181 ± 25	230 ± 35
	Control (Sham)	213 ± 38	261 ± 40
Duration of sperm motility (s)	GnRHa	208 ± 15	183 ± 10
	GnRHa + Pimozide	201 ± 9	192 ± 8
	Control (Sham)	189 ± 14	178 ± 11
Percentage of motile sperm (%)	GnRHa	≥ 75 %	≥ 75 %
	GnRHa + Pimozide	≥ 75 %	≥ 75 %
	Control (Sham)	≥ 75 %	≥ 75 %
Spermatocrit (%)	GnRHa	44.2 ± 4.0	37.4 ± 2.9
	GnRHa + Pimozide	38.9 ± 4.0	37.9 ± 5.1
	Control (Sham)	43.8 ± 4.0	42.0 ± 4.6
Spermatozoa ($x10^{10}/ml^{-1}$)	GnRHa	7.92 ± 1.6	7.52 ± 1.74
	GnRHa + Pimozide	8.64 ± 2.2	9.55 ± 3.27
	Control (Sham)	9.02 ± 1.8	7.41 ± 1.60

4. Discussion

Despite the importance of male reproductive potential in recruitment success this question has not been studied in common snook therefore, the present work focused on the effects of GnRHa-based hormonal treatment on gonadal sex steroids and milt production in captive male broodstock. In the first study, treatment of male common snook with a single dose of either slow or regular release GnRHa at 100 $\mu g/kg^{-1}$ bw had no effect on milt production. Similarly, the second experiment showed that repeated administration of GnRHa combined with the dopamine antagonist (pimozide), had no effect on the quality or volume of milt produced by male snook broodstock when compared with GnRHa alone. In contrast, a single treatment of regular release GnRHa administered at 50 $\mu g/ kg^{-1}$ bw, induced and synchronized ovulation and spontaneous spawning of captive common snook females up to 72 h post-implantation.

The control and synchronization of reproduction in captive fishes constitutes the first prerequisite to attain sustainable commercial aquaculture production. The absence of adequate environmental cues and the stressors imposed by confinement can directly impact the neuroendocrine regulation of gametogenesis and therefore, the application of exogenous hormonal treatments are routinely used as an effective method to induce spawning in captive fishes (Mylonas et al., 2010). In aquaculture, treatment with GnRHa-delivery enhanced gamete production in a number of commercially important species including European sea bass *Dicentrarchus labrax* L. (Forniés et al., 2001; Prat et al., 2001), gilthead seabream *Sparus aurata* L. (Barbaro et al., 1997) and turbot *Scolphtalmus maximus* (Muginer et al., 2000). Studies have also shown hormonal

induction with GnRHa increased volume and quality of male milt in a variety marine species including Atlantic cod *Gadus morhua* (Garber et al., 2009); greenback flounder *Pleuronectes ferrugineus* (Lim et al., 2004) and Senegalese sole *Solea senegalensis* (Guzmán et al., 2011). GnRHa acts directly at the level of the pituitary to induce release of the endogenous LH which, in turn act at the level of the gonad to induce steroidogenesis.

Despite the success of GnRHa treatments among a variety of fish species, these have sometimes been shown to be ineffective when used alone. This is due to a strong dopaminergic inhibition of gonadotropin secretion leading to incomplete or arrested gametogenesis at the later steps of oocyte maturation in females and spermation for males (Dufour et al., 2010). In some, but not in all teleosts species, dopamine exerts inhibitory actions on both the brain and pituitary through reduction of GnRH synthesis and release, down regulation of GnRH receptors and interference with the GnRH signal transduction pathways, thus affecting secretion from the pituitary (Chang et al., 1984). Results obtained from milt evaluation and plasma steroid analysis conducted in this study clearly showed a lack of effect of PIM on males, suggesting again the absence of an inhibitory dopaminergic action on the reproductive axis of male common snook breeders.

Sex steroids are of prime importance in the feedback control of reproductive development and provide an indicator to the brain and pituitary as to the reproductive status of the individual (Diotel et al., 2011). In females E_2 and T have important roles in reproduction. As ovarian recrudescence begins E_2 is secreted by the ovarian follicle and stimulates the hepatic synthesis and secretion of vitellogenin which is taken up by

the oocyte (Kime, 1993). Testosterone and 11KT, produced in the Leydig cells within the testis, are the dominant sex steroids involved in spermatogenesis and spermiogenesis in male teleosts (Schulz et al., 2010). In the present study, the annual plasma sex steroid profile levels among captive male and female common snook broodstock were higher in year one than year two of the trials. Overall, peak levels of T and E_2 were lower in captive female broodstock when compared with than those previously reported among wild female common snook (Roberts et al., 1999). When compared with wild males (Roberts et al., 1999), peak levels of 11-KT in captive male broodstock were similar in the first year of the trial but almost 2-fold lower in the second year. The significantly lower estrogen and androgen levels taken together with the reduced number of consecutive spawns and shorter spermiation period for males in all treatment groups suggests that these factors such as may have led to diminished reproductive performance.

Results from these studies confirmed the suitability of the environmental conditioning for matiring both male and female snook broodstock. Although females were successfully induced to spawn using hormonal therapies, only one spawn was documented from broodstock in tank B compared to multiple spawns observed from the other broodstock group (tank A). Despite the use of the same ambient natural photo-thermal conditioning regime during both trials, females failed to undergo oogenesis in the second trial and were unable to be implanted and no spawns occurred. Over the study period, broodstock in tank B were blood sampled monthly to monitor the seasonal reproductive cycle through sex steroid profiling, leading them to be handled almost two times more frequently than broodstock in tank A. The absence of reproductive activity in tank B during the second trial could have been caused by the

stress of monthly handling which involved cannulation biopsies in addition to the repeated blood sampling. Males were also repeatedly handled during the hormonal manipulation trials. In trial one, milt production was reduced (400 to 200 µl) by 50% in all three treatment groups.

Research has shown tolerance and physiological response to stress varies among fish species (Schreck et al., 2001). Stress factors, such as handling and hormonal induction, are known to have a negative effect on maturation and spawning in fish (Barton, 2002; Wendelaar Bonga, 1997). Such effects may have altered gamete and offspring viability (Bobe and Labbé, 2010); impacted androgen production and spermiation response in males (Castranova et al., 2005); altered spawning behavior (McConnachie et al., 2012; Morgan et al., 1999); advanced or delayed oocyte maturation and ovulation in females (Watanabe et al., 2005), induced follicular atresia (Micale et al., 1999), and reduced egg production (Bogevik et al., 2012; Milla et al., 2009). In captive common snook, extensive handling appears to have a negative impact on maturation and spawning and further investigations are needed to define the limits so optimal broodstock management strategies can be implemented.

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CHAPTER 4

RESEARCH ARTICLE

PARENTAL CONTRIBUTION AND SPAWNING PERFORMANCE IN CAPTIVE COMMON SNOOK *CENTROPOMUS UNDECIMALIS* BROODSTOCK

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Keywords: common snook, parental analysis, microsatellites, reproduction

Abstract

Common snook are a species relatively new to aquaculture and to date, virtually no information is available on captive broodstock spawning characteristics. Understanding basic and fundamental data such as broodstock contribution of captive mass spawning snook is important, not only for the development of a successful selective breeding program for the species, but also for restocking wild fisheries and maintenance of local genetic variation. A scoping study was undertaken to explore the potential of DNA profiling for monitoring mating outcomes in captive snook. Spawning success was monitored among wild harvested broodstock that were undergoing hormonal treatment to induce spawning. The broodstock were maintained in three separate tanks (tank A 18 males and 15 females; tank B 22 males and 11 females; tank C 40 males and 16 females) and were subject to different handling stresses. Sixteen mass spawning events were studied across the three tanks over a 15 month period. DNA profiling of eight microsatellite markers was employed to detect and quantify individual parental contributions for 2,154 larvae obtained from the three tanks. The panel of loci was generally robust and allowed unambiguous assignment of 89% of larvae to a single family. All spawns occurred within approximately 24 to 72 hours post-implantation and only females implanted with gonadotropin releasing hormone analogue (GnRHa) were found to contribute. Overall, spawning performance among the three tanks was highly variable in terms of the total number of eggs produced (from 86,300 to 2,378,000 per spawn), fertilization success (from 17.0 to 87.3 %) and hatch rate (from 47.8 to 98.1%). Three-day larval survival ranged from approximately 25.9 to 90.1 % in tank A and 19.9 to 74.2 % in tank C. During this study, new information regarding requirements for broodstock husbandry, mating

patterns and spawning periodicity of captive common snook broodstock were obtained. Spawn contribution data 1) provided a confirmation of GnRHa treatment efficacy in female snook with a minimum stage of oogenesis (late secondary growth-SGl) required for successful spawning; 2) identified a potential impact of handling on maturation and spawning in male and female broodstock; 3) confirmed that, through photothermal conditioning and hormone manipulation, captive common snook broodstock can spawn over consecutive days and several times per year, including outside of their natural spawning season.

1. Introduction

Saltwater recreational fishing is a multibillion (US) dollar industry in the United States, with the economic output in Florida alone valued at $4.9 billion in 2011 (American Sport Fishing Association, 2013). The common snook *Centropomus undecimalis* is one of the three most popular gamefish in Florida, making them a vital part of an economically important sport fishery (Muller and Taylor, 2012). However, red tide events, cold kills, habitat loss and increased sport fishing pressure have left wild stocks vulnerable, prompting the Florida Fish and Wildlife Conservation Commission (FWC) to regularly assess the condition of wild stocks (Muller and Taylor, 2012). Bag limits and limitations on size and seasons for snook harvest have been implemented and a sizeable investment has been apportioned for fisheries managers to develop an effective marine fish stocking technology for rapid restoration of depleted stocks (Tringali and Leber, 1999; Tringali et al., 2008a). In Florida, aquaculture technologies are being developed at marine fish hatcheries to increase the production of fingerlings for release (Alvarez-Lajonchère and Taylor, 2003) and investigate stock enhancement as a fisheries management tool (Brennan et al., 2008). In Central and South America, common snook, along with several other *Centropomus* species, are also popular food fish and as such considered as emerging species for intensive aquaculture in North and South America. Collectively, they support high value commercial fisheries in those regions (Alvarez-Lajonchère and Tsuzuki, 2008). Despite recent breakthroughs in the spawning of captive common snook broodstock (Yanes-Roca et al., 2009; Ibarra-Castro et al., 2011; Rhody et al., 2013, Chapter 2) and advances in larval rearing protocols (Wittenrich et al., 2009; Rhody et al., 2010; Ibarra-Castro et al., 2011; Yanes-Roca et al., 2012b; Barón-Aguilar et al., 2013), to date, there

is still no established large scale production of this species for food or restocking for recreational fisheries. To date, major reproductive bottlenecks of captive snook broodstock include the failure of females to ovulate without hormonal manipulation, reduced milt production in males and inconsistent supply of high quality eggs and larvae.

Common snook mating behavior in the wild is believed to be complex and is still not fully understood (Taylor et al., 2000; Yanes-Roca et al., 2009; Trotter et al., 2012). Initial successes in developing spawning procedures with wild common snook involved the induction of ovulation with hormones and strip spawning to obtain eggs (Neidig et al., 2000). In more recent years, studies focused on photothermal conditioning and hormonal manipulation to induce oocyte maturation, ovulation and volitional spawning of captive broodstock. Snook broodstock were successfully induced to spawn in captivity by implanting females with gonadotropin releasing hormone agonists (GnRHa) (Rhody et al., 2010; Ibarra-Castro et al., 2011; Rhody et al., 2013). To improve aquaculture technologies and increase the production of common snook fingerlings for food consumption or fisheries enhancement studies, a better understanding of the environmental, behavioral and social conditions that promote spontaneous spawning is required.

Genetic management in aquaculture and supplemental stocking programs also requires careful consideration, since maintenance of genetic diversity is a key biological requisite for population resilience and productivity (Duchesne and Bernatchez, 2002; Brown et al., 2005; Tringali et al., 2008a). When a restricted number of breeding individuals are available, conservation of molecular and quantitative genetic variation in managed populations requires maintenance of sufficiently large

(genetic) effective population sizes (Tave, 1993; Lorenzen et al., 2010). Common snook are mass spawners with spawning naturally taking place in relatively large breeding groups. Although this reproductive strategy can allow for the production of a large quantity of offspring, individual mating patterns and reproductive success cannot be easily monitored or assessed under standard captive culture conditions (Gruenthal and Drawbridge, 2012). Further, this mating tactic can potentially lead to highly skewed levels of individual parental contributions and large variability in family size. In such conditions, census population size is extremely unlikely to be a reliable indicator of the underlying effective population size.

Maintaining genetic variation and pedigree information has proven to be a critical component of the successful commercial-scale culture of many freshwater and marine fish species. The development of DNA-based genetic markers has had a revolutionary impact on terrestrial and aquatic animal breeding and selection (Georges 2001; Liu and Cordes, 2004; Migaud et al., 2013). In the field of fisheries and aquaculture, microsatellite markers have been used successfully in a number of ways including characterization of genetic stocks (Cushman et al., 2012; Heard 2012), broodstock selection (Hayes et al., 2007), mapping economically important quantitative traits and identifying genes responsible for these traits (Chistiakov et al., 2006). DNA profiling of parents and offspring have allowed for individuals to be assigned to family groups in many mass spawning species including barramundi, *Lates calcarifer* (Frost et al., 2007; Loughnan et al., 2013; Domingos et al., 2014), European sea bass, *Dicentrarchus labrax* (Massault et al., 2010) and Atlantic cod, *Gadus morhua* (Herlin et al., 2008). Genetic markers have also been used extensively as a management tool to identify and monitor hatchery fish reared for stock replenishment including species

such as brown trout, *Salmo trutta* (Taggart and Ferguson 1986), red drum, *Sciaenops ocellatus* (Tringali et al., 2008b; Saillant et al., 2009; Gold et al., 2010) and white seabass, *Atractoscion nobilis* (Gruenthal and Drawbridge, 2012). A number of DNA microsatellites suitable for genotyping have been developed for use in population assignment studies of common snook in Florida (Wilson et al., 1997; Seyoum et al., 2005). The isolation and characterization of 27 polymorphic loci for common snook was initially to allow potential genetic differences among wild populations originating from Florida's Atlantic and Gulf of Mexico waters (Seyoum et al., 2005; Tringali et al., 2008a).

To date, no data on spawning dynamics, including individual spawning performance, have been reported for common snook in captivity. As a first step in this direction, we initiated a scoping study employing microsatellite-based DNA profiling to assign parentage within a subset of captive broodstock maintained at Mote Marine Laboratory, Sarasota, Florida, USA. Screening was carried out on parents and offspring from three brood tanks in which hormonal treatments for volitional spawning were being trialed. In addition to an assessment of the DNA profiling technique, the study provided useful insights into the efficacy of hormonal treatments and fish husbandry on the spawning performance of individuals and its implications for monitoring and maintaining genetic variation within the captive stock.

2. Materials and Methods

2.1. Broodstock collection and maintenance

Wild adult common snook were collected from three locations in Sarasota County (Florida, USA) and transported to Mote Aquaculture Research Park (Sarasota,

Florida, USA) in 2009. Following a 40-day quarantine period, broodstock were PIT-tagged and a fin tissue sample was taken from each individual for DNA analysis. Tissue samples were stored in 95 % ethanol until the time of processing.

Collected broodstock were divided among three separate, indoor, photoperiod (10-15H light) and temperature (20-30°C) controlled recirculating tank systems (A, B, C) (Figure 1). Tank A contained 18 males and 15 females; Tank B had 22 males and 11 females; Tank C had 40 males and 16 females (Table 1). Systems A and B each consisted of a 4.6 m diameter, green, fiberglass tank with a total system volume of 28 m^3 whereas tank C was 6.1 m in diameter and had a total system volume of 48 m^3. Salinities were maintained at 35 ‰. Temperature was controlled in each tank system by cycling water through an individual heater/chiller unit (AquaCal, St. Petersburgh, FL, USA). Filtration included a drop filter (Aquaculture Systems Technologies, New Orleans, LA, USA) for the collection of fine solids, 900 L moving bed for bio-filtration, protein skimmer and an ultraviolet light (UV) sterilization unit.

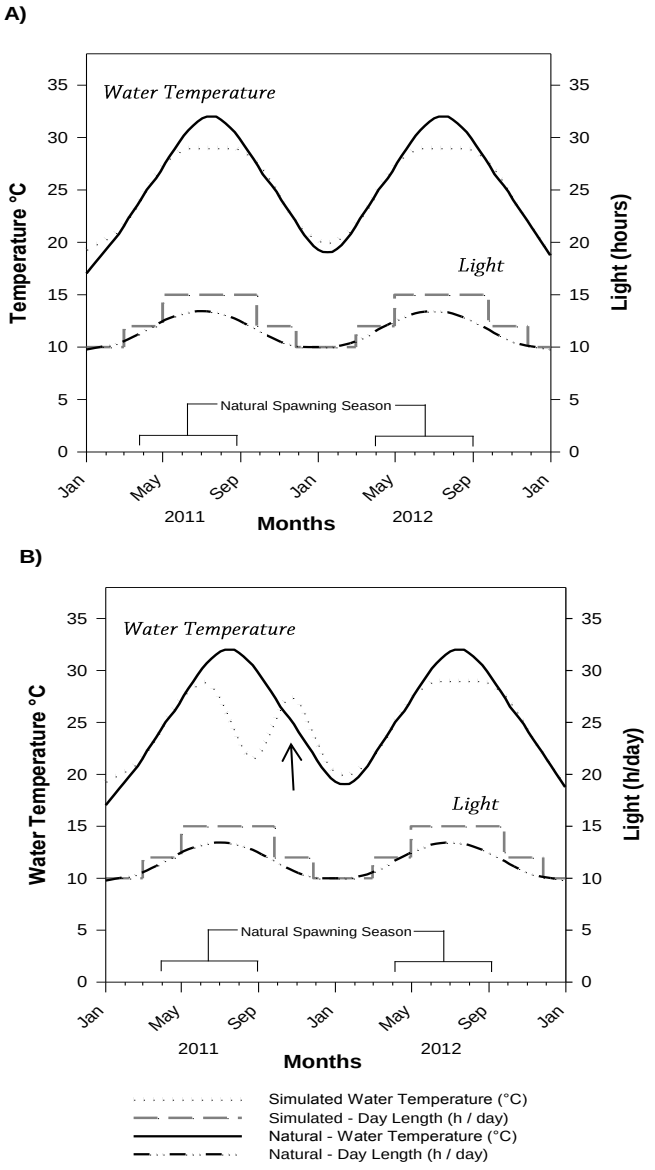

Figure 1. Schematic representation of natural and artificial (simulated) environmental conditions associated with the annual reproductive cycle of wild common snook located on the Gulf coast of Florida and captive common snook broodstock held at Mote Aquaculture Research Park, Sarasota, FL from the years 2011 to 2012. Simulated photo-thermal cycle used to mature and spawn captive broodstock in (A) Tanks A and B. Simulated photo-thermal cycle used to mature and spawn captive broodstock in (B) Tank C showing out-of-season spawning (→). Natural ambient cycle of day length (light h/day) (▬ ▬) and water temperature (°C) (▬) in Tampa Bay, FL. Imposed photo-thermal cycle used to mature and spawn captive broodstock including day length (light h/day) (▬ ·· ▬) and water temperature (°C) (▪▪▪)

Table 1. Description of captive common snook broodstock populations held at Mote Aquaculture Research Park. Weight and fork length (FL) ± SEM.

Broodstock Tank	Total No. Fish/Tank	Total No. Males/Females	Male: Female Sex Ratio	Female Mean Weight (kg)	Female Mean Length (cm)	Male Mean Weight (kg)	Male Mean Length (cm)	Tank Biomass (kg/m^3)
A	33	18 / 15	1.2 : 1	3.4 ± 0.4^{a}	68.4 ± 2.4^{a}	2.1 ± 0.1^{a}	60.1 ± 1.1^{a}	3.2
B	33	22 / 11	2 : 1	2.5 ± 0.3^{a}	60.9 ± 2.3^{a}	2.1 ± 0.2^{a}	59.1 ± 1.5^{a}	2.6
C	56	40 / 16	2.5 : 1	3.4 ± 0.3^{a}	68.9 ± 2.0^{a}	2.7 ± 0.2^{b}	63.5 ± 1.6^{a}	3.4

Means with different letters in the same column are significantly different ($P < 0.05$).

2.2. Broodstock sampling and hormonal induction

Over the study period (from April 2011 to June 2012, 15 months), snook in tanks A and C were handled a total of seven times. In contrast, snook in tank B were handled much more frequently (monthly intervals), to obtain blood samples from each individual for seasonal sex steroid profiling. Snook in tanks A and C were not blood sampled during the study. The procedures described below were conducted at all handling events.

To sample the broodstock the tank water level was lowered. Two dividers, made from plastic mesh stretched across a polyvinyl chloride (PVC) pipe frame, were used to corral the fish into a section of the tank. From this restricted section, individual fish were netted into a 500 L tank containing 200 L of water and anesthetized with tricaine methanesulfonate (MS-222) at a concentration of 300 ppm for approximately 1-2 minutes. All male and female common snook were weighed (to the nearest gram) and measured (total length, TL, mm). Female broodstock were biopsied and the reproductive status of each individual was assessed using a classification proposed by Grier et al. (2009) and adapted to common snook according to Rhody et al. (2013, Chapter 2). Not all handling events corresponded with the hormonal induction of females. In total, two sampling events in tank A, one in tank B and five in tank C utilized hormonal implantation. Only females determined to have oocytes in early secondary growth (SGe) or the later stages of the oogenetic cycle were implanted with GnRHa (Institute of Marine and Environmental Technologies, University of Maryland, Baltimore, MD, USA) at a dosage of 50 µg/kg bodyweight. Males were not implanted during this study.

2.3 Larval sampling

A total of 16 mass spawning events were documented during the study. Broodstock typically spawned during early to late evening where a spawning event or 'spawn' was defined as the sum of eggs or offspring produced during a single evening. Following each nightly spawning event, eggs were transferred from the broodstock tank to 100 L hatching tanks. At 4-6 h post fertilization (blastula stage), aeration was removed and non-viable (sinking) eggs were discarded. At 17 h post fertilization, approximately 250 newly hatched larvae (volumetrically measured) were stocked into individual microcosms. Accuracy of initial stocking ranged from 231 to 268 larvae per microcosm. The microcosms (25 for each spawn), made of PVC (2.6 cm diameter), were held in a shallow rectangular recirculating raceway tank system equipped with UV sterilization. The base of each microcosm was covered with a 200 µm nylon mesh to prevent the escape of larvae, while allowing circulation of the water. Temperature in the raceway tank was maintained at 28 ± 1 °C.

To assess daily larval survival for each spawn, the total number of live larvae from each of five microcosms were counted on days 1, 2 and 3 post-hatch (total of 15). An additional 150 three-day-old snook larvae were randomly sampled from the remaining ten microcosms (20 larvae per microcosm) and individually stored in 95 % ethanol until they could be genotyped.

2.4 DNA extraction

Genomic DNA was extracted from all 122 snook broodstock (fin clip biopsy) within the three spawning tanks and approximately 2,200 three-day-old snook larvae (whole animal) using the PureGene DNA Extraction kit (Qiagen, USA) according to

the manufacturer's instructions. Each sample was digested with 300 µl of cell lysis buffer, 2 µl of proteinase K (20 mg/µl), and incubated overnight at 55 °C. To increase DNA yield, 5 µl of glycogen (5 mg/ml) was added to the precipitation step and the DNA re-suspended with 30 µl of hydration solution. DNA was quantified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, USA). Adults and larvae yielded an average of 500 ng/µl and 10 ng/µl of pure DNA respectively. All samples were stored at 4 °C prior to PCR amplification.

2.5 DNA microsatellites and PCR amplification

Eight polymorphic microsatellite markers, Cun01, Cun08, Cun19, Cun10A, Cun18, Cun11, Cun14 and Cun16 (Seyoum et al., 2005) were assayed in three robust PCR multiplexes (detailed in Table 2). Each 12.5 µl PCR reaction consisted of 0.3 U of GoTaq (Promega, Madison, WI, USA), 2.5 µl 5 x GoTaq Buffer, 0.2 mM each dNTP, 3 mM $MgCl_2$, 1.25 mg/ml BSA, 0.8 µM each primer, and 5 to 20 ng DNA template (150 ng/µl for adults). Thermal cycling parameters for all amplifications were: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C 30 s, and a final extension of 7 min at 72 °C. PCR reaction products were stored at 4 °C until genotyped.

Table 2. Details of the eight polymorphic microsatellite markers used in the present study.

Locus	Primer sequence (5′ – 3′)	Annealing Temperature (°C)	Repeats	No. Of Alleles	Allele Size Range	Multiplex	Primer Label	H_E (unbiased)	H_o	Fis *
Cun01	F: AAGTTCGCTCCCTCTCACTT R: GATACACATTGCCCTCAAG	55	$(AC)_{26}$	12	117-149	1	NED	0.767	0.779	-0.015
Cun08	F: TTCTTTTCACTGCTTCTGTCTG R: GTAAACCCGGTCGATTCTTC	55	$(TG)_9(TA)_{10}$	13	168-202	1	HEX	0.799	0.803	-0.006
Cun19	F: AGCCAGCGAAGGCCAATGT R: AGCCAGCGAACACACACTCA	55	$(TG)_{16}/(TG)_9$	6	152-164	1	FAM	0.516	0.533	-0.033
Cun10A	F: CCCCAAGGATCGTCTATCTC R: TAACATTTCACACGGTGCTG	58	$(CA)_{18}$	23	158-216	2	FAM	0.922	0.959	-0.040
Cun18	F: CTGACAGCTCAGTGCGTCT R: TGTAAACAGAAGGCGGTCAT	58	$(TG)_{12}$	9	111-135	2	HEX	0.757	0.836	-0.104
Cun11	F: CACATGCAAAGAGACTGCAC R: GGAGGGAAAAACGACTTGAT	55	$(AC)_6(ATGT)(GC)_3(AC)_{13}$	17	150-204	3	NED	0.795	0.754	0.052
Cun14	F: GCCTGTCATCTCACTGGGTA R: GTCTTTGATCTGCCCGTTTA	55	$(TG)_{36}$	13	173-215	3	FAM	0.738	0.746	-0.011
Cun16	F: TATTGCTGTGAGCAGATGGA R: ATAGCCTGCAGTTCTTGGAA	55	$(TG)_6(CG)(TG)_3/(TG)_4$	7	119-141	3	HEX	0.752	0.721	0.041

* Fis: Computed as in Weir & Cockerham (1984).

2.6 Genotyping and parentage analyses

Each PCR multiplex was screened with 1 µl of each PCR product being added to 12 µl Hi-Di formamide containing 0.86 µl Home Made ROX DNA size standard (De Woody et al., 2004). Microsatellite alleles were detected and sized on an ABI 3130xl genetic analyzer (Applied Biosystems, Carlsbad, CA, USA) and scored with GeneMapper v4.0 (Applied Biosystems, Carlsbad, CA, USA). The potential presence of null alleles and genotype errors in the broodstock were tested with Micro-Checker version 2.2.3 (Van Oosterhout et al., 2004). All adult samples were screened twice to maximize genotyping accuracy. The genotyping data were initially interpreted using

the exclusion based program Probmax (Danzmann, 1997) and further confirmed with the Family Assignment Program, FAP (Taggart, 2007; Herlin et al., 2007). Both programs provided the user with an assignment mode that allows for the identification of all possible parental combinations for each offspring; however, a predictive mode to calculate the resolving power of specific genotypic data sets was only available in FAP.

3. Results

3.1 Genotyping

All broodstock were genotyped across the eight loci on two separate occasions. No genotyping inconsistencies were found between these two screenings. FAP predictive mode was used to compute the power of the eight locus microsatellite panel to unambiguously assign parentage in each of the three experimental tanks (Table 3). Mean assignment rates among families within tanks ranged from 95% - 99%. Similar statistics were also calculated for seven loci (dropping the problematic Cun14 locus; see below). Mean predicted resolving power was reduced to 91% - 97% among tanks (Table 3).

Table 3. Computation of the resolving power of microsatellite panels within the three experimental tanks. The proportion of offspring per family that should be unambiguously assignable to a single family are given. Eight and seven loci options are considered. The calculations, performed using FAP (Taggart, 2007), were based on the known parental genotypes within each spawning tank and assume that all sire x dam combinations were equally likely to occur. Figures in parentheses refer to the number of potential different mating combinations possible, given the numbers of sires and dams present in each tank.

		Tank A (270)	Tank B (242)	Tank C (640)
All 8 loci	Mean:	0.99	0.99	0.95
	SD:	0.02	0.02	0.06
	Min:	0.87	0.88	0.67
	Max:	1.00	1.00	1.00
7 loci (exc. Cun14)	Mean:	0.97	0.96	0.91
	SD:	0.04	0.04	0.10
	Min:	0.77	0.82	0.41
	Max:	1.00	1.00	1.00

Of 2,200 offspring that were initially screened for eight loci, complete genetic profiles were obtained for 2,154 individuals. Of these 74 % assigned to at least one family under the stringent exclusion model (i.e. assuming no genotyping error at all) – with 26% not assigning to any expected family. Using a more realistic model for practical genotyping, i.e. allowing up to one allele mismatch across the 8 loci composite genotype per progeny, 98% of individuals were assigned to families with 89 % unambiguously assigned to a single family. Thus, actual assignment rates were in line with predicted expectations (see above). Inspection of the data for these single match assignments showed that 54% of the identified genotyping errors occurred for

locus Cun14, and involved miscalling of alleles from adjacent bins. The remainder of genotyping miscalls were spread evenly across all other loci and again involved either alleles resolving on bin boundaries or weak samples where allelic dropout resulted in heterozygous individuals being mis-scored as homozygous for the smaller allele. There was no evidence of null alleles segregating. A further 9% of progeny assigned to multiple families. In all these multiple-match cases, at least one of the families implicated was a confirmed spawning pair (from unambiguous single family matches). Finally, approximately 2% of progeny (n=44) required mismatches at 2-4 alleles to be permitted in order to assign to potential parental crosses. Multiple families were identified for each offspring and, in all cases, implicated at least one confirmed spawning pair. Invariably, inspection of trace files revealed poorly resolved / ambiguous profiles at one or more loci. These individuals were not considered further. Assignments were made using both PROBMAX and FAP software, and results concurred.

3.2 Hormonal induction

During the study period, all spawns occurred within approximately 24-72 hours post implantation and only females implanted with GnRHa were found to contribute (Table 4). Progeny testing revealed that offspring sampled at 3 days post-hatch were only spawned from females that, at the time of hormonal implantation, had a minimum oocyte developmental stage of late Secondary Growth (SGl). Total parental contribution from females with oocytes in SGl was 20.0 % and 74.2 % for females with oocytes staged as Secondary Growth full grown (SGfg). Progeny were identified from 35 of 48 (73 %) hormonally treated females (Table 4). No progeny were detected from

the five implanted females identified as having the majority of their oocytes in early Secondary Growth (SGe). Total spawn contribution from females implanted at different stages of oocyte development is presented in Table 4.

Table 4. Relationship between the timing of hormonal implantation, oocyte stage of development and total female spawn contribution for sixteen mass spawning events observed in three captive common snook broodstock populations.

Oocyte Development (Stage)	Oocyte Development (Step)	Abbreviation	No. Females Implanted (*n*)	No. Females Spawned (*n*)	Females Implanted (%)	Females Spawned (%)	Total Spawn Contribution (%)	No. Fish Spawned 1 day post-implantation (*n*)	No. Fish Spawned 2 days post-implantation (*n*)	No. Fish Spawned 3 days post-implantation (*n*)
SECONDARY GROWTH (SG)	early	SGe	5	0	10.4	0	0	0	0	0
	late	SGl	9	7	18.8	77.8	20.0	6	7	5
	full-grown	SGfg	32	26	66.7	81.3	74.2	23	13	3
OOCYTE MATURATION (OM)	eccentric germinal vesicle	OMegv	1	1	2.1	100.0	2.9	1	0	0
	germinal vesicle migration	OMgvm	1	1	2.1	100.0	2.9	1	1	1
	preovulatory	OMpov	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

3.3 Spawn performance

From April 2011 to June 2012, five spawning events occurred in tank A, one in tank B and 10 in tank C (Table 5). In tank A, successive spawning over a maximum of three consecutive days was observed in six female and eight male broodstock with one female and four males contributing in both years 2011 and 2012 (Table 6A, B). In tank C, successive spawning over consecutive days was observed in five females and eighteen males with > 50% spawning at multiple times in the same year (Table 7A, B). Results from the November 2011 spawning event in tank C showed photo-thermal conditioning of snook outside their natural spawning season (April to September in Florida) is possible (Table 7A, B; Figure 1)

Table 5. Common snook broodstock spawning performance in three mass spawning tanks following hormone induction. Data presented represents number of eggs collected, fertilization rate, hatch rate and three day larval survival ± SEM (n=3).

Broodstock Tank	Spawning Event (mm/dd/yyyy)	Number of Males / Females Spawned	Total Male / Female Spawn Contribution (%)	Number of Eggs Collected	Fertilization Rate (%)	Hatch Rate (%)	Survival 1 DPH (%)	Survival 2 DPH (%)	Survival 3 DPH (%)
A	Jun 15, 2011	9 / 4	50 / 26	190,000 ± 11,000	69.3 ± 2.1	47.8 ± 5.5	91.8 ± 2.1	87.6 ± 4.3	79.3 ± 2.6
	Jun 16, 2011	10 / 6	55 / 40	204,700 ± 31,700	17.0 ± 3.0	78.2 ± 3.7	97.3 ± 0.5	95.8 ± 1.9	90.1 ± 1.9
	May 22, 2012	3 / 2	17 / 13	272,000 ± 15,159	60.7 ± 0.5	56.3 ± 2.9	92.2 ± 1.2	61.4 ± 4.1	25.9 ± 2.3
	May 23, 2012	2 / 3	11 / 20	239,100 ± 5,253	65.1 ± 2.3	50.6 ± 3.9	67.5 ± 3.6	60.9 ± 2.0	60.7 ± 2.3
	May 24, 2012	2 / 2	11 / 13	79,500 ± 4,642	70.1 ± 4.1	91.0 ± 0.6	97.0 ± 0.8	77.3 ± 3.2	76.3 ± 3.1
B	May 24, 2012	2 / 1	9 / 9	86,300 ± 4,439	34.4 ± 4.8	64.3 ± 6.2	80.6 ± 3.9	74.6 ± 4.8	68.5 ± 3.8
C	May 12, 2011	19 / 10	48 / 63	2,075,300 ± 60,103	62.5 ± 2.1	78.8 ± 0.2	76.4 ± 7.7	74.2 ± 4.1	59.9 ± 2.5
	Jul 6, 2011	6 / 4	15 / 25	964,600 ± 39,443	68.8 ± 1.9	84.0 ± 2.3	81.1 ± 2.6	78.9 ± 2.0	53.0 ± 3.1
	Jul 7, 2011	12 / 5	30 / 31	1,310,000 ± 47,849	55.7 ± 1.0	86.0 ± 2.0	90.2 ± 1.8	59.7 ± 1.9	27.5 ± 2.7
	Jul 8, 2011	11 / 3	28 / 19	656,730 ± 8,806	40.4 ± 2.7	98.1 ± 0.4	72.3 ± 4.9	64.4 ± 3.2	49.4 ± 5.2
	Nov 10, 2011	13 / 2	33 / 13	583,400 ± 22,385	63.9 ± 0.4	93.0 ± 1.9	65.3 ± 5.1	45.9 ± 3.5	28.5 ± 1.6
	Apr 6, 2012	15 / 4	38 / 25	906,100 ± 8,662	22.8 ± 1.4	90.9 ± 2.2	93.4 ± 1.9	88.1 ± 3.1	19.9 ± 2.1
	Apr 7, 2012	10 / 4	25 / 25	145,500 ± 6,936	55.4 ± 2.7	53.2 ± 1.8	76.7 ± 0.5	43.9 ± 7.2	27.7 ± 5.9
	Jun 6, 2012	10 / 4	25 / 25	2,378,000 ± 53,655	78.7 ± 1.4	69.8 ± 2.4	79.9 ± 3.3	68.7 ± 1.1	52.0 ± 5.4
	Jun 7, 2012	10 / 4	25 / 25	1,043,200 ± 15,247	63.6 ± 1.0	97.1 ± 0.5	85.5 ± 4.3	79.0 ± 1.4	74.2 ± 2.3
	Jun 8, 2012	5 / 2	13 / 13	375,000 ± 27,221	87.3 ± 1.7	83.6 ± 2.2	79.7 ± 3.0	77.8 ± 5.7	22.2 ± 1.9

Table 6. Female (A) and male (B) spawn contribution observed from 2011 to 2012 in a single captive common snook broodstock population (Tank A). Results were determined by exclusion based parentage using 8 DNA microsatellites for genotyping individual larvae (n =5 spawns).

A)

Date	Jun 15, 2011	Jun 16, 2011	May 22, 2012	May 23, 2012	May 24, 2012	Total No. Offspring	Percentage (%)
No. of larvae sired by females							
Female ID - Tank A							
F4	….	91	….	….	….	91	10.9
F5	….	….	25	2	….	27	3.2
F6	106	5	….	8	1	120	14.4
F9	11	14	….	….	….	25	3.0
F11	14	28	….	….	….	42	5.0
F12	39	10	….	….	….	49	5.9
F14	….	….	150	147	175	472	56.7
F15	….	6	….	….	….	6	0.7

B)

Date	Jun 15, 2011	Jun 16, 2011	May 22, 2012	May 23, 2012	May 24, 2012	Total No. Offspring	Percentage (%)
No. of larvae sired by males							
Male ID - Tank A							
M1	17	1	….	….	….	18	2.2
M3	1	8	….	….	….	9	1.1
M4	3	2	….	….	….	5	0.6
M6	1	3	10	….	….	14	1.7
M8	….	4	19	….	….	23	2.8
M9	17	57	….	….	….	74	8.9
M10	102	15	146	149	151	563	67.7
M13	9	14	….	….	….	23	2.8
M15	17	10	….	….	….	27	3.2
M17	….	40	….	8	….	48	5.8
M18	3	….	….	….	25	28	3.4

Table 7. Female (A) and male (B) spawn contribution observed from 2011 to 2012 in a single captive common snook broodstock population (Tank C). Results were determined by exclusion based parentage using 8 DNA microsatellites for genotyping individual larvae (n = 10 spawns).

A)

Date	May 12, 2011	Jul 6, 2011	Jul 7, 2011	Jul 8, 2011	Nov 10, 2011	Apr 6, 2012	Apr 7, 2012	Jun 6, 2012	Jun 7, 2012	Jun 8, 2012	Total No. Offspring	Percentage (%)
No. of larvae sired by females Female ID Tank C												
F29	…	…	1	…	…	…	…	…	…	…	1	0.1
F30	3	1	…	…	…	1	9	…	…	…	14	1.3
F31	…	…	9	…	…	9	150	1	6	…	175	16.1
F32	9	…	…	…	…	…	…	…	…	…	9	0.8
F34	13	37	8	80	42	…	1	65	47	41	334	30.7
F36	13	20	21	3	…	5	…	6	5	…	73	6.7
F37	12	…	…	…	…	…	…	…	…	…	12	1.1
F38	2	…	…	…	…	…	…	…	…	…	2	0.2
F39	1	…	…	…	…	…	…	…	…	…	1	0.1
F40	3	…	…	…	…	…	…	…	…	…	3	0.3
F41	4	…	…	…	…	…	…	…	…	…	4	0.4
F42	22	36	24	37	112	9	2	61	72	85	460	42.3

B)

Date	May 12, 2011	Jul 6, 2011	Jul 7, 2011	Jul 8, 2011	Nov 10, 20	Apr 6, 2012	Apr 7, 2012	Jun 6, 2012	Jun 7, 2012	Jun 8, 2012	Total No. Offspring	Percentage (%)
No. of larvae sired by males												
Male ID Tank C												
M39	6	20	…	3	…	…	…	…	…	…	29	2.5
M40	3	…	…	…	…	…	…	…	3	4	10	0.9
M42	…	…	…	1	…	…	…	…	…	…	1	0.1
M45	3	32	…	61	20	…	1	…	…	…	117	10.0
M46	3	19	2	5	34	8	…	…	…	…	71	6.1
M48	…	…	…	…	…	2	…	…	…	…	2	0.2
M49	…	…	…	…	…	1	…	…	6	7	14	1.2
M50	…	…	…	…	1	…	…	…	…	…	1	0.1
M51	…	…	2	…	…	1	2	79	…	…	84	7.2
M52	17	…	4	…	4	14	2	…	…	…	41	3.5
M54	…	…	…	…	1	…	…	6	72	…	79	6.8
M55	…	9	8	33	…	48	2	…	…	…	100	8.6
M56	2	…	…	1	48	…	…	3	26	3	83	7.1
M58	4	…	…	…	8	2	…	…	…	…	14	1.2
M59	5	…	21	…	5	…	…	…	…	…	31	2.7
M60	16	…	2	…	…	2	7	1	6	…	34	2.9
M62	…	…	…	…	…	1	6	…	…	…	7	0.6
M64	1	…	6	…	…	1	…	…	…	…	8	0.7
M65	2	…	…	2	…	…	…	29	3	…	36	3.1
M66	3	…	1	3	…	…	…	…	…	…	7	0.6
M67	1	…	6	…	…	…	…	…	…	…	7	0.6
M68	…	…	…	…	1	…	…	…	…	…	1	0.1
M69	…	…	…	…	12	…	…	1	…	…	13	1.1
M70	…	…	…	7	…	2	24	…	…	…	33	2.8
M71	2	…	2	…	…	…	3	12	2	…	21	1.8
M72	1	…	…	…	…	…	…	…	…	…	1	0.1
M73	6	…	…	…	…	…	…	…	…	…	6	0.5
M74	7	13	10	3	…	5	…	…	…	…	38	3.3
M76	1	…	…	…	…	5	116	…	8	43	173	14.8
M77	…	1	…	1	3	1	…	…	5	39	50	4.3
M78	…	…	…	…	…	…	…	2	…	…	2	0.2
M80	…	…	…	…	17	24	…	…	…	…	41	3.5

Overall, spawning performances between the three tank groups were highly variable in terms of the total number of eggs produced (from 86,300 to 2,378,000 eggs/spawn), fertilization (from 17.0 to 87.3 %) and hatch rate (from 47.8 to 98.1 %) (Table 5). The mean number of eggs produced per spawn in tank A was 197,060 ± 32,643 (SEM) whereas in tank C production averaged almost five times that at 1,043,783 ± 224,999 (SEM) eggs per spawn. Three day larval survival ranged from approximately 25.9 to 90.1 % in tank A and 19.9 to 74.2 % in tank C (Table 5). Mean larval survival in tanks A and C was recorded as 66.5 % and 41.4 % respectively. Only a single spawn was obtained from tank B therefore, no comparisons could be made with tanks A and C.

3.4 Parental contribution

Tank A: Approximately 50% of males had a detectable contribution in 2011 but only 11-17 % contributed in 2012 (Table 5). A similar pattern was observed for females with many more spawning in 2011 than in 2012. Overall, parental contributions were highly skewed, particularly in 2012. Two dams were responsible for 47.6 % of the offspring (202 out of 424 sampled) accounted for in 2011 from tank A (Table 6A). Similarly, in 2012, a total of 93.0 % (472 out of 508 sampled) were assigned to a single dam. Neither of the two females that were predominant contributors in year one contributed in year two. The opposite was found among the male broodstock in tank A. A single male sired up to 36 % of assigned fry in year one (117 out of 324) and the same male sired 88 % (446 out of 508) in year two (Table 6B). Five males made substantial contributions (siring 18-74 offspring) in 2011 while low levels of contribution (1-8 offspring) were detected for another five males (Table 6B).

Offspring were identified from two to six (13 to 40 %) of the total fifteen female broodstock and from two to ten (11 to 50 %) of the total eighteen male broodstock in tank A (Table 6A, B).

In contrast to tank A, only one spawn occurred in tank B with 100 % of the offspring assigned to a single female and two males (data not shown). Tank C: Male and female total spawn contribution was highly variable between spawning events in tank C. Throughout 2011 and 2012 spawning seasons, offspring were assigned to ≤ 63 % of female and ≤ 48 % of male broodstock in tank C (Table 5). Four of twelve females were repeatedly the top contributors, siring 1,042 larvae or 95 % of the total larvae assigned in tank C (Table 7A). Low levels of contribution were identified from the additional eight spawning female broodstock (1-14 offspring). Based on results obtained from preliminary studies evaluating the effect of hormonal therapy on milt production in male common snook broodstock (Rhody, Chapter 3), the number of males spawning in tank C was much higher than expected despite low fertilization rates observed across some spawning events (Table 5). Thirty-two of forty males spawned (80 %) overall; although the average proportion of males spawning per event was 28 % (Table 7B).

3.5 Impact of broodstock size on reproductive success

A single factor one way ANOVA (SPSS 19, IBM, USA) showed the average weight of captive female snook broodstock in tanks A, B and C were not significantly different (Table 1, P=0.11). In contrast, a statistical difference in weight was detected among males. Variations in these metrics were determined using Duncan's post hoc test where males in tank C were significantly heavier than males in tanks A and B (P =0.04,

F=3.2, df =2). Lengths were not significantly different for females (*P*=0.06) and males (*P*=0.10) in all tanks (Table 1). Size distributions of contributing males and females were varied and no size-assortative mating was observed in tank A or tank C (Figure 2A, B). In tank A, there was no significant correlation between male or female size and contribution to offspring (male regression, *P*=0.92, R^2=0.001, n=11; female regression, *P*=0.09, R^2=0.39, n=8) (Figure 3A, B). In tank C, there was no correlation between male size and number of offspring sired (regression, *P*=0.30, R^2=0.03, n=32; Figure 3C) however; a correlation between female size and contribution to offspring (regression, *P*=0.01, R^2=0.45, n=12) was observed (Figure 3D).

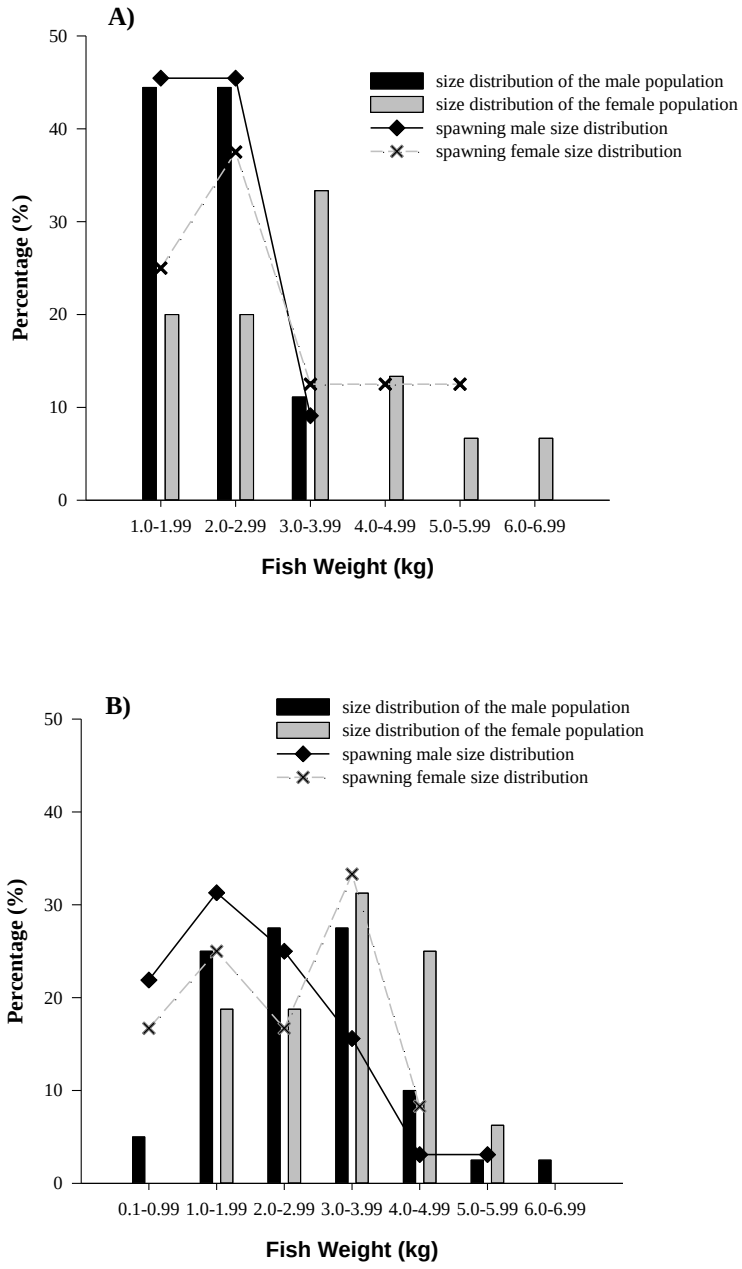

Figure 2. Relationship between the total numbers of offspring produced from 2011 to 2012 plotted against parental size distribution of female/male population (sire weight−dam weight in kg) for common snook broodstock in (A) Tank A and (B) Tank C.

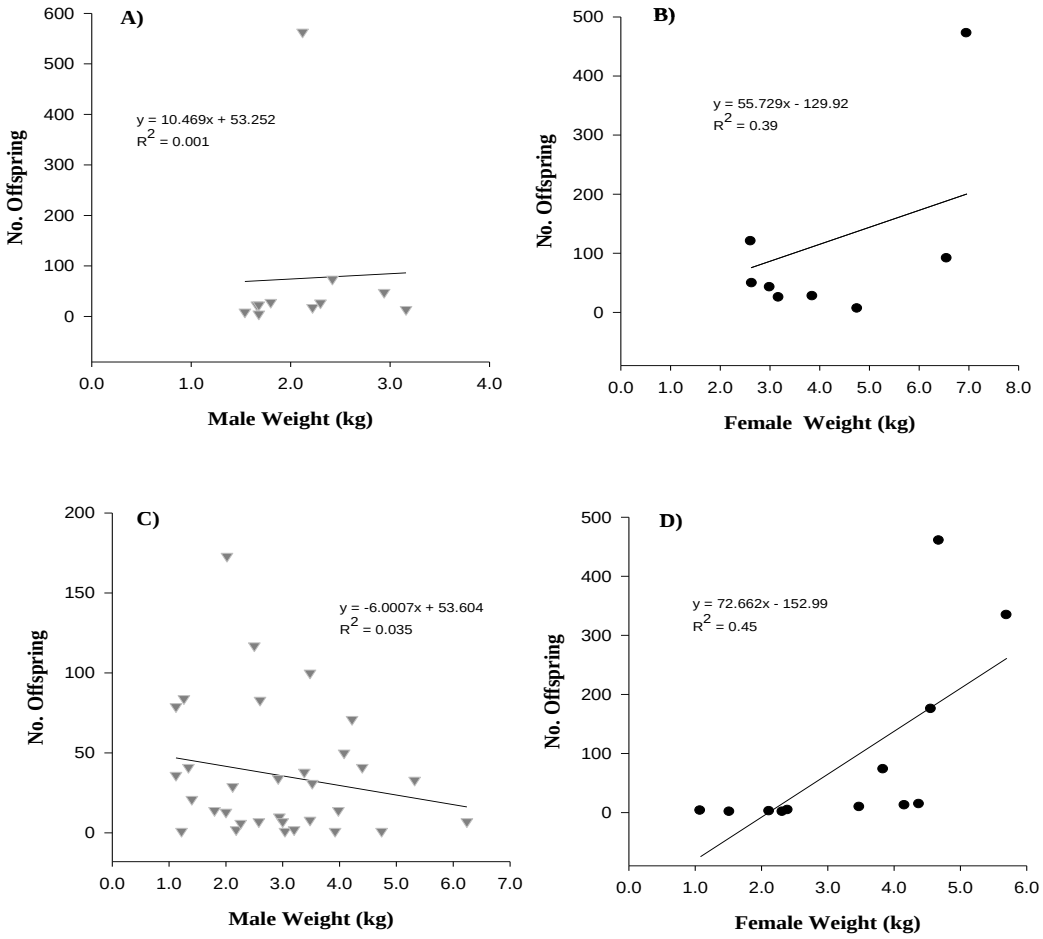

Figure 3. Scatterplot of male (▼) and female (●) body size (kg) versus contribution to offspring. Male (A) and female (B) body size (kg) versus contribution to offspring in Tank A (n=11, n=8). Male (C) and female (D) body size (kg) versus contribution to offspring in Tank C (n=32, n=12).

4. Discussion

While most genetic studies on snook populations have been directed toward conservation of wild stocks (Seyoum et al., 2005; Tringali et al., 2008a), the present study focused on using genetic markers for the improved management of captive common snook broodstock. In this molecular based assessment, new information on requirements for broodstock husbandry, mating patterns and spawning periodicity of captive common snook broodstock were obtained. More specifically, spawn contribution data 1) provided a confirmation of GnRHa treatment efficacy in female snook with a minimum stage of oogenesis (late secondary growth-SGl) required for successful spawning; 2) identified a potential impact of handling on maturation and spawning in male and female broodstock; 3) confirmed that, through photothermal conditioning, captive common snook broodstock can spawn over consecutive days and several times per year including outside of their natural spawning season.

Overall, the eight loci microsatellite panel performed well, giving robust data for all the parents and vast majority of offspring. The rate of genotyping errors within the offspring was 1.7%, mostly attributable to one locus (Cun14) where there was ambiguity between some adjacent allele bins. In future work, it may be prudent to replace Cun14 with an alternative marker. The exclusion based assignment worked well when one allelic mismatch was tolerated, an approach that is routinely taken (Pompanon et al., 2005), given the inevitable low level of error expected.

Since fish are held under enclosed conditions, reproductive bottlenecks observed in common snook broodstock, such as lack of spontaneous spawning, poor fertilization and low fecundity could be linked to one or more missing environmental

cues. Bottlenecks include few individuals initiating and completing the gametogenic cycle even with the use of hormonal therapies and low spermatogenic activity in males, with reduced milt production evidenced by inability or difficulty in stripping male snook (Rhody, Chapter 3). The relationship between the developmental stage of the gonads and required timing of hormonal stimulation in teleosts is well described (Zohar and Mylonas 2001; Mylonas et al., 2010); however, few studies in aquaculture species have linked hormonal treatment to the effective spawn contribution from individual broodstock during mass spawning events. Hormonal treatments given to immature individuals or to adults too early in the reproductive cycle are usually ineffective (Mylonas et al., 2010). In the present study, parentage assignment of the progeny showed GnRHa hormonal stimulation was effective when performed with females whose oocytes were classified in the later stages of vitellogenesis including Late Secondary Growth (SGl) or beyond. These results confirmed that the timing of implantation must be correlated with the stage of oocyte development in order to optimize broodstock spawning potential in common snook. Captive common snook males were not implanted during the spawning trials. Average fertilization rate from all spawns combined was 56.9 % indicating hormonal stimulation is not required for male spermiation to occur; however, future work may aim to investigate the effect of hormonal manipulation on male spawn performance and quality.

Parental contribution has already been studied in a range of teleost species with many reports showing skewed contribution (Coleman and Jones 2011), especially in captive male broodstock, potentially leading to a reduction in genetic diversity (Borrell et al., 2011; Liu et al., 2012). This was confirmed in the present study, although in some of the spawning events and tanks, up to 50 % of the female and/or male snook

broodstock significantly contributed to the progeny. This is a very interesting finding. First, results confirmed the suitability of the environmental conditioning of captive snook broodstock and the efficacy and requirement of hormonal therapies in female broodstock. Second, results showed multiple males fertilized egg batches in the absence of hormonal treatments. These results are important for future aquaculture and restocking programs where genetic variability must be maximized. Selection programs in all livestock require the generation of as many families as possible to select from and improve commercially important traits, such as growth, disease resistance and yield. During the present study, progeny were identified from 35 of 48 (73 %) hormonally treated females (Table 4). A total of 40 families were produced in tank A and 87 families in tank C, all from a relatively small broodstock population of males and females (Table 1). The most represented family was from tank A with 337 progeny, 40.5 % of the total assigned. This type of dominance has been seen in other mass spawning fish species, such as Atlantic cod (Herlin et al., 2008) and Japanese flounder *Paralichthys olivaceus* (Sekino et al., 2003).

It must be acknowledged that parental contribution to individual spawns may have been much higher given the variable fertilization rates (17 to 83 %), hatch rates (50 to 97 %) and survival to 3 DPH (20 to 90 %). This variability is similar to most non-domesticated marine fish studied. In well domesticated aquaculture fish species, such as European sea bass (Vandeputte et al., 2009) and Atlantic salmon, *Salmo salar* (Vasemagi et al., 2012) domestication and selection has reduced such variability along with better husbandry and captive spawning technologies.

Parental contribution was highly variable between spawning seasons, events and tanks with fewer individuals contributing towards the end of the season (Tables 6 and

7). Previous field studies conducted with wild spawning snook showed that as the spawning season advances, egg quality decreases, thus having a direct impact on spawn quality including fertilization and hatch rate as well as larval survival (Yanes-Roca et al., 2009). Further investigation is needed to document patterns of egg quality across spawning seasons in captive common snook broodstock to better understand this relationship. Data presented here shows a lack of size-assortative mating, probably due to sexual dimorphism in snook, as they are protandric hermaphrodites (Taylor et al., 2000). Interestingly, during the course of the study no sex change in males was observed in any of the captive broodstock populations. Overall, the spawning population matched the size distribution of the entire captive broodstock population, suggesting reproductive success in captive common snook is not linked to this factor. A positive correlation between female size and contribution to offspring was observed. These findings are similar to other studies documenting that larger females have higher fecundity; a possible mechanism that could allow enhanced contributions by some individuals to population replenishment (Beldade et al., 2012).

Broodstock contributions varied among the three snook populations. Only one spawn was documented from broodstock in tank B compared to multiple spawns observed from the other two broodstock groups (A and C). Over the study period, broodstock in tank B were blood sampled monthly as part of another study to monitor the seasonal reproductive cycle through sex steroid profiling, leading them to be handled almost two times more frequently than broodstock in tanks A and C. In tank B, a lack of spermiation in males and oocyte maturation in females was observed throughout most of the trial. The single spawn obtained from broodstock in tank B was recorded near the end of the study following a four month period where no handling

occurred. The absence of reproductive activity in tank B could have been caused by the stress of monthly handling which involved cannulation biopsies and repeated blood sampling. Research has shown tolerance and physiological response to stress varies among fish species (Schreck et al., 2001). Stress factors, such as handling and hormonal induction, are known to have a negative effect on maturation and spawning in fish (Wendelaar Bonga 1997; Barton 2002). Such effects may have altered spawning behavior (Morgan et al., 1999; McConnachie et al., 2012), advanced or delayed oocyte maturation and ovulation (Watanabe et al., 2005), induced follicular atresia (Micale et al., 1999), altered gamete and offspring viability (Bobe and Labbé, 2010) and reduced egg production (Bogevik et al., 2012; Milla et al., 2009). In captive common snook, extensive handling appears to have a negative impact on maturation and spawning and further investigations are needed to define the limits so optimal broodstock management strategies can be implemented.

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CHAPTER 5

RESEARCH ARTICLE

INFLUENCE OF TIDAL CYCLES ON THE ENDOCRINE CONTROL OF REPRODUCTIVE ACTIVITY IN COMMON SNOOK (*CENTROPOMUS UNDECIMALIS*)

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In Review: General and Comparative Endocrinology

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Keywords: common snook; gene expression; gonadotropins; lunar cycle; maturation

Abstract

The aim of this study was to confirm the role of tidal pattern on the coordination of oocyte maturation and spawning in common snook *Centropomus undecimalis*. To do so, we studied gonadal development during the reproductive season in both males and females by means of gonad histology and levels of major endocrine control components along the pituitary-gonadal axis. LH plasma levels, as well as transcript levels of gonadotropin genes (*fshβ* and *lhβ*) from the pituitaries of sexually mature male and female common snook, were determined using a heterologous ELISA and quantitative RT-PCR, respectively. The *fshβ* and *lhβ* cDNAs were isolated and phylogenetic analysis of the deduced amino acid sequence revealed strong identity with other teleosts (75-90%). A significant link was found between tide and follicular development. Female snook sampled on the rising tide were all found to have oocytes in the secondary growth stage, whereas females sampled at high tide or on the falling tide had oocytes in the later stages of maturation and ovulation. LH plasma and mRNA levels of *fshβ* and *lhβ* gradually increased during vitellogenesis peaking at ovulation in females. In addition, plasma estradiol and testosterone gradually increased during the later stages of ovarian development. However, while estradiol significantly increased in late vitellogenesis, testosterone levels showed a dramatic increase at oocyte maturation. In males, *lhβ* mRNA expression as well as plasma levels of testosterone (T) and 11-ketotestosterone (11-KT) peaked at the mid germinal epithelium stage while *fshβ* expression and plasma LH levels peaked at late germinal epithelium stage. Taken together, the morphological and endocrinological changes occurring along the tidal cycle confirm the role played by tide on the entrainment of gametogenesis of common snook and provides a better understanding of the link between environmental and

endocrine control of reproduction in this species. The new tools developed and knowledge gained will assist with the management of captive broodstock.

1. Introduction

In recent years, there has been an increasing scientific effort to understand circadian and seasonal environmental cues and their molecular basis that entrain (initiation and synchronization) reproduction in a variety of marine organisms. This, however, still remains largely unknown in many fish species probably due to the complexity and range of abiotic factors involved (light, temperature, tide, moon). In the field of aquaculture, understanding the environmental and endocrine control of reproduction is a key component to improve spawning of captive fish broodstock. In fisheries, this information is important in developing tools to monitor wild stock recruitment and for designing and implementing effective fisheries management strategies.

Research has shown in temperate fish species, the brain-pituitary-gonad (BPG) axis is entrained by external cues, such as seasonal variation in environmental conditions including mainly photoperiod and temperature (Migaud et al., 2010). Photoperiod manipulation, through the application of artificial lighting, can therefore be used to control the timing of seasonal reproductive patterns in fish and is now widely adopted in aquaculture to alter spawning season, delay or inhibit maturation during ongrowing and stimulate growth (Pankhurst and Porter, 2003; Rad et al., 2006; Migaud et al., 2010; Taranger et al., 2010). However, while most research has focused on photo-thermal requirements of temperate fish species, other environmental signals like lunar and tidal cycle or flooding during the rainy season are involved in the entrainment of reproduction in tropical and sub-tropical fish species (Yamahira 2004). This is due to the relatively small seasonal changes in temperature and photoperiod that occur in the tropics (Naylor 2010). The importance of lunar cycle, in particular on fish reproduction

has been reported in a range of fish species, although, to date, few physiological studies have investigated how changes in tidal cycle can act as an ultimate factor that entrains and synchronizes reproductive functions (Takemura et al., 2004; Ando et al., 2013). The simulation of tidal cycle in land based systems is difficult to achieve, possibly explaining why the control of reproduction in captivity remains problematic for a number of key species, which rely on this environmental factor to spawn. This is the case in the common snook *Centropomus undecimalis* although available data on the influence of tidal cycle on snook mating behavior remains anecdotal at this stage.

Common snook are protandric hermaphrodites (Taylor et al., 2000) and have an annual reproductive cycle with peak spawning activity from May to August when water temperatures (>24°C) and day length (≥ 13.5 h) are maximal (Taylor et al., 1998). Early observations suggested that sexually mature adults travel from fresh and euryhaline water to coastal marine areas to spawn in aggregations (Lowerre-Barbieri et al., 2003). Common snook are multiple batch spawners and their mating behavior in the wild is believed to be complex and is still not fully understood (Trotter et al., 2012). Recent studies with captive common snook broodstock revealed that, using photothermal conditioning and hormonal manipulation, spawning can be induced in captivity, although it remains unpredictable in terms of quantity and quality of egg production (Ibarra-Castro et al., 2011; Rhody et al., 2014). Spawning appears to be strongly linked to the tidal cycle, with wild spawning that occurs during the late afternoon and evening predominantly coinciding with the new and full moon although further confirmation is required (Yanes-Roca et al., 2009; Young et al., 2014). While the biology of wild common snook has been studied for over 50 years, the description of their reproductive biology is still incomplete and to date, no published information is

available on its control and function along the BPG axis in this species, especially in relation to tidal cycle.

In fish, as in other vertebrates, it is well known that reproduction is regulated by an intricate network of endocrine, paracrine and autocrine regulatory signals along the BPG axis (Zohar et al., 2010). Among these, gonadotropin releasing hormone (GnRH) neurons have long been considered as the starting point of the BPG axis, although other neuropeptides (e.g. kisspeptin) now appear to be involved upstream (Tena-Sempere et al., 2012). These neurohormones regulate the synthesis and secretion of two pituitary gonadotrophins (e.g. follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which in turn regulate gonadal steroidogenesis and gametogenesis (Peter and Yu 1997; Swanson et al., 2003; Zohar et al., 2010). The mode of action and regulation of FSH and LH has been investigated in a number of fish species (Yaron et al., 2003; Levavi-Sivan et al., 2010; Kah and Dufour 2011). For example, in rainbow trout *Onchorhynchus mykiss*, gonadotropic regulation in the early stages of gametogenesis (vitellogenesis or spermatogenesis) is typically characterized by an elevation in *fshβ* mRNA, whereas *lhβ* peaks at the time of ovulation and spermiation (Bobe et al., 2004; Sambroni et al., 2013a). Alternatively, in some batch spawning fish species, *fshβ* and *lhβ* (and their receptors) mRNA expression increase simultaneously, while oocytes are developing at different rates to reach a peak at spawning as in European sea bass *Dicentrarchus labrax* (Migaud et al., 2012), Atlantic cod *Gadus morhua* (Mittelholzer et al., 2009a and b; Almeida et al., 2011; Cowan et al., 2012) and Atlantic halibut *Hippoglossus hippoglossus* (Weltzien et al., 2003).

The aim of the present study was to confirm the role of tidal pattern on the synchronization of oocyte maturation and spawning in common snook and to determine

the chain of endocrine events that support it. To do so, we studied the temporal levels of plasma sex steroids and LH, as well as expression patterns of *fshβ* and *lhβ* during gonadal development with special focus on the events surrounding the tidal cycle.

2. Materials and methods

2.1 Source of the animals and sample collection

Adult male (mean body weight: 2143.8 ± 234.0 g, fork length: 512.8 ± 16.0 mm) and female (mean body weight: 2929.5 ± 268.1 g, fork length: 630.8 ± 22.4 mm) common snook were collected from three locations in Tampa Bay (Florida, USA) using a 92-m seine net deployed from a research vessel (Figure 1). The study was conducted in the months of April through August in years 2011 and 2012, during which fish were sampled monthly to track gonadal development. All sampling events took place 1 or 2 days following the new or full moon between 08:00 and 21:00 hours to track oocyte maturation (based on previous unpublished work detailing seasonal spawning patterns in common snook). Due to harvest restrictions on a protected game fish and efforts to minimize the number of fish sacrificed, a limited number of individuals (38 males and 56 females) were sampled over the study period. The time of sampling from start to finish was recorded for each individual. Data were pooled for both years from all three locations.

Fish were weighed (± 0.1 g) and fork length (± 1 mm) was measured. An ovarian biopsy was taken from each female. Biopsies were placed on ice in the field then photographed and measured (n = 100/individual) within 3-6 hours post collection using an Olympus BX53 microscope fitted with a DP-72 digital camera using Olympus cellSens (version 1.3) imaging software (Olympus, USA). Blood was withdrawn from

the caudal vein using a heparinized 3 ml syringe and 21G sterile hypodermic needle (0.8 mm x 40 mm) and kept on ice from the time of collection until return to the lab for processing (up to 6 hours). Blood samples were then centrifuged at 1500 *g* for 10 minutes and plasma stored at - 70 °C for steroid and gonadotropin analyses. Fish were then dissected, the pituitary and brain were collected, then frozen separately using dry ice and stored at -70 °C for later total RNA extraction. Gonads were fixed for histological examination by immersion in Trump's fixative consisting of 4% formaldehyde: 1% glutaraldehyde (McDowell and Trump 1976). Gonadosomatic indexes (GSI) were calculated as GSI = (gonad weight / total body weight) x 100.

Figure 1. Map of sampling locations. Male and female common snook were collected from three sampling locations (●) in Tampa Bay (Florida, USA) from April through August in years 2011 and 2012. Google Earth Pro 7.0 (2014). Tampa Bay, Florida, USA. 27°42'57.6"N 82°33'40.7"W. NOAA, Digital Globe 2013. http://www.google.com/earth/index.html (Accessed February 10, 2014).

2.2 Histological preparation and analysis

Fixed tissue samples were embedded in glycol methacrylate (JB-4, Electron Microscopy Sciences, Ft. Washington, PA, USA) and sectioned (6 µm) on an LKB Bromma 2218 Historange Microtome (Sweden). Tissue sections were stained with periodic acid-Schiff, metanil yellow and Weigert's hematoxylin and eosin according to Quintero-Hunter et al. (1991), then viewed on an Olympus BX53 microscope (Olympus, Center Valley, PA, USA). Using light microscopy, the stage of testicular development was classified according to Grier and Uribe-Aranzábal (2009): early, mid and late germinal epithelium development. The oocyte stages of development in female snook (Primary Growth, Secondary Growth and Oocyte Maturation) were determined using the staging described in Rhody et al. (2013, Chapter 2).

2.3 Hormone analysis

The level of plasma testosterone (T) and 17β-estradiol (E_2) were measured by radioimmunoassay (RIA) according to methods described by Duston and Bromage (1987). For testosterone and estradiol, tritiated T (GE Healthcare, UK) and E_2 (PerkinElmer, Boston, MA, USA) radiolabels along with anti-testosterone and anti-estradiol antisera (CER group, Marloie, Belgium) were used. Plasma levels of 11-ketotestosterone (11-KT) were measured by RIA according to Fostier et al. (1982). A parallelism test was performed for all three hormonal assays to confirm that the serial dilutions of common snook plasma extracts and hormone standards were immunologically comparable. Radiolabel and antibody were provided by Dr. Alexis Fostier (Department of Animal Physiology and Livestock Systems, Rennes Research Centre, France). Intra- and inter-assay coefficients of variation were; 4.2, 5.3, and 5.4

%; 7.5, 8.6 and 8.9 %; for T, E_2, and 11-KT respectively (n = 2 assays for each hormone). All standards and samples were assayed in duplicate.

Luteinizing hormone (LH) levels in the plasma were measured using a heterologous LH ELISA developed for striped bass (*Morone saxatilis*) as previously described (Mañanós et al., 1997). In order to validate the assay for application with common snook, displacement curves for plasma and pituitary samples were generated by serial dilutions of the sample in the ELISA buffer and compared with the standard curve. For the parallelism analysis, whole pituitary glands were collected from sexually mature female snook. Pituitaries were homogenized on ice in PBS pH 7.4 containing 0.02M phenylmethylsulfonyl fluoride and 0.05 M EDTA using a Polytron homogenizer. The homogenate was centrifuged 15,000 *g* for 30 min. The resulting supernatant was used as the pituitary extract. The displacement curves obtained were parallel to the standard curve, indicating that they were immunologically comparable. The sensitivity of the assay was 80 pg/ml. Plasma FSH levels were not measured during this study due to the lack of an available assay developed for use with common snook.

2.4 Gene expression

2.4.1 RNA extraction and cDNA synthesis

For the analysis of *fshβ* and *lhβ* expression, total RNA was extracted from individual pituitary samples in 1 ml TRI Reagent® (Applied Biosystems/Ambion, Warrington, UK) in accordance with the manufacturer's guidelines and pellets were reconstituted in 10 µl of MilliQ water. Non-reduced samples of common snook pituitary extract were electrophoresed and RNA concentration and quality checks were

performed. All RNA samples were DNase treated (DNA-*free*™, Applied Biosystems/Ambion, Warrington, UK) and cDNA was then synthesized with 1 µg of total RNA and reversed transcribed in a 20 µl reaction containing 10X RT Random primers, MultiScribe™ Reverse Transcriptase (50 U/µl), 10mM dNTP mix, provided buffers and nuclease free H_2O (High-capacity cDNA reverse transcriptase kit, Applied Biosystems/Ambion, Warrington, UK). Thermal cycling conditions were 10 min at 25 °C, 120 min at 37 °C and 5 min at 85 °C and then samples were stored at -20 °C.

2.4.2 Isolation of fshβ and *lhβ cDNAs from the pituitary of common snook*

Snook specific LH and FSH sequences were obtained using 5´- and 3´ - RACE PCR (SMART Rapid Amplification of cDNA Ends kit; BD Biosciences) according to the manufacturer's protocol. Sequences were generated based on iterative rounds of RACE PCR using the primers as listed in Table 1. All generated fragments were cloned into a PGEM-T vector (Promega, Madison, WI, USA) for further analysis and sequencing. The sequences from all PCR rounds were combined to obtain the full *lhβ* and partial *fshβ* coding sequence (CDS). For the *fshβ* and *lhβ* assays linearized plasmids containing partial snook cDNA sequences were first generated. The cDNA sequences for target genes were generated with primers (Eurofins MWG Operon, Edersberg, Germany) designed using Primer Select ver.6.1 program (DNASTAR, https://www.dnastar.com) (Table 1). Primers were designed based on the conserved region of open reading frames (ORF) from common snook sequences obtained during this study (GenBank accession numbers: KF314818 and KF314819). PCR reactions were performed using 2.0 µl 10X reaction buffer, 1 mM $MgCl_2$, 100 µM dNTPs, 0.4 µM forward and reverse primers, 1 unit of Klear Taq DNA polymerase (KBiosciences,

Hertfordshire, UK) and 2 ng of synthesized cDNA (1:10 dilution). Thermal cycling consisted of initial denaturation at 95 °C for 15 mins followed by 40 cycles at 95 °C for 30 s, X °C for 15 s, 72 °C for 30 s and a final extension at 72 °C for 4 min. Annealing temperatures "X" are listed in Table 1 for each primer pair. Prior to cloning, the PCR product was checked on 1 % agarose gel then purified using GeneJET PCR Purification Kit (Thermo Scientific, UK). All primer pairs generated a single PCR product and those products, which were to be used for qPCR standards, were cloned into a pGEM®-T Easy Vector (Promega, Southhampton, UK). The inserted fragment was sequenced using a CEQ-8800 Beckman Autosequencer (Coulter Inc., Fullerton, USA) and the identities of the cloned PCR products were then verified (100 % overlapping) using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Sequence analysis was performed using BioEdit Sequence Alignment Editor Software (Ibis Biosciences, Carlsbad, CA, USA) to edit and assemble DNA sequences. ClustalW (Thompson et al., 2000) was used to generate multiple alignments of deduced protein sequences. MEGA version 4 (Tamura et al., 2011) was used to deduce and bootstrap phylogenetic trees using the neighbor joining method (Saitou and Nei, 1987).

Table 1. List of primers, procedure for which primers were used, sequence and annealing temperature for different genes studied.

Primer Name	Procedure	Direction	Sequence	Product size (bp)	Annealing Temperature (°C)
FSH1	ORF cloning primary	Forward	5′-AYACNACNATHTGYRYNGG-3′	300	48
FSH2	ORF cloning nested	Forward	5′-GGNYWBTGYTWYHVNVRNGA-3′		48
LHR1	ORF cloning primary	Reverse	5′-GTRCANSWNGTRTCCAT-3′	380	46
LHR2	ORF cloning nested	Reverse	5′-GCNACNGGRTANGTNAC-3′		48
3SnookLH1	3' RACE primary	Forward	5′-CACCACCTGCACACACCTGGAG-3′	589	62.5
3SnookLH2	3' RACE nested	Forward	5′-GCGATGGCTGTACGGATCAGCAG-3′	566	62.1
3SnookLH3	3' RACE nested	Forward	5′-GCGTTCCAGCTGCCGCCCTGCC	470	70.3
5SnookFSHR1	5' RACE primary	Reverse	5′-AGGTCCAGTCCCCGTTACAG-3′	177	58.7
5SnookFSHR2	5' RACE nested	Reverse	5′-CAGCCCAGTCATGGTTGCCGATG-3′	144	62.7
SNKfshF1	FSH qPCR	Forward	5′-TGTCCAGTGGGCGTCACCTA-3′	387	59
SNKfshR1	FSH qPCR	Reverse	5′-GGCCGCAGTACGTGTTTCCT-3′		
SNKlhF1	LH qPCR	Forward	5′-TCCTCCTGGTGTGGACCCAGTT-3′	637	62
SNKlhR1	LH qPCR	Reverse	5′-TCGGGTTGCAGGCTCTCAAAGG-3′		
ActinF	PCR	Forward	5′-AGTGTTGTCCCTGTACGCCTCTG-3′	681	57
ActinR	PCR	Reverse	5′-CTCGTCGTACTCCTGCTTGCTGAT-3′		
ActinqpcrF	qPCR reference gene	Forward	5′-ATCCTGACAGAGCGCGGTTACAGT-3′	112	61
ActinqpcrR	qPCR reference gene	Reverse	5′-TGCCCATCTCCTGCTCAAAGTCCA-3′		

2.4.3 Quantitative RT - PCR (*QPCR*)

Expression of the genes of interest was measured by absolute quantification. Snook specific *fshβ* and *lhβ* assays had to be optimized, *β-actin* was selected as the house-keeping gene for normalization using a generic teleost *β-actin* assay originally designed on Atlantic salmon *β-actin* (AF012125), following verification of sequence identity of the amplified product and confirmation of suitable amplification efficiency.

Absolute quantification was achieved by a parallel set of reactions containing standards consisting of a serial dilution of spectrophotometrically determined linearized plasmid containing partial snook cDNA sequences generated as described above ultimately allowing levels to be expressed in copies per µg total RNA. qRT-PCR was performed using 0.7 µl (10 pMol) of each forward and reverse primer (Table 1), 5ng cDNA , 1X SYBR Green qPCR mix (ABsolute™, Abgene, Surrey, UK) consisting of Thermo-Start™ DNA polymerase, a proprietary reaction buffer, dNTP's and SYBR Green I with 3 mM of $MgCl_2$ and 3.0 µl MilliQ for a total reaction volume of 20 µl. The thermal cycling protocol run in a Techne Quantica (Cambridge,UK) thermocycler consisted of 15 min at 95 °C followed by 45 cycles of 95 °C for 15 s, X °C for 15 s, and 72 °C for 30 s, followed by a temperature ramp from 60 to 95 °C for melt-curve analysis. Melt-curve analysis verified the primer sets for each qPCR assay generated one single product and no primer-dimer artifacts. The annealing temperatures "X" were changed as follows: 61 °C *fshβ*, 62 °C *lhβ* and 61 °C for *β-actin*. All samples and standards were run in duplicate together with non-template controls.

2.5 Statistical analysis

Statistical analysis of data was performed with SPSS Statistics, Version 19.0 (IBM Corp., Armonk, NY, USA) unless otherwise stated. All data sets were tested for normality using Kolmogorov-Smirnov test and homogeneity of variances using Levene's test. Where necessary, data were log or arcsine transformed before further statistical analysis. The strength of the association between pairs of parameters (gene expression levels and plasma hormone levels) was evaluated by calculating the Pearson product moment correlation coefficient (r). Gene expression data, plasma sex steroid levels and GSI were analyzed by one-way ANOVA followed by Duncan's post hoc test for multiple comparisons to identify significant differences ($P \leq 0.05$). Results are presented as mean ± standard error of the mean (SEM).

The relationship between tidal cycle and spawning was investigated using stage and step of oocyte development along with the location, date and time at which oocytes were collected. To account for daily or seasonal variation in tidal cycles, data were transformed so sample collection times could be standardized and expressed irrespective of tidal length. Using a standardized tidal pattern (12 hours), data are expressed as percentages and plotted with respect to the time in hours from high tide. Information on the timing of high and low tide for each specific date and collection site was obtained via the Tampa Bay Operational Forecast System (TBOFS), a division of the National Oceanographic and Atmospheric Association's Center for Operational Oceanographic Products and Services (http://www.co-ops.nos.noaa.gov/), which compiles observed meteorological, oceanographic and flow rate data.

Generalized linear models (GLM) were used to model LH plasma levels and pituitary mRNA expression of *fshβ* and *lhβ* as a function of time (year), space (sample

collection site), female reproductive phase of development and tidal cycle. GLM is particularly suited to ascertain whether certain variables have any predictor power and have been employed successfully to evaluate similar types of data sets in a variety of disciplines (Meyer and Laud, 2002). When the main effects were significant, Tukey's post hoc test was used to compare differences between groups. All GLM analyses were conducted using SAS® 9.2 software.

3. Results

3.1 Common snook fshβ and *lhβ cDNA sequences and phylogenetic analysis*

Common snook specific sequences for *fshβ* and *lhβ* were isolated and sequenced. The partial *fshβ* sequence produced a 387 bp fragment from common snook pituitary cDNA samples using primer pair SNKfshF1 and SNKfshR1 (Figure 2). This partial sequence consists of a 327 bp coding sequence (cds) and 59 bp 3´untranslated region (UTR). Phylogenetic analysis of the partial deduced amino acid sequence (109 aa) shows the snook *fshβ* protein has a comparable identity (> 75 %) with other teleost *fshβ* sequences including, Southern flounder (*Paralichthys lethostigma*), Japanese rice fish (*Oryzias latipes*) and Senegalese sole (*Solea senegalensis*) (Figure 3). The complete *lhβ* sequence generated a 637 bp product from common snook pituitary cDNA samples using primer pair SNKlhF1 and SNKlhR1 (Figure 4). This complete cds translates into 148 aa protein. A tblastn search of the deduced amino acid sequence shows the common snook *lhβ* protein having comparable identity with other teleost *lhβ* sequences including European sea bass *Dicentrarchus labrax* (90 %), striped bass *Morone saxatilis* (89 %) and chub mackerel *Scomber japonicus* (84 %) (Figure 5).

A

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1      ACG GCG GGG GCG GGG CAG GGC TGC AGC TTC GGC TGC AAT CCA ACC   45
1       T   A   G   A   G   Q   G   C   S   F   G   C   N   P   T   15

46     AAC ATC AGC ATC CCC GTG GAG AGC TGT GGC AGC ATC GAG TTC ATC   90
16      N   I   S   I   P   V   E   S   C   G   S   I   E   F   I   30

91     TTC ACC ACC ATA TGT GCA GGA CAG TGC TGC CAC GAG GAT CCG GTC   135
31      F   T   T   I   C   A   G   Q   C   C   H   E   D   P   V   45

136    TAC ATC GGC AAC CAT GAC TGG GCT GAA CAG AAG ATC TGT AAC GGG   180
46      Y   I   G   N   H   D   W   A   E   Q   K   I   C   N   G   60

181    GAC TGG ACC TTT GAG GTG AAG CAC ATT AAA GGA TGT CCA GTG GGC   225
61      D   W   T   F   E   V   K   H   I   K   G   C   P   V   G   75

226    GTC ACC TAC CCC GTG GCC AGA AAC TGG GAG TGC ATG GCA TGT AAT   270
76      V   T   Y   P   V   A   R   N   W   E   C   M   A   C   N   90

271    GCA GGA AAC ACG TAC TGC GGC CGC TTT CCT GGA GCT GTG CCC AGC   315
91      A   G   N   T   Y   C   G   R   F   P   G   A   V   P   S   105

316    TGC CTG TCC TAA ACA ACC CCT GGT GTC ACT GCA TTT GGG AGC GAA   360
106     C   L   S   *

361    ATA AAC AGG CAT CAC TTA AAA AAA AAA   387
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B

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                          10        20        30        40        50        60
                  ....|....|....|....|....|....|....|....|....|....|....|....|
Common snook      -------------------TAG-AGQGCSFGCNPTNISIPVESCGSIEFIFTTICAGQCC
European sea bass --------MQLVVMVAVLALAR-AGQGCSFGCHPTNISIQVESCGLTEVIYTTICEGQCY
Striped bass      --------MQLVVMVAVLALAG-AGQGCSFGCHPTNISIQVESCGLTEVIYTTICEGQCY
Chub mackerel     --------MQLVVMVAVLAMAG-AGQGCSLDCHPKNVSIPVESCGRTEFIFTTMCEGQCY
Southern flounder --------MKLVVMVAVLAVAG-AGQGCSFDCRPTNISIPVESCGSTEYIYTTVCAGQCY
Atlantic salmon   ------------VMAMLWVTPVRAGTDCRYGCRLNSMTITVEREDCHGSITITTCAGLCE
Chum salmon       MYCTHLMTLQLVVMAMLWVTPVRAGTECRYGCRLNNMTIIVEREDCHGSITITTCAGLCE

                          70        80        90       100       110       120
                  ....|....|....|....|....|....|....|....|....|....|....|....|
Common snook      HEDPVYIGNHDWAEQKICN-GDWTFEVKHIKGCPVGVT---YPVARNWECMACNAGNTYC
European sea bass HEDLVYLSHYERPEQRICN-GDWSYEVKHIKGCPVGVT---YPVARNCECTTCNTENTDC
Striped bass      HEDLVYISHYERPEQRICN-GDWSYEVKHIKGCPVGVT---YPVARNCECTTCNTENTDC
Chub mackerel     HRDPVYGQEE----QQICS-GDWTYEVKHIDGCPVGVT---YPVARHCACTACNTGNTYC
Southern flounder YEDPVYISETGPAKQRICN-GDWTYEVKHINGCPVAVT---YPVARHCHCTICNPGNTDC
Atlantic salmon   TTDMNYQSTWLPRSQGACNFKDWSYEKVYLEGCPSGVDPFFIPVAKSCDCIKCETDNTDC
Chum salmon       TTDLNYQSTWLPRSQGVCNFKEWSYEKVYLEGCPSGVEPFFIPVAKSCDCIKCKTDNTDC

                          130
                  ....|....|....|..
Common snook      GRFPGAVPSCLS-----
European sea bass GRFPGDIPSCLSF----
Striped bass      GRFPEDIPSCLSF----
Chub mackerel     GRLP-YVPSCPSF----
Southern flounder GRFPGDIPSCLPF----
Atlantic salmon   DRISMATPSCIVNPL--
Chum salmon       DRISMATPSCIVNPLEM
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Figure 2. Molecular characterization of common snook *fshβ*. (A) Nucleotide and deduced amino acid sequence of common snook *fshβ* partial cDNA fragment. The mature peptide is identified by the solid black line and the signal peptide by the broken line. The stop codon is marked by the * symbol. (B) Alignment of the deduced protein sequence for common snook, European sea bass, striped bass, chub mackerel, southern flounder, Atlantic salmon and chum salmon *fshβ*. The conserved amino acid residues are shaded.

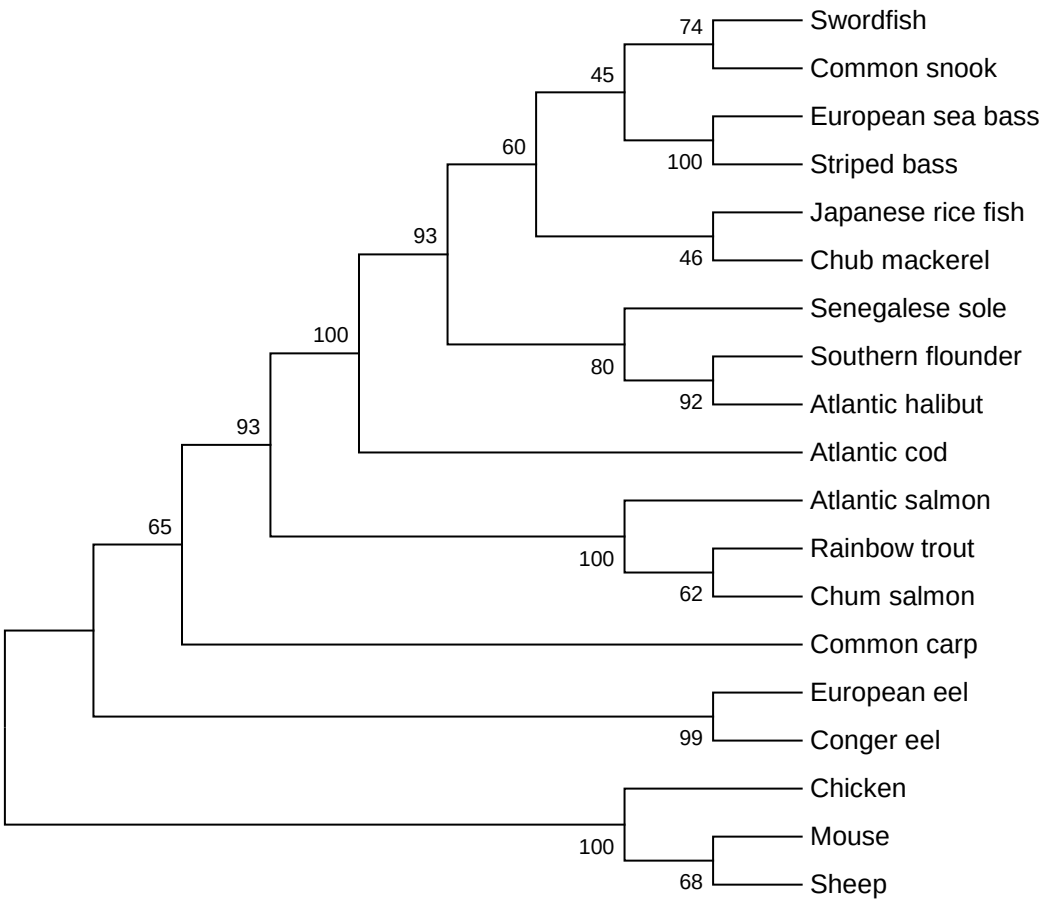

Figure 3. Phylogenetic tree analysis of *fshβ* sequences in vertebrates. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (5000 replicates) is shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Accession numbers: Swordfish (EC11223), Common snook (KF314819), European sea bass (AF543314), Striped bass (L35070), Japanese rice fish (AB541981), Chub mackerel (JF495132), Senegalese sole (EU100409), Southern flounder (JX002657), Atlantic halibut (16604709), Atlantic cod (DQ402373), Atlantic salmon (AF146152), Rainbow trout (185135807), Chum salmon (M27153), Common carp (2114095), European eel (AY169722), Conger eel (8250135) , Chicken (AB077362), Mouse (40254640) and Sheep (57619323).

A

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2     CGC GGG AAC AGT GAG GTT ACA GAC AAA TCT AAT TAG ATG GAG AGA   46
47    TGG ACA CCA CCT GCA CAC ACC TGG AGG GCG ATG GCT GTA CGG ATC   91
                                          M   A   V   R   I    5

92    AGC AGA ATG ATA TTA CCC CTG ATG TTG AGT CTG TTT TTG GGA GCC   136
6      S   R   M   I   L   P   L   M   L   S   L   F   L   G   A    20

137   TCA TCT TCA ATT TGG TCC CTG GCT CTG GCA GAT GCG TTC CAG CTG   181
21     S   S   S   I   W   S   L   A   L   A   D   A   F   Q   L    35

182   CCG CCC TGC CAA CTC ATC AAC CAG ACG GTG TCT CTG GAG AAG GAG   226
36     P   P   C   Q   L   I   N   Q   T   V   S   L   E   K   E    50

227   GGC TGT CCC AAA TGT CAC CCA GTG GAA ACA ACC ATC TGC AGT GGT   271
51     G   C   P   K   C   H   P   V   E   T   T   I   C   S   G    65

272   CAC TGC ATC ACC AAG GAC CCT GTC ATC AAG ATA CCG TTC AGT AAT   316
66     H   C   I   T   K   D   P   V   I   K   I   P   F   S   N    80

317   GTG TAC CAG CAC GTG TGC ACG TAC CGG GAC TCG TAC TAC AAG ACG   361
81     V   Y   Q   H   V   C   T   Y   R   D   S   Y   Y   K   T    95

362   TTT GAA CTC CCT GAC TGT CCT CCT GGT GTG GAC CCA GTT GTC ACC   406
96     F   E   L   P   D   C   P   P   G   V   D   P   V   V   T   110

407   TAC CCT GTG GCT CTG AGC TGC CAC TGT GGC CGG TGT GCG ATG GAC   451
111    Y   P   V   A   L   S   C   H   C   G   R   C   A   M   D   125

452   ACA TCT GAC TGC ACC TTT GAG AGC CTG CAA CCC GAC TTC TGC ATG   496
126    T   S   D   C   T   F   E   S   L   Q   P   D   F   C   M   140

497   AAT GAC ATA CCT TTC TAC TAC TAG TCT CAA GAA CTA GCA GGA TAA   541
141    N   D   I   P   F   Y   Y   *

542   AAA ACA CAA TAC ATA GAG GAA GCG TCC TGA CTT TTA GCA TCA ACT   586
587   GTA GTA AAT AAA GAT TGT TTC AAA TAC CAA AAA AAA AAA AAA AAA   631
632   AAA AAA   637
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B

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                          10        20        30        40        50        60
                  ....|....|....|....|....|....|....|....|....|....|....|....|
Common snook      -MAVRIS-RMILPLMLSLFLGASSSIWSLALADAFQLPPCQLINQTVSLEKEGCPKCHPV
European sea bass -MAVQAS-RVMFPLVLSLFLGATSSIWPLATAEAFQLPPCQLINQTVSLEKEGCPKCHPV
Striped bass      -MAVQAS-RVMFPLVLSLFLGATSDIWPLAPAEAFQLPPCQLINQTVSLEKEGCPKCHPV
Chub mackerel     -MVVKVT-RVMFPLMLSLFLAVSSPIWPLTPAVAFQLPPCQLINQTVSVEKEGCATCHPV
Southern flounder -MSMQLSLRVKIPLTLIFFL---SSMWPLAPAEAFQLPTCLLIKQMVSLEKEGCPKCHTV
Atlantic salmon   MLGLHVG--TLISLFLCILL---------EPVEGSLMQPCQPINQTVSLEKEGCPTCLVI
Chum salmon       MLGLHVG--TLISLFLCILL---------EPVEGSLMQPCQPINQTVSLEKEGCPTCLVI

                           70        80        90       100       110       120
                  ....|....|....|....|....|....|....|....|....|....|....|....|
Common snook      ETTICSGHCITKDPVIKIPFSNVYQHVCTYRDSYYKTFELPDCPPGVDPVVTYPVALSCH
European sea bass ETTICSGHCITKDPVIKIPFSNVYQHVCTYRNSHYKTFELPDCPPGVDPTVTYPVAQSCH
Striped bass      ETTICSGHCITKDPVIKIPFSNVYQHVCTYRDLHYKTFELPDCPPGVDPTVTYPVAQSCH
Chub mackerel     ETTICSGHCITKDPVIKIPYSKVYQHVCTYRDFYYKTFELPDCPSGVDPMVTYPVALSCH
Southern flounder ETTICSGHCKTKDPVAKIPFLNMYQHVCTYQELYYKTFELPDCPPGVDPTVSYPVAVSCY
Atlantic salmon   QTPICSGHCVTKEPVFKSPFSTVYQHVCTYRDVRYETIRLPDCPPWVDPHVTYPVALSCD
Chum salmon       QTPICSGHCVTKEPVFKSPFSTVYQHVCTYRDVRYETIRLPDCPPWVDPHVTYPVALSCD

                          130       140       150
                  ....|....|....|....|....|....|...
Common Snook      CGRCAMDTSDCTFESLQPDFCMNDIPFYY----
European Seabass  CGRCAMDTSDCTFESLQPNFCMNDIPFYY----
Striped Bass      CGRCAMDTSDCTFESLQPNFCMNDIPFYY----
Chub Mackerel     CSRCAMDTSDCTFESLQPDFCMNDIPFYY----
Southern Flounder CGRCALNTSDCTFQSLQPDFCMNDIPFYD----
Atlantic Salmon   CSLCNMDTSDCTIESLQPDFCITHRALMDGNMW
Chum Salmon       CSLCNMDTSDCTIESLQPDFCITQRVLTDGDMW
```

Figure 4. Molecular characterization of common snook *lhβ*. (A) Nucleotide and deduced amino acid sequence of common snook *lhβ* partial cDNA fragment. The mature peptide is identified by the solid black line and the signal peptide by the broken line. The stop codon is marked by the * symbol. (B) Alignment of the deduced protein sequence for common snook, European sea bass, striped bass, chub mackerel, southern flounder, Atlantic salmon and chum salmon *lhβ*. The conserved amino acid residues are shaded.

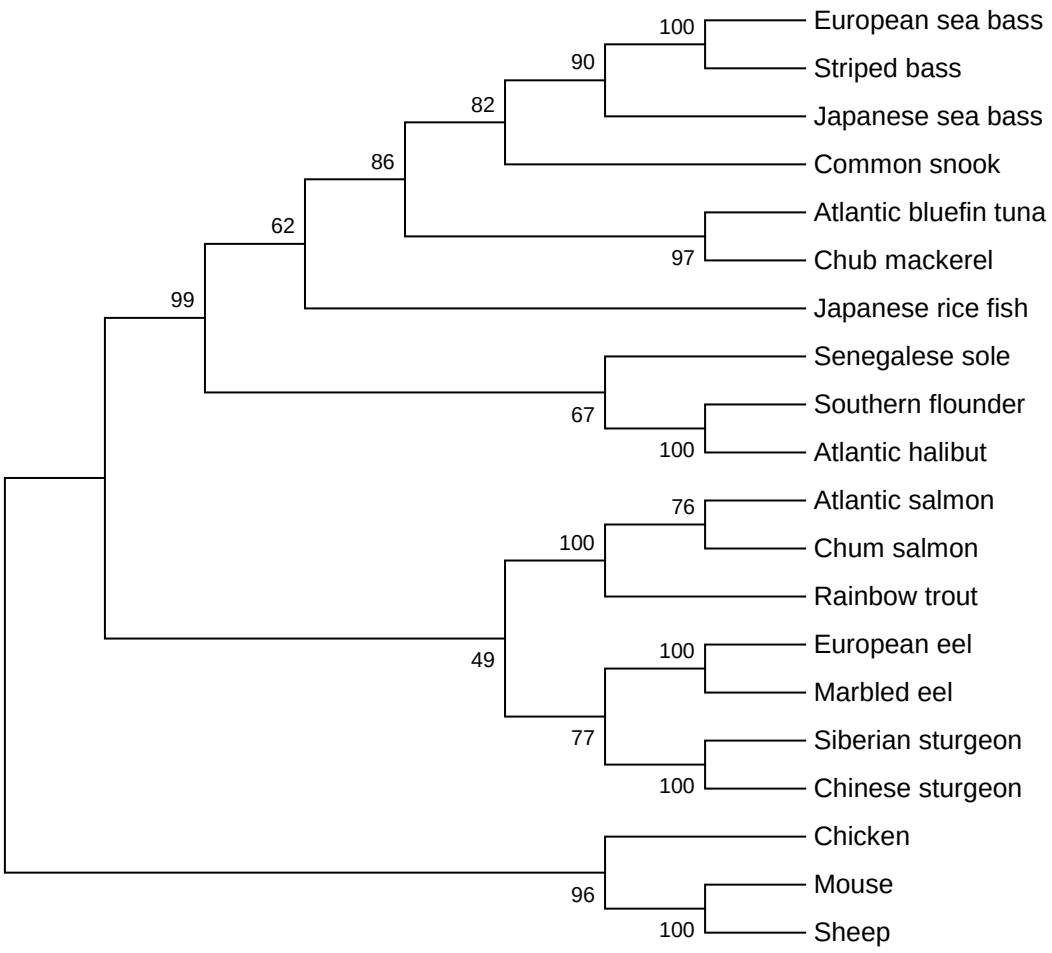

Figure 5. Phylogenetic tree analysis of *lhβ* sequences in vertebrates. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (5000 replicates) is shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Accession numbers: European sea bass (AF543315), Striped bass (L35096), Japanese sea bass (JX185719), Common snook (KF314818), Atlantic bluefin tuna (EF205591), Chub mackerel (JF495133), Japanese rice fish (212549679), Senegalese sole (EU100410), Southern flounder (JX002656), Atlantic halibut (16604711), Atlantic salmon (291190577), Chum Salmon (M27154), Rainbow trout (1853502), European eel (62395), Marbled eel (FJ490347), Siberian sturgeon (8250127), Chinese sturgeon (EU523733), Chicken (HQ872606), Mouse (1772991) and Sheep (57164374).

3.2 Gonadal development and synchronization of spawning with tidal cycle

In females, GSI level usually was correlated with the different stages of ovarian development: Lower in individuals whose ovaries contained oocytes in Primary Growth (PG) stage (< 1.0 %) when compared with those classified as being in the advanced stages of growth (Secondary Growth (SG) or vitellogenesis) or Oocyte Maturation (OM), except for the time of ovulation when the GSI decreased due to the evacuation of ova (Figure 6A). Female snook sampled in the spring (April and May) had oocytes that were previtellogenic; classified as Primary Growth Stage, Perinucleolar Step (PGpn) and having a mean oocyte diameter of 87.6 ± 0.6 µm (Figure 6B). Females collected in the summer months (June, July and August) were found to have oocytes in the later stages of oocyte development including Secondary Growth and Oocyte Maturation (Figure 6C) where mean oocyte diameter ranged from 356.1 ± 1.7 to 501.1 ± 2.8 mm, respectively (Figure 6B).

Concomitant with the morphological changes that occurred in testicular lobules during the annual reproductive cycle, GSI was found to be significantly different (P<0.05) among males that were sampled in this study (Figure. 6D). Males collected in spring were found to be in early recrudescence (Early Germinal Epithelium Development) and their testes contained mainly spermatogonia (Figure. 6E). By summer, male testis had progressed into Late Germinal Epithelium Development where the testes were filled with spermatozoa and sperm could be collected when a slight abdominal pressure was applied (spermiating).

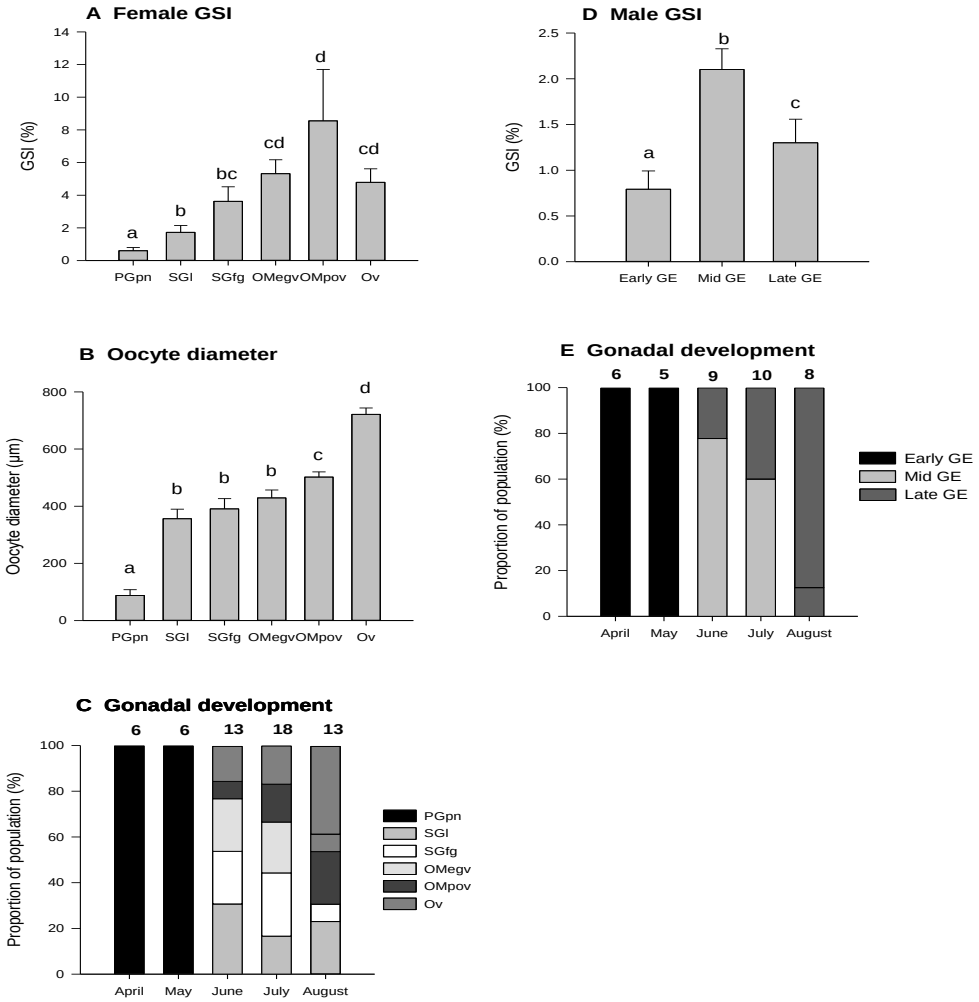

Figure 6. Seasonal changes in the gonadosomic index (GSI) of wild common snook. Monthly changes in the gonadosomic index (GSI%) of wild female common snook during the reproductive cycle (A). Changes in oocyte diameter (µm) in the ovaries of wild female common snook (n = 50 oocytes measured per female) classed according to stage of maturity (B). Gonadal development in wild female common snook shown as proportion of the total population sampled each month (C). Monthly changes in the gonadosomic index (GSI%) of wild male common snook during the reproductive cycle (D). Gonadal development in wild male common snook shown as proportion of the total population sampled each month (E). All data are presented as mean ± SEM. Letters denote significant differences between reproductive phases (P = 0.05). Graphs are labeled as non-significant (NS) where no significant differences between gonadal stages were observed. Numbers above bars indicate the number of individuals sampled at each time point. Females: PGpn, Primary Growth Stage, Perinucleolar Step (n = 12); SGl, Secondary Growth Stage, Late Step (n = 10); SGfg, Secondary Growth Stage, Full-grown Step (n = 9); OMegv, Oocyte Maturation Stage, Eccentric Germinal Vesicle Step (n = 10); OMpov, Oocyte Maturation Stage, Preovulatory Step (n = 5); Ov, Ovulated (n = 10). Males: Early GE, Early Germinal Epithelium Development (n = 11); Mid GE, Mid Germinal Epithelium Development (n = 14); Late GE, Late Germinal Epithelium Development (n = 13).

Histological observations of gonads collected during the latter stage (around spawning time) of the reproductive cycle showed a circa-tidal rhythm of follicular development in female common snook (Figure 7). When the data were plotted, two distinct groups emerged. The first group, comprised of female snook sampled on the rising tide, was all found to have oocytes that were staged in Late Secondary Growth (SGl) and Secondary Growth Full–Grown (SGfg). In the second group, oocytes in the later stages and steps of Oocyte Maturation (including ooplasmic [clearing yolk] and germinal vesicle migration) and ovulation were only observed in females sampled at high tide or on the falling tide.

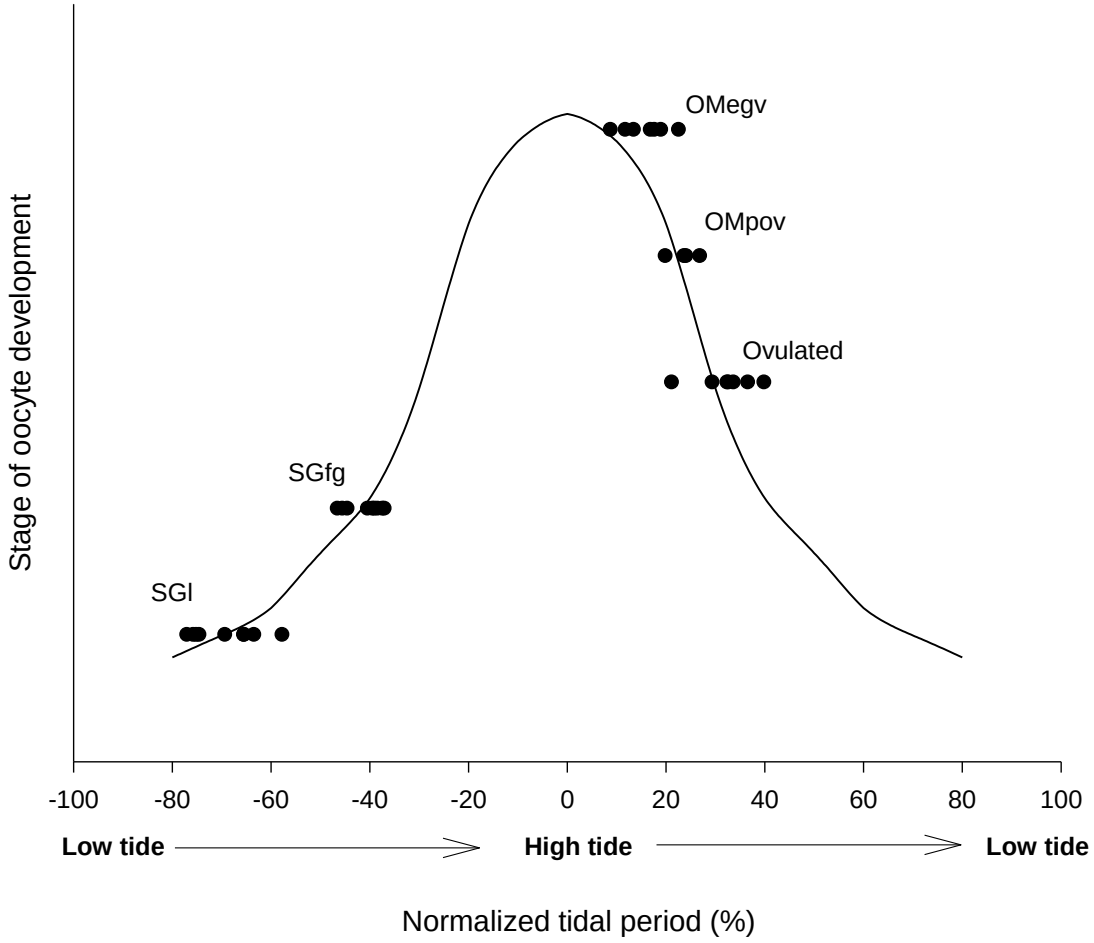

Figure 7. Influence of tidal cycle on oocyte development in common snook. Diurnal rhythm of follicular development observed in wild common snook. Each dot represents a single fish collected from one of three locations in Manatee County (Florida, USA) at different time points throughout a spring tidal cycle. Some dots many overlap. To account for daily or seasonal variation in tidal cycles, a generalized tidal pattern (12 hours) was used where data are expressed as percentages and plotted with respect to the time in hours from high tide. Oocyte development was assessed based on the most developmentally advanced cohort. Stages and Steps of oocyte development: SGl, Secondary Growth Stage, Late Step (n = 10); SGfg, Secondary Growth Stage, Full-grown Step (n = 9); OMegv, Oocyte Maturation Stage, Eccentric Germinal Vesicle Step (n = 10); OMpov, Oocyte Maturation Stage, Preovulatory Step (n = 5); Ov, Ovulated (n = 10).

3.3 Hormone analysis

Among the individuals sampled, plasma testosterone was only significantly elevated (P = 0.04) in females whose ovaries contained oocytes at the Oocyte Maturation Stage, Eccentric Germinal Vesicle Step (OMegv) (Figure 8A). Plasma 17β-estradiol (E_2) remained at basal levels (<0.1 pg/ml) in the months prior to the spawning season with a highly significant (P = 0.001) increase observed in females with oocytes classified as Secondary Growth Full-Grown (Figure 8B). In males, significantly higher (P = 0.001) levels of plasma T and 11-ketostestosterone (11-KT) were found among individuals with testes in Mid Germinal Epithelium Stage when compared to those in Early and Late Germinal Epithelium Stages (Figure 8C and D).

During primary growth and secondary growth plasma LH values in female common snook remained low. Levels started to increase in the Oocyte Maturation Stage and peaked during ovulation (Figure 9A). A highly significant (P = 0.001) elevation in LH plasma level was observed in male snook from the Early Germinal Epithelium (16 ng/ml) to Late Germinal Epithelium (103 ng/ml) Stage (Figure 9B).

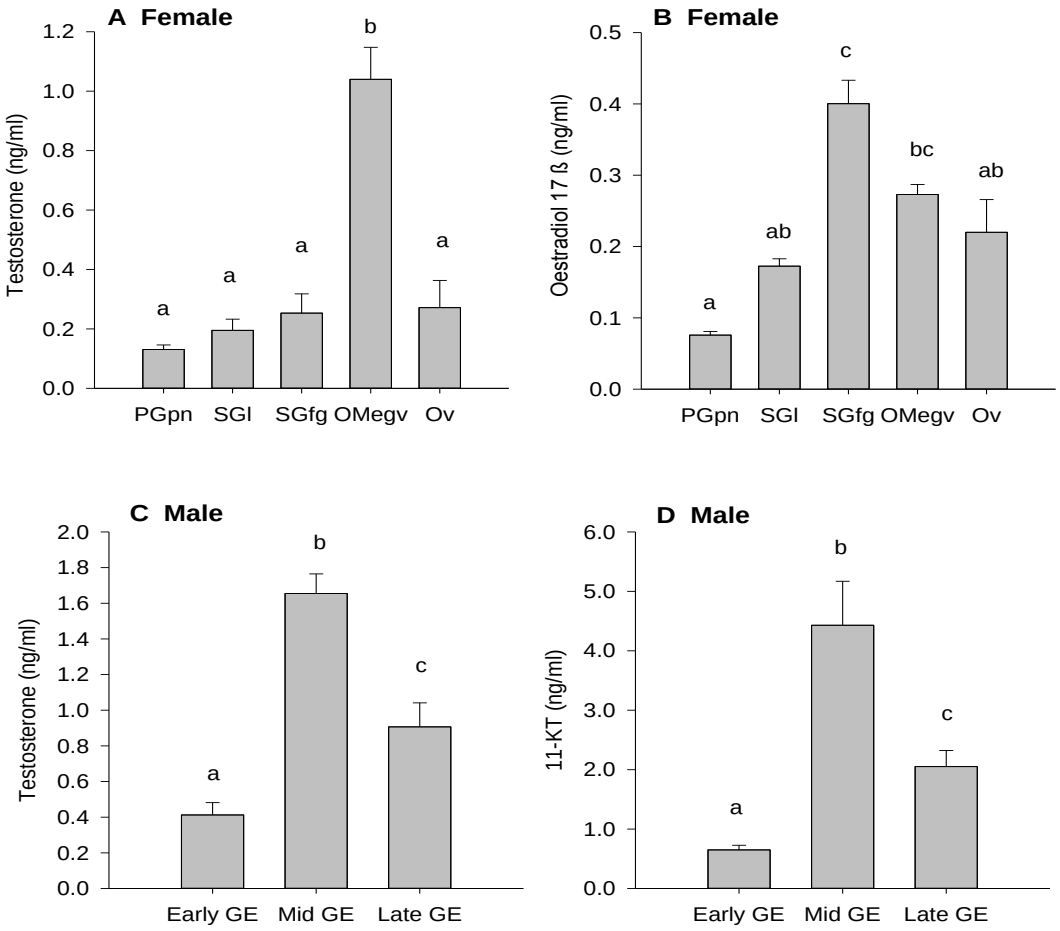

Figure 8. Changes in plasma sex steroid concentration during the reproductive cycle. Changes in plasma testosterone (A) and 17β-estradiol (B) in wild females and testosterone (C) and 11-ketotestosterone (D) in wild males collected during the reproductive period of common snook. Data are presented as mean ± SEM. Different letters denote significant differences between gonadal stages (P =0.05). Females: PGpn, Primary Growth Stage, Perinucleolar Step (n = 12); SGl, Secondary Growth Stage, Late Step (n = 10); SGfg, Secondary Growth Stage, Full-grown Step (n = 9); OMegv, Oocyte Maturation Stage, Eccentric Germinal Vesicle Step (n = 10); OMpov, Oocyte Maturation Stage, Preovulatory Step (n = 5); Ov, Ovulated (n = 10). Males: Early GE, Early Germinal Epithelium Development (n = 11); Mid GE, Mid Germinal Epithelium Development (n = 14); Late GE, Late Germinal Epithelium Development (n = 13).

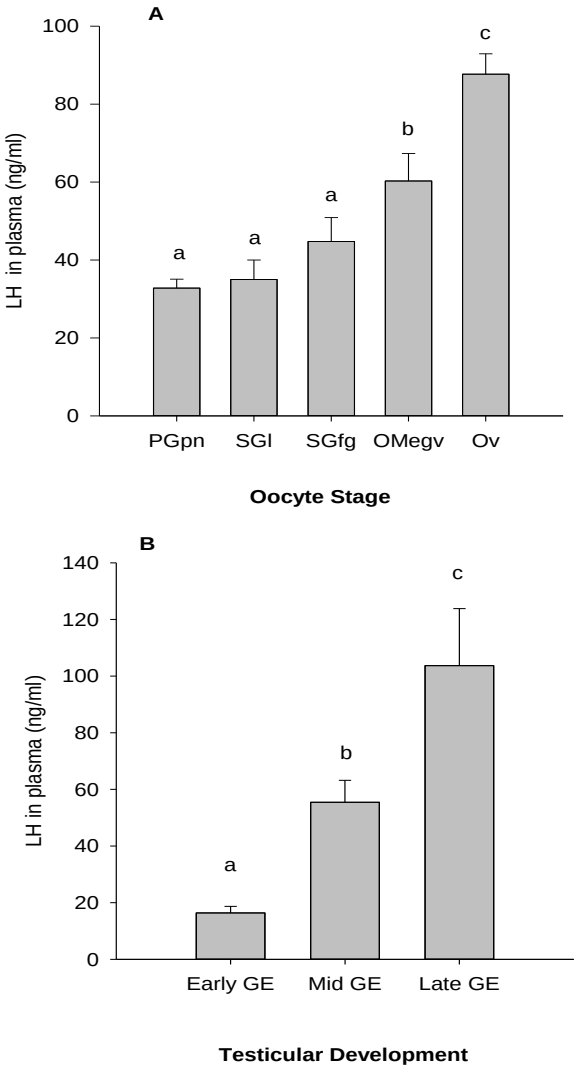

Figure 9. Change in LH plasma levels during maturation. Change in the concentration of plasma LH (A) in wild female and (B) male common snook in relation to reproductive phase of development as determined by histology. Values, shown as the mean ± SEM, Different letters denote significant differences between gonadal stages (P =0.05). Females: PGpn, Primary Growth Stage, Perinucleolar Step (n = 12); SGfg, Secondary Growth Stage, Late Step (n = 10); SGl, Secondary Growth Stage, Full-grown Step (n = 9); OMegv, Oocyte Maturation Stage, Eccentric Germinal Vesicle Step (n = 10); OMpov, Oocyte Maturation Stage, Preovulatory Step (n = 5); Ov, Ovulated (n = 10). Males: Early GE, Early Germinal Epithelium Development (n = 11); Mid GE, Mid Germinal Epithelium Development (n = 14); Late GE, Late Germinal Epithelium Development (n = 13).

3.4 Pituitary expression of fshβ and *lhβ*

In female common snook, the expression of *fshβ* and *lhβ* was very low (<25,000 copies/µg total RNA) in individuals collected in the spring (April and May), which had oocytes in the Primary Growth Stage (previtellogenesis), and it was not until the beginning of vitellogenesis (Secondary Growth Stage) that a slight but non-significant increase (4-fold) of the expression was observed (Figure 10A, B). A significant increase (P = 0.03) was observed in individuals whose oocytes were classified in the later stages of vitellogenesis including Secondary Growth Stage, Full-grown Step and Oocyte Maturation Stage, Eccentric Germinal Vesicle Step. Throughout the reproductive season, from the primary growth stage to the time of ovulation, a 6 and 10-fold increase was documented in *fshβ* and *lhβ* mRNA expression levels respectively. In male common snook, *fshβ* expression profile across the reproductive cycle showed levels gradually increased (2-fold difference between the highest and lowest levels) from early spermatogenesis (Early Germinal Epithelium Stage) through until males were fully spermiating (Mid and Late Germinal Epithelium) (Figure 10C, D). In contrast, expression levels of *lhβ* significantly increased (~2-fold) during the Mid Germinal Epithelium stage and fell again at the Late Germinal Epithelium Stage. Overall, maximum mean *lhβ* mRNA expression levels in females were almost 2-fold greater than in males; whereas, *fshβ* mRNA expression levels were similar between the sexes (Figure 10). Regression analysis showed a significant correlation (P = 0.005, r^2 = 0.58) between the *fshβ* and *lhβ* mRNA levels in female common snook (Figure 11).

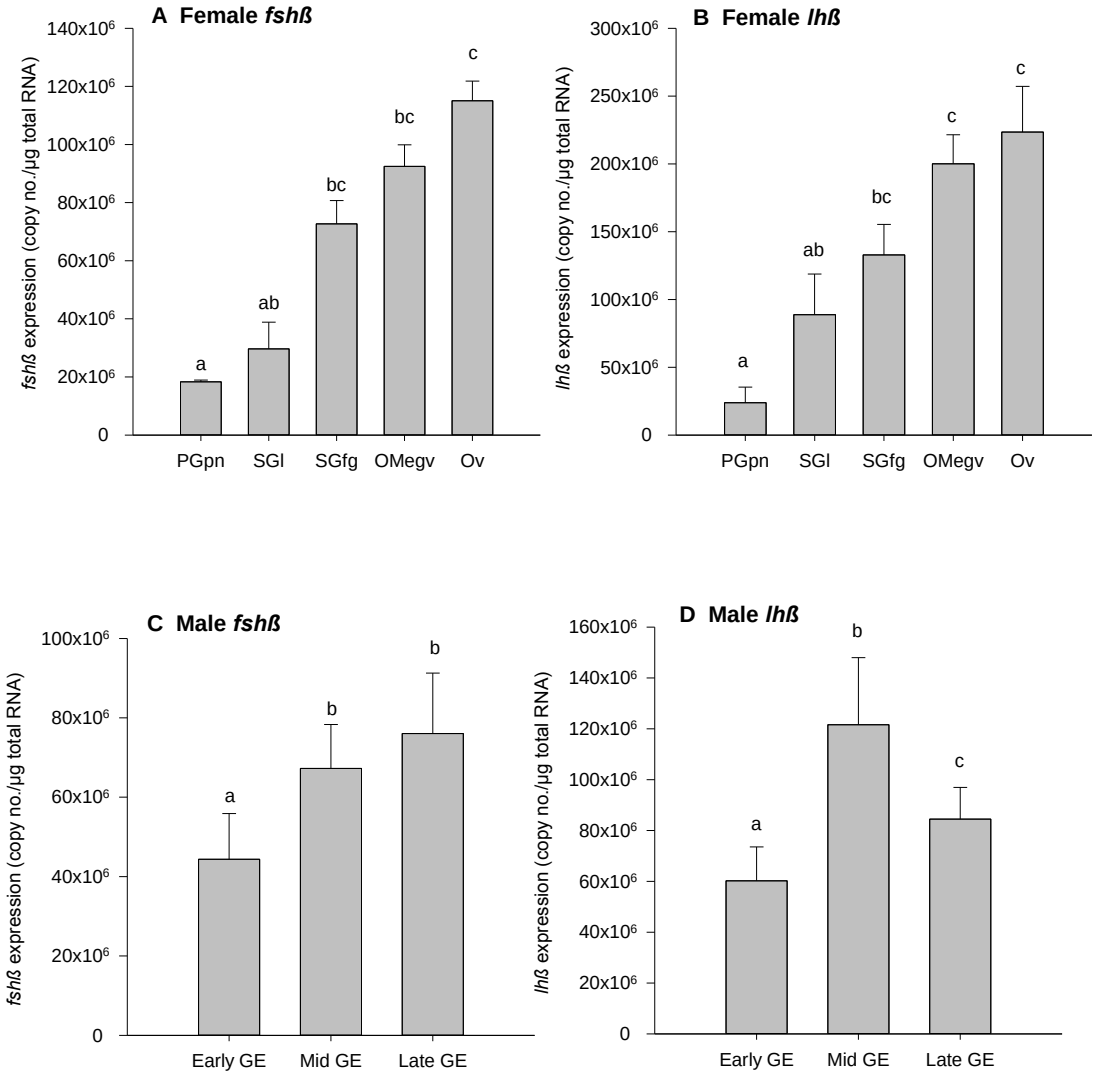

Figure 10. Mean absolute copy numbers of *fshβ* and *lhβ* based on gonadal staging. Mean absolute mRNA expression of *fshβ* and *lhβ* in the pituitary of female (A, B) and male (C, D) common snook sampled throughout the reproductive season. Values, shown as the mean ± SEM, are represented by stages of gonadal development as determined by histology. Females: PGpn, Primary Growth Stage, Perinucleolar Step (*n* = 12); SGl, Secondary Growth Stage, Late Step (*n* = 10); SGfg, Secondary Growth Stage, Full-grown Step (*n* = 9); OMegv, Oocyte Maturation Stage, Eccentric Germinal Vesicle Step (*n* = 10); OMpov, Oocyte Maturation Stage, Preovulatory Step (*n* = 5); Ov, Ovulated (*n* = 10). Males: Early GE, Early Germinal Epithelium Development (*n* = 11); Mid GE, Mid Germinal Epithelium Development (*n* = 14); Late GE, Late Germinal Epithelium Development (*n* = 13).

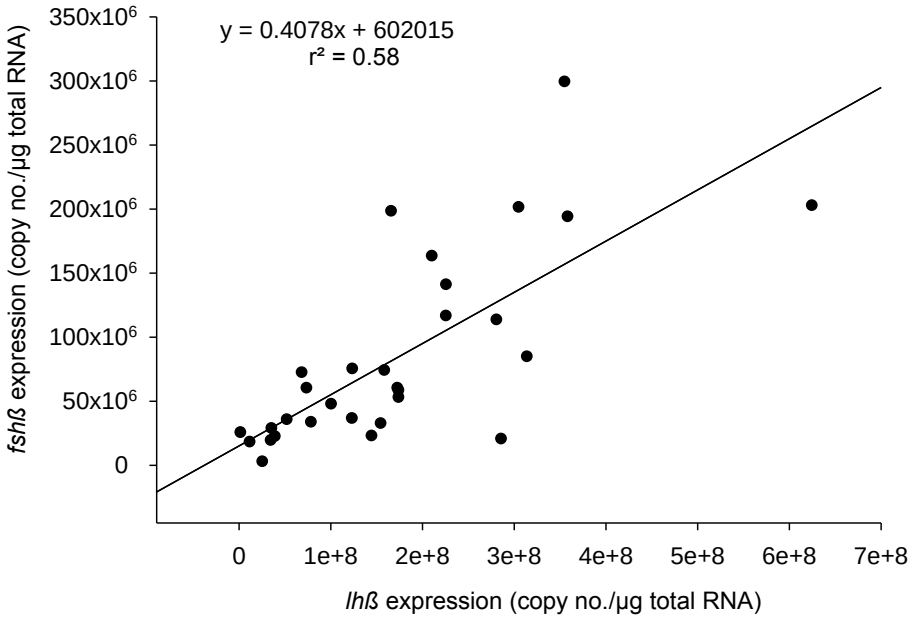

Figure 11. Linear regression lines between expression levels of *fshβ* and *lhβ* in female wild common snook collected throughout a reproductive season (*n* = 36).

The profiles of *fshβ* (Figure 12A) and *lhβ* (Figure 12B) in the pituitary of female common snook collected during this study showed a clear circa-tidal pattern of expression concomitant with histological changes in oocyte development. When the data were plotted, two distinct groups appeared and a strong circa-tidal influence was observed. Females in the first group, all sampled on the rising tide, had oocytes which were classified as Late Secondary Growth (SGl) and Secondary Growth Full–Grown (SGfg) and mean expression levels of *fshβ* (~110,000,000 copies/µg total RNA) and *lhβ* (~50,000,000 copies/µg total RNA) were shown to increase as oocyte maturation progressed. The second group of females, comprised of individuals sampled at high tide or on the falling tide, was found to have oocytes in the later stages and steps of maturation (Oocyte Maturation Step, Eccentric Germinal Vesicle Step (OMegv);

Oocyte Maturation Step, Preovulatory Step (OMpov). Mean expression levels of *fshβ* and *lhβ* were 2-fold higher (>100,000,000 copies/µg total RNA) than those observed in the first group and again increased in accordance with oocyte development peaking just prior to ovulation (Ov) (Figure 12 A, B). Regression analysis showed there was no correlation between the expression profile of *lhβ* mRNA and plasma LH level for male or female common snook ($P > 0.05$).

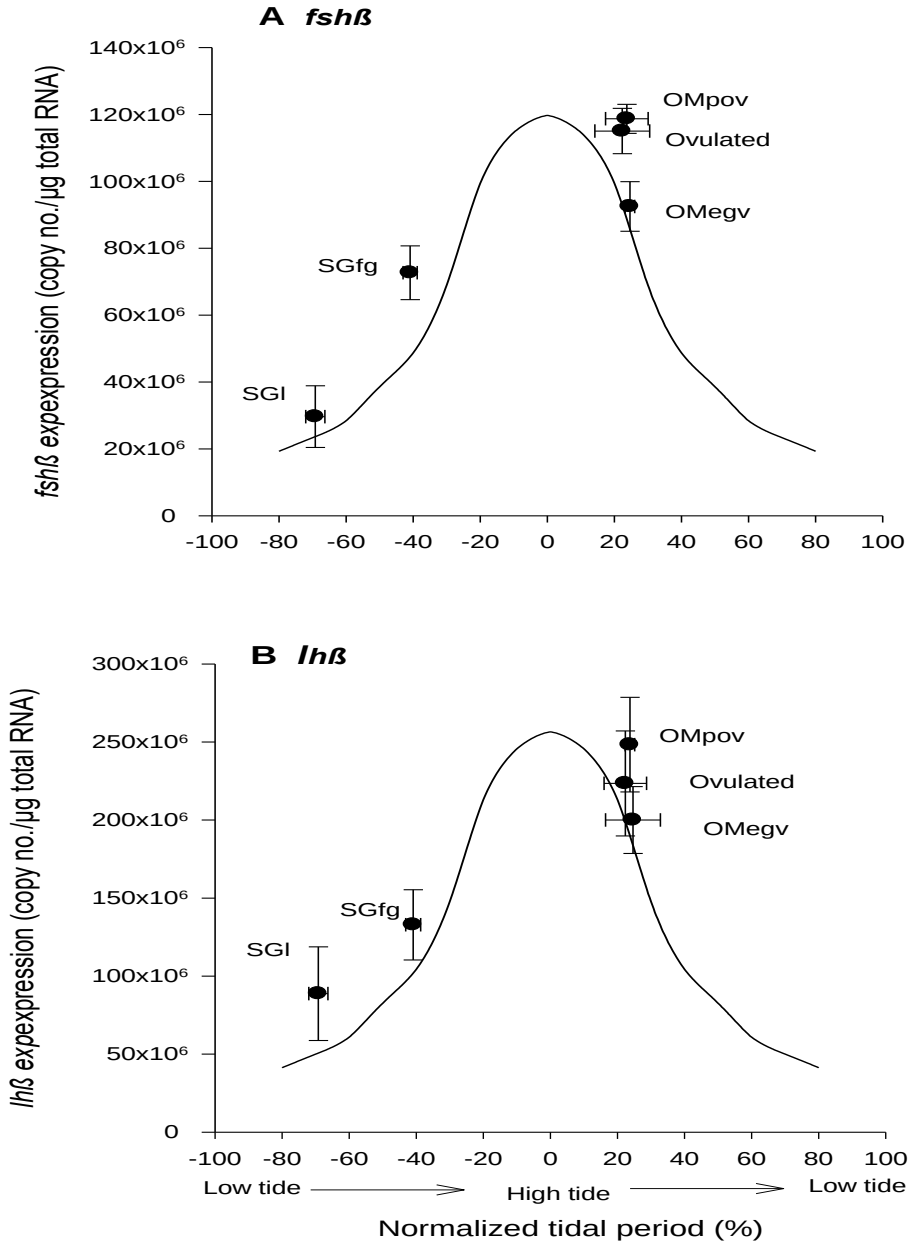

Figure 12. Influence of tidal cycle on expression of *fshβ* and *lhβ*. Each dot represents the mean ± SEM. Absolute copy numbers of *fshβ* (A) and *lhβ* (B) in the pituitary of female common snook collected from one of three locations in Manatee County (Florida, USA) at different time points throughout a spring tidal cycle. To account for daily or seasonal variation in tidal cycles, a generalized tidal pattern (12 hours) was used. Data are plotted in relation to the stage of oocyte development (verified by histology) with respect to the time in hours from high tide calculated as a percentage. Values, shown as the mean ± SEM. Stages and Steps of oocyte development: SGl, Secondary Growth Stage, Late Step (n = 10); SGfg, Secondary Growth Stage, Full-grown Step (n = 9); OMegv, Oocyte Maturation Stage, Eccentric Germinal Vesicle Step (n = 10); OMpov, Oocyte Maturation Stage, Preovulatory Step (n = 5); Ov, Ovulated (n = 10).

3.5 Predictive models

The overall GLM showed a significant effect (P = 0.03) and revealed varying influences of year, sample collection site, reproductive phase of development and tidal cycle on LH plasma level and pituitary mRNA expression of *fshβ* and *lhβ* in wild common snook. The model also revealed that among the four aforementioned predictor variables investigated, only tidal cycle and reproductive phase of development was found to significantly ($P \leq 0.05$) affect plasma LH levels and the mRNA expression of pituitary *fshβ* and *lhβ* (Table 2).

Table 2. Predictors used to model LH plasma levels and pituitary mRNA expression of fshβ and lhβ as a function of time (year), space (sample collection site), female reproductive phase of development and tidal cycle.

Response Variable	Effects	df	F	*P*
LH plasma	year	1	1.560	0.222
	collection site	2	0.130	0.875
	reproductive phase	4	4.170	0.009
	tidal cycle	42	6.010	0.021
fshβ	year	1	0.250	0.624
	collection site	2	0.100	0.903
	reproductive phase	4	2.910	0.040
	tidal cycle	42	4.520	0.043
lhβ	year	1	0.710	0.406
	collection site	2	1.090	0.351
	reproductive phase	4	5.910	0.001
	tidal cycle	42	5.070	0.032

4. Discussion

The environmental synchronizers of reproduction are not well understood in tropical and subtropical teleosts. This study shows a clear correlation between tidal cycle and oocyte maturation in wild common snook as evidenced by tidal patterns. Histological observations of gonads showed a circa-tidal rhythm of follicular development and levels of pituitary *fshβ* and *lhβ*, thus confirming the key role of this environmental factor in the control of spawning in common snook. The present study also correlates sex steroids and LH blood plasma levels with specific stages of maturation in male and female common snook. Fluctuations in plasma sex steroid levels reported in this study corresponded to their known roles in female and male gonadal maturation; where estradiol (E_2) promotes oogonial proliferation and vitellogenesis whereas the androgens, mainly 11-ketotestosterone (11-KT), regulate spermatogenesis and spermiogenesis. The range of steroid levels measured in this study was similar to those measured in Atlantic cod *Gadus morhua* (Cowan et al., 2012), red grouper *Epinephelus morio (Johnson et al., 1998)* and other marine teleosts (Pankhurst and Carragher 1991). Also, as expected, when all females were grouped according to their gonadal stage of development, the lowest levels of T and E_2 were measured in fish whose oocytes were in primary growth; whereas, the highest levels of T were measured in females just prior to ovulation (OMegv) and for E_2 in females with oocytes in secondary growth (SGfg). The highest androgen levels and GSI were measured in males during the spawning season when spermatocytes, spermatids and spermatozoa were present (Mid GE Development). Overall, findings confirmed those reported in Roberts et al. (1999) characterizing the complete annual reproductive cycle of wild common snook.

A partial *fshβ* and complete *lhβ* sequence was isolated from the pituitaries of wild individuals. Comparison of the amino acid sequences of the common snook *fshβ* and *lhβ* subunits with those of other fish showed higher similarities between *lhβ* sequences than between *fshβ* sequences as already suggested (Swanson et al., 2003). As in mammals, both gonadotropins are heterodimic glycoproteins, sharing an identical and distinct α while the β subunits differ and give them their biological specificity. The amino acid sequences of fish *lhβ* subunits are highly conserved, particularly in regions thought to be important for receptor interaction (Moyle et al., 1994). In contrast, the primary structures of fish *fshβ* subunits are more variable than that of fish *lhβ* subunits even in regions thought to confer ligand specificity (Swanson et al., 2003). One hypothesis would suggest that *fshβ* subunits have diverged more rapidly than *lhβ* during teleost evolution, and therefore, *fshβ* became the most species specific gonadotropic gene. This acceleration in the rate of evolution of *fshβ* was evidenced by discrepancies between relative similarity scores during phylogenetic comparisons addressing the split between two major clades of osteichthyans; actinopterygians (ray-finned) and sarcopterygians (fish lobe-finned fishes) (Quérat et al., 2000).

Although extensively covered in higher vertebrates, in comparison, the precise functions of LH and FSH in teleosts have only recently been understood in teleosts (Sambroni et al., 2013b). The mode of action and regulation of gonadotropins differs among species. This is often attributed to the phylogenetic diversity associated with the range of reproductive strategies found in fish (Levavi-Sivan et al., 2010; Kah and Dufour 2011). The analysis of gonadotropin gene expression in the pituitary of male and female common snook during the reproductive cycle showed both *fshβ* and *lhβ* subunits fluctuated in parallel with gonadal growth (GSI), peaking at the initiation of

spermiation in males and just prior to ovulation in females. These observations suggest that both gonadotropins are actively involved in the regulation of all processes of the reproductive cycle; a profile of expression similar to that of other teleosts that are multispawners, such as gilthead seabream (Gothilf et al., 1997) and goldfish (Yoshiura et al., 1997). These species, like common snook, exhibit various levels of asynchronous ovarian development (including group-synchronous [model] development), where several generations of oocytes are present simultaneously, each at a different stage of development (Wallace and Selman 1981; Brown-Peterson et al., 2011). In this model, synthesis of both FSH and LH β subunits are most likely to be required for vitellogenesis as well as for the end stages of oocyte maturation both of which occur at the same time in fish that exhibit this reproductive strategy (Yaron et al., 2003). In contrast, in synchronous spawners (whereby all oocytes develop and ovulate simultaneously) like salmonids, FSH synthetized in the pituitary and released into the blood stream is generally considered to regulate early phases of gametogenesis, such as vitellogenesis and spermatogenesis, whereas LH would be involved in the regulation of oocyte maturation, ovulation and spermiation (Yaron and Sivan, 2006). Mateos et al. (2003) reported a similar correlation to gonadal development in sea bass to that in the present study concluding that pituitary expression of both *fshβ* and *lhβ* is an accurate representation of pituitary protein abundance. In our work with common snook, no such analysis was conducted due to the lack of an available assay for measuring plasma FSH. Homologous immunoassays for both FSH and LH have only been developed for a few salmonids (Suzuki et al., 1988; Govoroun et al., 1998) and more recently tilapia (Aizen et al., 2007) therefore, restricting measurements to LH in most other species, a fact that has hampered studies on the functional duality of FSH and LH in fish (Molés et al. 2011).

Analysis of LH plasma concentrations in female and male common snook showed an increase throughout oogenesis and spermatogenesis, peaking at ovulation and spermiation. These results would support the already suggested role of LH in the regulation of the later steps of oocyte maturation and spermiation (Swanson et al., 2003). Endocrine (sex steroid profiles) and morphological (oocyte diameter GSI, histological staging) assessments performed during this study showed common snook collected on the Gulf coast of Florida had a seasonal pattern of gonadal growth and development.

The synchronization of recruitment into maturation, gonadal development and release of gametes are key reproductive events in vertebrates, which directly impact reproductive performance and success. Temperature and photoperiod have been shown to act as proximate (and/or ultimate) environmental cues stimulating maturation in a number of temperate teleosts (Migaud et al., 2010). Although their roles may be less critical in tropical species due to nominal seasonal photo-thermal changes in the natural habitat, studies have confirmed these exogenous cues can still impact reproductive timing and success (Hilder and Pankhurst 2003). There are however, a number of additionally important environmental factors showing daily and annual variations like rainfall, lunar phase and food supplies that alone or in combination may entrain the biological rhythms of living organisms (Yamahira, 2004; Naylor, 2010). In the present study, when compared with a variety of biotic and abiotic factors including year of capture and sample location, only tidal cycle and reproductive phase of development were among the predictors found to significantly ($P \leq 0.05$) affect plasma LH levels and pituitary *fshβ* and *lhβ* mRNA expression, all of which are directly linked to the timing of spawning. These results are contradictory to those reported in an earlier study

by Taylor et al. (1998) where oocyte maturation in common snook was determined to occur independently of either tidal cycle or lunar phase. However, the authors of this same study acknowledged that tidal amplitude may have influenced spawning (P = 0.057) despite the lack of statistical significance found during their analysis.

While most studies have concentrated on the seasonal rhythmicity associated with spawning, few physiological studies have investigated the role of tidal cycle on reproductive activity in fish (Naylor 2010). The lunar or semilunar-synchronized reproductive cycle has been seen in a wide variety of organisms, particularly those living in shallow waters and reef areas. In rabbitfishes, of the family Siganidae, studies showed oocytes develop synchronously in the ovaries with mature gametes being released with species-specific lunar periodicity during the reproductive season (Rahman et al., 2003; Takemura 2004; Park et al., 2006). A similar trend was observed where seasonal reproductive and lunar-related spawning cycles were documented in the honeycomb grouper *Epinephelus merra* (Lee et al., 2002). Grass puffer *Takifugu niphobles* were found to spawn in semilunar cycles during the spring tide and exhibit similar spawning behavior to snook where fish have been observed aggregating to certain near shore locations several hours before high tide with spawning occurring 1-2 h during the rising tidal phase (Ando et al., 2013). In the threespot wrasse *Halichoeres trimaculatusi,* the significant role of tidal cycle in gonadal development and spawning was demonstrated in the *Halichoeres trimaculatus* where daily spawning reportedly peaked around daytime high tides (Takemura et al., 2008). The current work provides the first empirical evidence of a circa-tidal control of the later phases of the gametogenesis cycle in common snook. These results highlight the need for further research on the role of environmental variables, such as tidal oscillations driven by

lunar gravity, in synchronizing rhythmic physiology in marine fish, potentially mediated via the biological clock mechanism.

Overall, our findings provide a better understanding of the environmental control of reproduction that in aquaculture can assist with the mitigation of reproductive bottlenecks, such as a lack of spontaneous spawning in captive broodstocks. In fisheries, an understanding of the synchronization of spawning events in wild common snook can lead to refined sampling regimes and focused capture efforts to ensure documentation of key unanswered questions regarding reproductive strategy. In conclusion, this new information about the influence of circa-tidal clock mechanisms will help to provide a better understanding of wild population reproductive strategy as well as how reproduction is entrained through environmental cues and the pathways leading to oocyte recruitment and maturation.

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CHAPTER 6

RESEARCH ARTICLE

EMBRYONIC AND EARLY LARVAL DEVELOPMENT IN HATCHERY REARED COMMON SNOOK

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Keywords: *Centropomus undecimalis*; oogenesis; egg development; larval growth

Abstract

To gain an improved understanding of the early life history of common snook *Centropomus undecimalis* and refine hatchery production techniques for this species, a combination of digital photography and histological techniques were used to document the embryonic and early larval development of hatchery-reared individuals. Embryo development from fertilization to hatching took 15 h at 28°C. Larvae at 2 d post-hatch showed fully pigmented eyes, and histological sections of the digestive tract revealed the presence of cellular structures indicative of a functional gut. This suggests that common snook larvae have the mechanical ability to detect, capture, and digest prey at 2 d post-hatch.

1. Introduction

The common snook *Centropomus undecimalis* is a diadromous, stenothermic, euryhaline, estuarine-dependent species found in the tropical and subtropical western Atlantic Ocean and Gulf of Mexico from about 34◦ N to about 25◦ S latitude (Howells et al. 1990). Snook *Centropomus* spp. are protandric hermaphrodites: some males develop into females between 1 and 7 years of age, having a maximum 20-year lifespan. The spawning of common snook has been studied for the last 55 years, but despite the importance of common snook as a popular game fish, the description of its reproductive biology is incomplete. Common snook in Florida have a daily spawning cycle in which spawning episodes occur during the late afternoon and the early evening hours during the lunar phases and during all tidal stages (Taylor et al. 1998).

The identification of critical embryonic and larval stages, such as eye and gut formation, first feeding, and swim bladder inflation, is essential for a better understanding of any fish species. This understanding may help to improve common snook larval rearing techniques and therefore increase larval survival rates.

Although less developed than in adults, the larval digestive tract is functional when feeding is initiated (Govoni et al., 1986). Additionally, the digestive tract develops as the larvae grow. This facilitates changes in the rates of ingestion, digestion, and the assimilation efficiency influence resulting in improved larval growth (Sarasquete et al. 1995). The development of the digestive system tract, as well as the possible abnormalities and deficiencies that result from the absence or inadequacy of food, has been studied in several teleosts (Cousin and Baudin-Laurencin, 1985; Avila and Juario, 1987; Eckman, 1987; Ferrais et al., 1987; Deplano et al., 1991; Boulhic

and Gabaudan, 1992) and is defined by the development of key digestive system structures.

Eye development for most fish species is critical for their survival, especially once exogenous feeding starts (Mani-Ponset et al., 1996), mainly due to their visual feeding nature (Blaxter, 1986; Batty, 1987). Prey capture, orientation, schooling and eluding predators are other basic activities that rely on vision (Paul, 1983; Blaxter, 1986; Porter and Theilacker, 1999). The role of vision in feeding has been investigated using varied light intensities. Significant differences in the consumption of prey were found among a variety of fish species including cisco *Coregonus artedi* (Jonh and Hasler, 1956), sole *Solea solea* and plaice *Pleuronectes platessa* (Blaxter, 1968), with most variations in foraging corresponding to periods of dusk and dawn. The decrease in rate of feeding corresponds with dusk and dawn periods. Some species can feed in the dark, e.g., cisco (Jonh and Hasler, 1956), especially when food is present in high concentrations. For example, plaice feed in the dark only at later stages around metamorphosis (Blaxter, 1968). Kawamura (1984), Pankhurst (1996), and Roo et al. (1999) reported major changes in the visual system in the lecithotrophic phase as preparation for onset feeding on sparids. At the same time, Roo et al. (1999) studied the relationship between gut and eye development in red porgy *Pagrus pagrus*, demonstrating that visual capability was developed before the onset of exogenous feeding.

No common snook eggs, embryos or early larvae have been documented from wild collections. This is a void in information on the life history and early feeding habits that generally forms the building blocks of aquaculture protocols. The objective

of this study was to describe the physiological, embryonic and larval developmental features of the common snook.

2. Materials and Methods

2.1 Egg collection and sampling procedures

All the samples were collected at the Mote Aquaculture Research Park located in Sarasota, Florida, from May to August 2007. The eggs were stripped from wild females from the field and fertilized with milt from wild males collected at the same time using a 92 m seine net deployed from a research vessel. Once fertilized, the eggs were transported to the main facilities and stocked in the rearing tanks.

One hour after fertilization, common snook embryos were stocked in a 50-L tank at a constant temperature of 28°C and salinity of 35‰. No aeration was used; dissolved oxygen was constant at 8 mg/L. The eggs were kept in the dark, simulating night time light characteristics. A sample of 10 embryos was collected every hour until hatching occurred. Once collected, samples were placed under a compound light microscope (Olympus 3500X) with a dark field. The egg diameter was measured and embryonic development was observed and documented using an attached 35 mm camera (Nikon 500). This sampling was repeated three times during three separate spawning events over the course of three nights.

2.2 Larval sampling procedures

Larvae were sampled from a 3,300-L production tank, where water temperature was maintained at 28° C, salinity at 35‰ and dissolved oxygen at 10 mg/L. Five larvae were randomly collected daily from day 0 to day 3 and every two days from day 4 to day 14 after hatching. Once collected larvae were placed under a light microscope (Olympus 4000), which had a digital camera mounted (Sony 600), larval pictures were taken under dark field conditions. Standard length (SL) and myomere height from the specimens were recorded using a calibrated microscope reticule (Table 1). This sampling regime was repeated for seven different spawning events, collecting a total of 280 larvae, between day 0 and day 14. Another 10 larvae were collected from day 1 to day 3 after hatching and fixed for transmission electron microscope (TEM) and scanning electron microscope (SEM) work.

Table 1. Mean ± SD standard length (mm) and myomere height (mm) of common snook during the first 14 d after hatching ($n = 35$).

Day	Standard length	Myomere height
0	1.785 ± 0.289	0.165 ± 0.015
2	2.311 ± 0.434	0.211 ± 0.024
4	2.266 ± 0.360	0.211 ± 0.059
6	2.520 ± 0.515	0.246 ± 0.036
8	2.556 ± 0.454	0.277 ± 0.040
10	3.137 ± 0.495	0.368 ± 0.047
12	3.580 ± 0.454	0.401 ± 0.055
14	4.433 ± 0.780	0.549 ± 0.025

2.3 Electron microscopy specimen fixation and preparation.

The larvae collected for the TEM were fixed in Karnovsky's fixative at 4◦ C for 2 hours and placed in cacodylate buffer rinse (pH 7.5) at 4°C until further processing. The tissues were then transferred to the Electron Microscopy Laboratory, Institute of Aquaculture, at the University of Stirling, Stirling, Scotland, where final processing was completed.

Block sectioning was carried out using a glass knife for semithin sections (0.5 µm) to be observed under the light microscope. For the TEM, block sectioning was carried out with a diamond knife for ultrathin sections (50 nm). Ultrathin sections were mounted on copper Formavar carbon-coated grids and metal stained with uranyl acetate and lead citrate using methods adapted from Venable and Coggeshall (1965) and Bozzola and Russell (1992). A drop of saturated uranyle acetate was placed on a piece of dental wax and the grid was floated over the drop for 30 min in the dark. The grid was rinsed with 50% alcohol and then with distilled water and floated on a drop of lead citrate for 25 min. The grid was thoroughly rinsed, dried on filter paper, and immediately removed to avoid dust accumulation on the grid. Sectioned samples were viewed and photographed at the Institute of Aquaculture on the TEM (FEI Tecnai E2 BioTWIN).

All fish were collected under a Florida Fish and Wildlife Conservation Commission Special Activity License (Contract No.10087, Permit # SAL 09-522-SR). Handling of the fish for routine management and experimentation was done in accordance with United States legislation concerning the protection of animals used for experimentation. All methods were conducted in accordance with Mote Marine Laboratory's Institutional Animal Care and Use Committee approved protocols (IACUC Approval No. 12-03- KM1).

3. Results

3.1 Embryonic development

The development of common snook embryos at 28° C took 15 h from fertilization to hatching. The fertilized eggs were spherical, with a homogenous yolk, a smooth chorion, and a single oil droplet. Egg diameters ranged from 0.65 mm to 0.72 mm (mean = 0.69 mm, n = 300) and the oil droplet diameter ranged from 0.15 to 0.30 mm (mean = 0.26 mm, n = 300). Fertilization occurred in the field; therefore, no embryonic development was documented prior to the blastodisc stage (first hour after fertilization). We estimated that the blastoderm separated from the yolk approximately 30 min after fertilization. Shortly after that, the blastoderm was then divided in two blastomeres.

The following is a description of development at approximate 1-h intervals. One hour and thirteen minutes after fertilization, the second segmentation or four-cell stage occurred. The second cleavage furrow developed on two blastomeres at a right angle to the first cleavage plane. It deepened until each blastomere divided into two cells of the same size. The oil droplet was larger and gathered toward the vegetal pole (Figure 1, a). One hour and thirty-two minutes after fertilization another division occurred. This time, the blastoderm divided in 16 blastomeres (Figure 1, b), also called the 16-cell stage, where the fourth cleavage plane, which parallels the second, divides the two rows of four blastomeres into four rows of four blastomeres (Figure 1, b).

Two hours after fertilization, another division occurred and the blastoderm divided into 32 blastomeres (Figure 1, c), the 32-cell stage, where the fifth cleavage plane divided the marginal 12 blastomeres meridionally into 24, and the central four

blastomeres horizontally into eight thereby forming two layers, an outer and an inner layer, in the central region. The number of marginal cells is 14 (Figure 1, c). Three hours after fertilization, the blastomeres were still distinct, but the rapid division was too advanced to observe the number of blastomeres; this is the late morula stage (Figure 1, d). At this time, the planes of the sixth and the later cleavages were difficult to precisely trace. The blastomeres (256–512) had different cleavage planes depending on their positions within the dome-shaped blastoderm and were arranged in three layers. The peripheral blastoderms (21–24) were flattened in shape. The cells were arranged in three to four layers but still easily dissociated from each other (Figure 1, d).

Four hours and ten minutes after fertilization, the blastomeres were no longer distinguishable and the blastocoel, or segmentation cavity, began to form. This was the blastula stage (Figure 1, e). Projection of the underside of the blastoderm (central cells) into the yolk sphere was observed. In this stage, some blastomeres began to cleave asynchronously and to migrate. Several rows of periblast nuclei were visible around the blastoderm (Figure 1, item e).

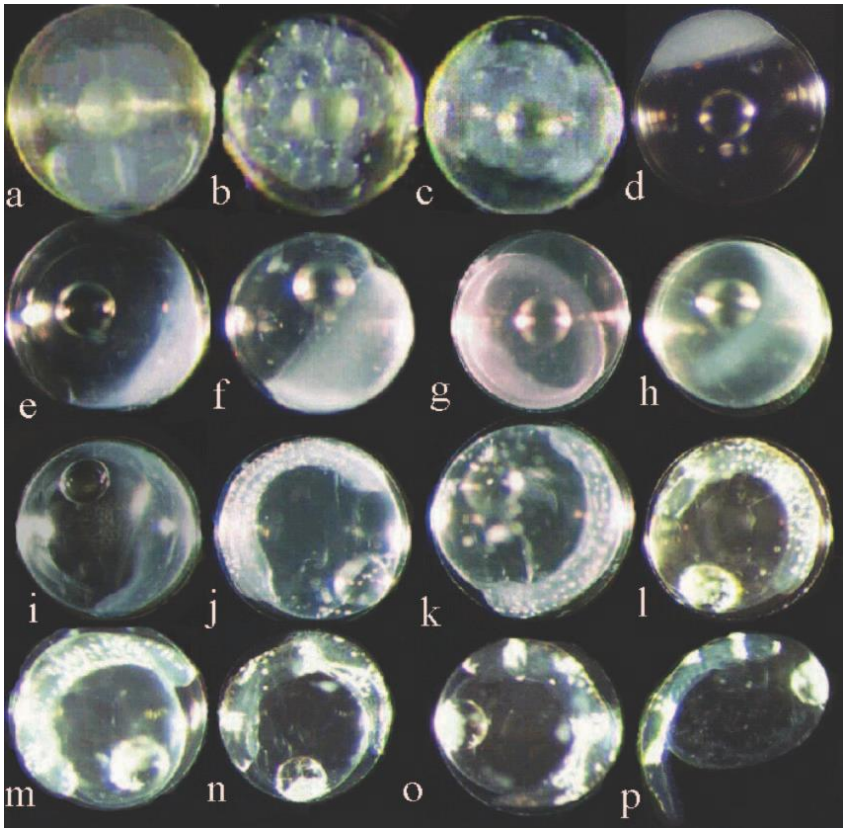

Figure 1. Embryonic development of common snook from fertilization to hatch (all times are postfertization): a = 1 h, 13 min; b = 1 h, 32 min; c = 2 h; d = 3 h; e = 4 h, 10 min; f and g = 5 h, 12 min; h = 6 h, 12 min; i = 7 h, 15 min; j = 8 h, 10 min; k = 9 h, 21 min; l = 10 h, 12 min; m = 11 h, 12 min; n = 12 h, 30 min; o = 13 h, 5 min; p = 14 h, 22 min.

Five hours and twelve minutes after fertilization, the blastoderm covered more than half the yolk (Figure 1, f) and the blastocoel were completely formed (Figure 1, f). This was the mid-gastrula stage, where a streak was visible in the midline of the embryonic shield projecting into the germ ring area (Figure 1, i g).

Six hours and twelve minutes post fertilization, the blastoderm covered three-fourths of the yolk sphere (Figure 1, h) and the embryonic shield became more clearly visible as a narrow streak. The enveloping layer extends uniformly over the yolk sphere through this stage (Figure 1, h).

Seven hours and fifteen minutes after fertilization, the blastoderm covered more than four-fifths of the yolk surface, leaving a small area around the vegetal pole exposed (Figure 1, i). This was the neurula stage, and the head could be clearly recognized anteriorly in the distinct embryonic body. A beak-like mass of cells was seen in front of the head, the embryo completes an arc of 150°. The brain and nerve cord in the arrow-shaped embryonic body developed as a solid rod of cells. A solid optic bud appeared on each side of the cephalic end. The beak-like cell mass was still visible and the blastopore was closed (Figure 1, i).

Eight hours and ten minutes after fertilization, melanophores were visible on the embryo for first time (Figure 1, j) and the optic vesicles were clearly outlined. This is the somite stage, where a pair of otic (auditory) vesicles appeared at the posterior region of the head. Depressions began to form at the dorsal surface of the eye vesicles (Figure 1, j).

Nine hours and twenty-one minutes after fertilization, the embryo was strongly pigmented (Figure 1, k), especially dorsolaterally. Melanophores on the yolk concentrated on the ventral surface on each side of the embryo. Some melanophores were also apparent on the oil droplet (Figure 1, k).

Ten hours and twelve minutes after fertilization (Figure 1, l), the optic vesicles differentiated to form the optic cup and the lenses began to form. The small otic vesicles appeared, but they lacked otoliths. The regions of the brain were well defined, and the neural fold was seen as a median line along the body (Figure 1, l).

Eleven hours and twelve minutes after fertilization (Figure 1, m), the tubular heart appeared under the head from the posterior end of the midbrain to the anterior

end to the hindbrain. The body cavity extended further toward the posterior end of the eye vesicles. The melanophores on the oil droplet were larger and more distinct; those on the head expanded to outline the olfactory lobes and optic vesicles. The tail separated from the yolk (Figure 1, m).

Twelve hours and thirty minutes after fertilization (Figure 1, item n), the melanophores were much larger and fewer, forming aggregations. The anterior portion of the heart, which exhibited a slow pulsation, extended upward to the anterior end of the forebrain. Cuverian ducts and the vitello-caudal vein were still incomplete. The embryonic body encircles nearly three-fourths of the yolk sphere (Figure 1, n).

Thirteen hours and five minutes after fertilization (Figure 1, o), the blood began to circulate and the heart beat was faster and more constant. At the same time, the embryo started moving with quick, short movements mainly produced by the tail. Melanophores were more compact and they accumulated in four places along the embryo's body (Figure 1, o).

Fourteen hours and twenty-two minutes after fertilization, the first larvae hatched (Figure 1, p). At this time, the melanophore accumulations could be observed clearly. Five groups spread along the newly fresh larvae. There were two on the head region, one over the olfactory region, and another around the eyes. The other two were situated in the midlateral and dorsolateral area, and another one in the postanal area (Figure 1, item p). Fifteen hours after fertilization massive hatching took place; 90% of the eggs hatched at that time.

3.2 Larval development

Common snook larvae hatched 15 h after fertilization at 28°C (Figure 1, p), measuring 1.71 mm in mean SL, ranging from 1.38 to 1.84 mm (n = 35). The body of newly hatched yolk sac larvae was elongated with an oval shape and a mean size of 0.91 mm in length, ranging from 0.85 to 1.02 mm (n = 300), and with a mean width of 0.58, ranging between 0.41 and 0.72 mm. A single oil droplet was present, located on the yolk sac's front side under the head. A transparent voluminous fin fold covered most of the body (Figure 1, p). The eyes and mouth were not formed, and no eye pigmentation was present. The larvae were concentrated on the surface, floating and moving mainly in circles due to their limited fin development.

3.3 Yolksac stage

On day 0 (24 h after fertilization), common snook larvae (Figures 2, 3, and 4, item a) measured 2.25 mm (mean SL), ranging from 1.90 to 2.45 mm (n = 35), and had a mean myomere height (M_H) of 0.164 mm, ranging from 0.152 to 0.171 mm (n = 35). The yolk sac was reduced in size with a mean length of 0.51 mm, ranging from 0.31 to 0.63 mm, and a mean width of 0.35 mm, ranging from 0.29 to 0.45 mm. Eyes were starting to form, and some pigmentation was observed. The mouth was not formed, although some definition was observed. The pectoral fins were starting to develop. In terms of larval behavior, larvae were mainly at the surface, although some larvae were distributed in the water column but close to the surface. Swimming movements were more directional.

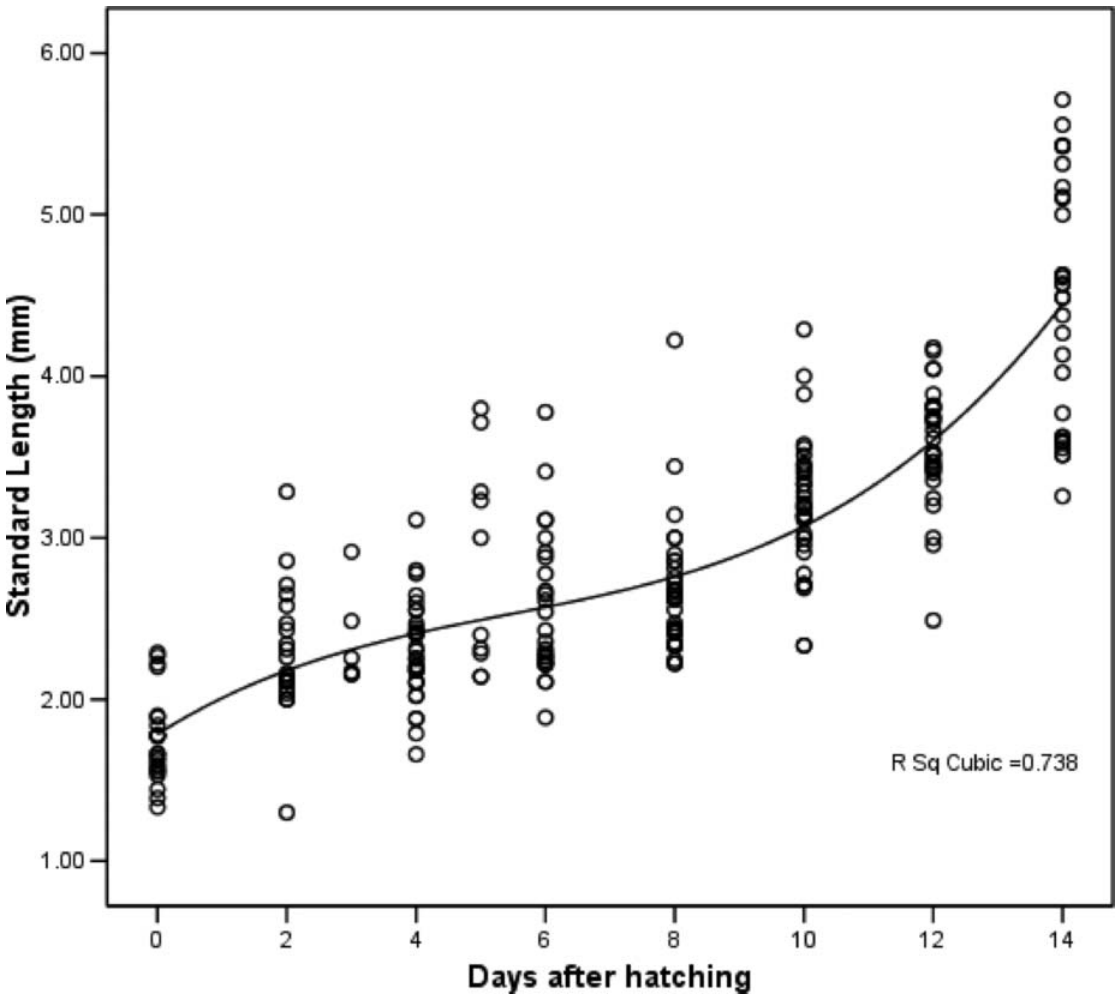

Figure 2. Common snook larval growth during the first 14 d after hatching.

Day 1 larvae (Figure 3) had a mean SL of 2.07 mm, ranging from 1.92 to 2.17 mm, and an average M_H of 0.202 mm, ranging from 0.180 to 2.071 mm (n = 35). The yolk sac, although reduced to half the size from the previous day, was still present with a mean length of 0.16 mm, ranging from 0.12 to 0.20 mm. The eyes were starting to gain definition and the retina could be differentiated with eye pigmentation observed but not fully completed. The optic tectum or primary optic center of the brain large (Figure 5A) as in most fish, which rely on their sense of vision. Lenses were fully

complete and visible (Figure 5A) and the internal layer organization was well established, although layer thickness is still low. The cornea and the cartilaginous ring were present, as well as the optic chiasma directly below the infundibulum. The retina layer organization could be observed (Figure 5B) and the main layers were visible, such as the outer limiting layer and the outer nuclear layer, where the columnar nuclear bodies were present although undeveloped. Also, the outer plexiform layer and inner nuclear layer could be observed, yet most of layers were not fully developed with some main organelles missing (Figure 5C), where the outer limiting layer and the outer nuclear layer were still lacking organelles definition. Some pigments in the epithelium were present, although still low in levels (Figure 5D). The alimentary canal of 1-d-old larvae showed some differentiation along its length. Cilia, which help to circulate the contents, could be observed in the lumen (Figure 6B–C), and at the same time some irregular small microvilli appeared along the digestive wall. Other organelles observed included the mitochondrion and nucleus of the epithelial cells, although these organelles were present in low numbers. Other structures, such as the nonvillous region and the terminal web, were clearly defined although they were lacking in thickness (Figure 6C).

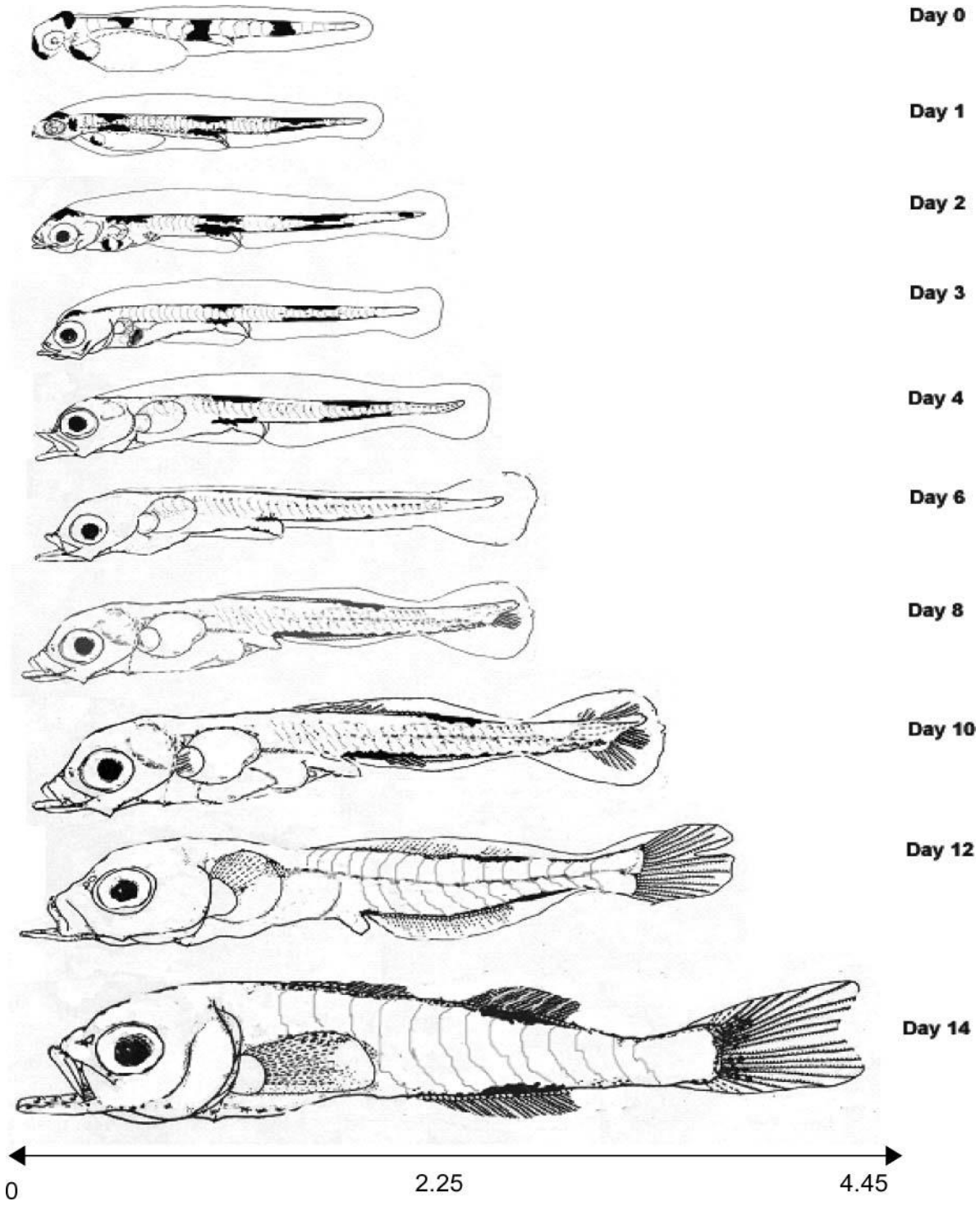

Figure 3. Common snook larval development from day 0 to day 14 after hatching. Length is shown in millimeters.

On day 2 (Figures 3 and 4, item b), SL had increased to a mean 2.31 mm, ranging from 2.12 to 2.71 mm, and with an average M_H of 0.211 mm, ranging from 0.185 to 0.282 mm ($n = 35$). The yolk sac had been nearly absorbed reducing its size to a mean length of 0.15 mm, ranging from 0.11 to 0.20 mm. The oil droplet was still present although reduced in size (Figures 3 and 4, item b). Eyes were formed and pigmented (Figure 5E–F) and the cornea had gained in thickness and was tight against the lens. The retina layer was more defined and each layer was thicker. The optical nerve was fully formed and connected to the main nervous system (Figure 5E–J). For the first time, the clear layer of pigment epithelium cells was present and the other layers, such as the outer nuclear layer, were gaining in complexity (Figure 5F–G) with the development of organelles such as the cones and rods (Figure 5H) nearly completed (Figure 5I). The mouth was formed and opened (Figures 3 and 4, item b) with the main cartilages, such as the Meckel's cartilage, the hyposynplectic cartilage, and the basihyal cartilage, already present (Figure 5E). Also the tongue could be observed (Figures 4, item b, and 5E). The digestive system was straight and long, extending past the posterior margin of the yolk sac and into the ventral fin fold, and although undeveloped, it had some food inside. The digestive system walls were defined, no cilia was observed in the lumen, and the microvilli layer was now well established along the walls (Figure 6D). Also the microvilli were long and compact (Figure 6E–F). The epithelium cell structure was forming and the main organelles were observed, such as the mitochondrion, the clear and dark apical cells, and the epithelium nucleus (Figure 6E–F); however, organelle numbers were low. Pectoral fins were well developed and functional. Slight caudal fin definition could be observed. Larvae showed a photopositive reaction, gathering at those areas with more light. The

distribution of larvae was more diverse, having larvae distributed in the water column and at the surface. Burst movements towards prey were commonly observed.

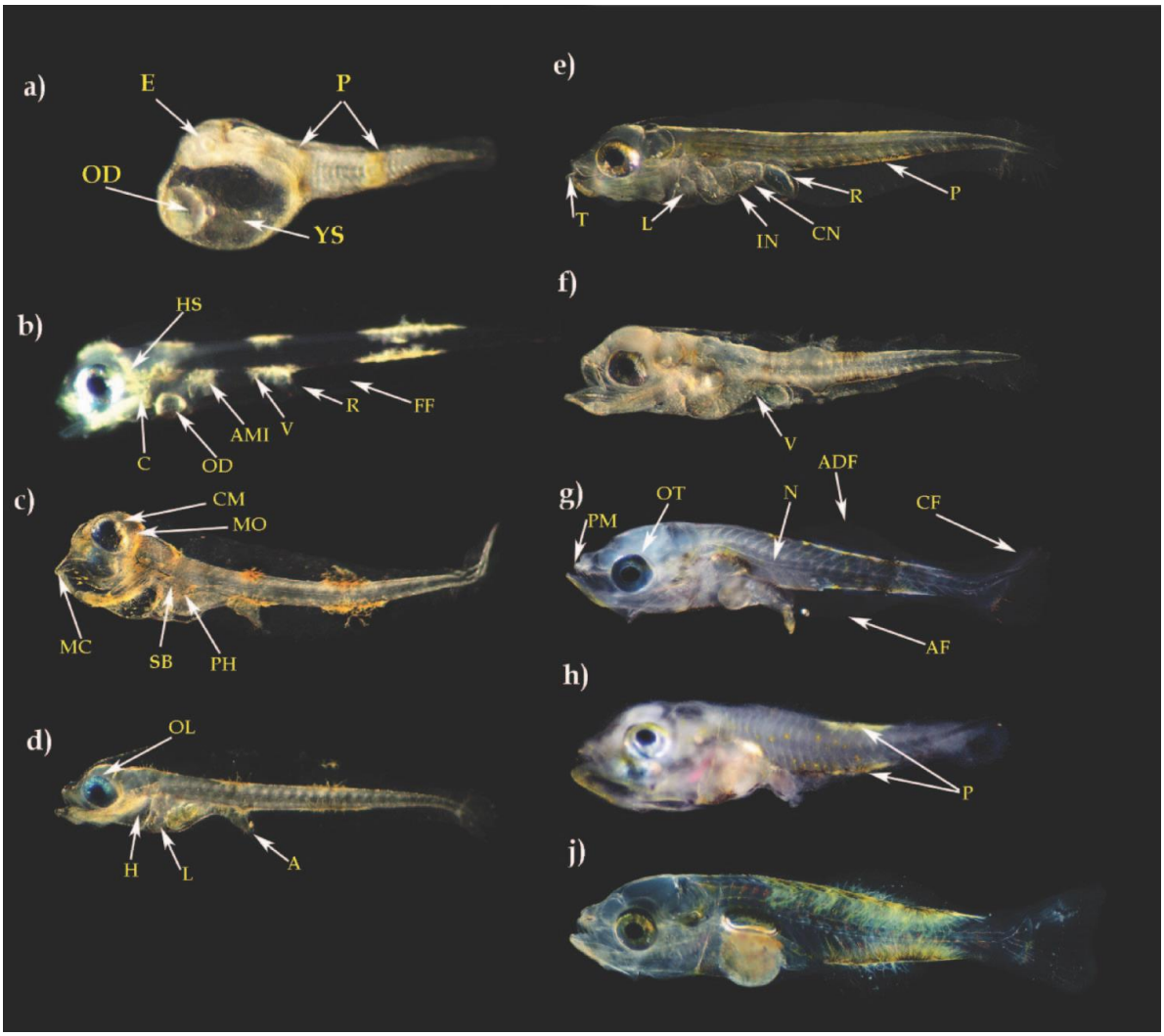

Figure 4. Common snook organ development from day 0 to day 14 after hatching: a = day 0, b = day 2, c = day 3, d = day 4, e = day 6, f = day 8, g = day 10, h = day 12, j = day 14. Abbreviations are as follows: A = anus, ADF = anterior dorsal fin, AF = anal fin, AMI = anterior median intestine, C = ceratohyal, CF = caudal fin, CM = cerebellum, CN = constriction, E = eye, FF = fin fold, H = heart, HS = hyposynplectic cartilage, IN = intestine, L = liver, MC = Merkel's cartilage, MO = medula oblongata, N = notochord, OD = oil droplet, OL = optic lobe of brain, OT = optic tectum, P = pigments, PH = posterior part of hind, PM = premaxilla, R = rectal area, SB = swim bladder, T = teeth, V = intestine-recto valve, and YS = yolk sac.

On day 3 (Figures 3 and 4, item c), snook larvae had a mean SL of 2.26 mm, ranging from 2.15 to 3.1 mm, and a mean M_H of 0.214 mm, ranging from 0.20 to 0.35 mm (n = 35). The yolk sac was almost completely absorbed and the oil droplet was still present, although severely reduced. The eyes had gained pigmentation and were developed. The retina layer was well structured due to the development of most of the layer organelles with the cornea fully formed (Figure 5K–L). All the different layers were clearly differentiated, such as the outer ganglion layer, the inner nuclear layer and the inner ganglion layer (Figures 5L, 6A). The pigment epithelium cell layer was fully formed with photoreceptor inclusions (Figure 6A), the bodies of the photoreceptors were also now well defined (Figures 5L, 6A). The maxillar jaws were developing and mouth gap was increasing. The alimentary canal was no longer a long straight tube; some structure could be observed especially in the anal region, which, by this stage, was well developed. Structural epithelium organelles, such as the nucleus, mitochondrion, and dark vesicles, were all present and in high numbers (Figure 6H– I). The microvilli layer had gained length and was more compact (Figure 6H). Other important organelles were also present, such as the Golgi apparatus, rough endoplasmatic reticulum, desmosome of apical junction and pinocytotic vesicle (Figure 6I). Food was observed in the gut, mainly rotifers and algae. The swim bladder was formed and showing signs of inflation. Swimming speed had increased, as well as larvae motility.

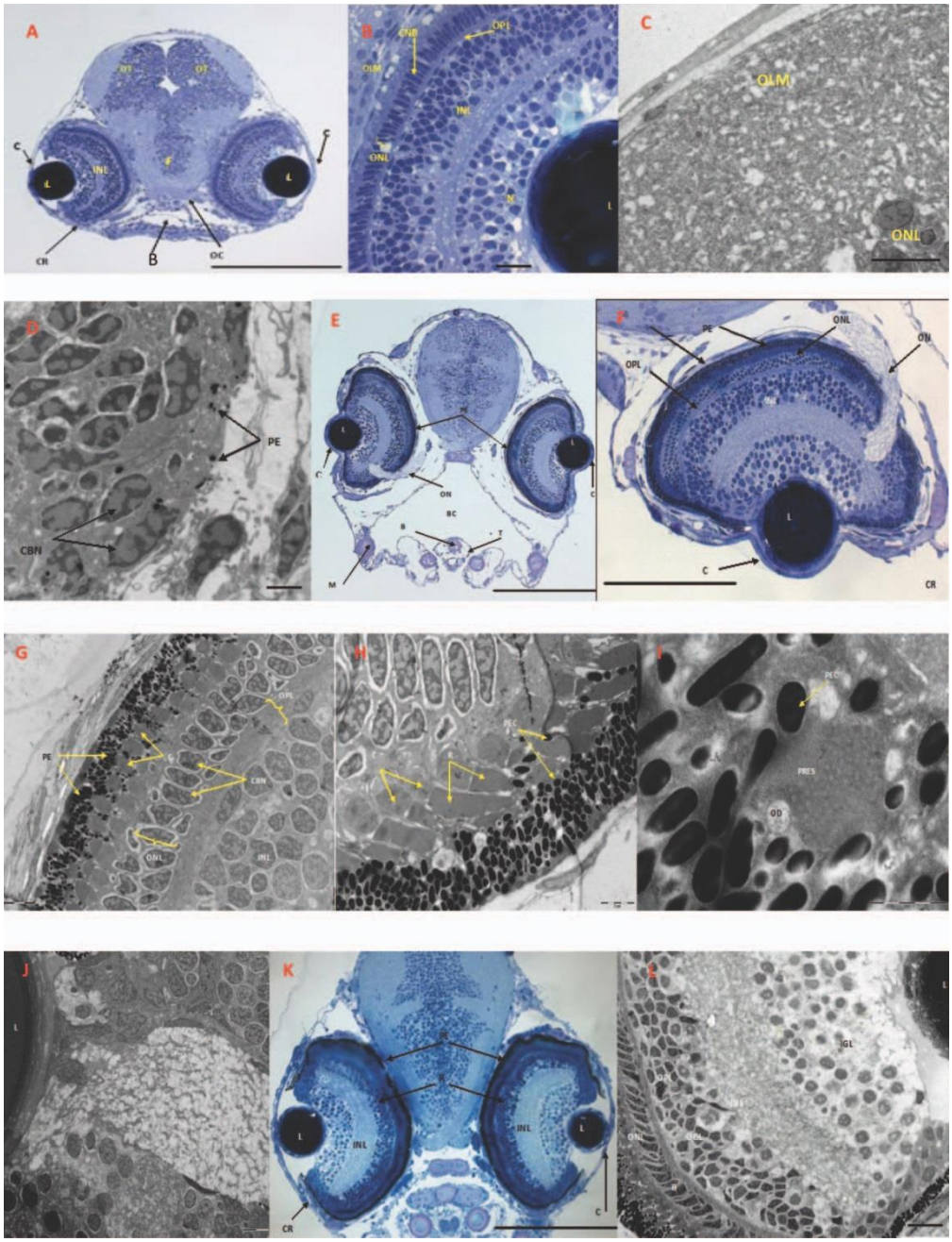

Figure 5. Optical SEM and TEM cross sections of common snook larva from day 1 to day 3 posthatch (dph): **(A)** semithin cross section from the head of a 1-dph common snook larva (scale bar = 100 µm; B = buccal cavity, C = cornea, CR = cartilaginous ring, F = infundibulum, INL = inner nuclear layer, L = lens, OC = optic chiasma, and OT = optic tectum); **(B)** cross section of the eye of a common snook larva at 1 dph (scale bar = 15 µm; CNB = columnar nuclear bodies [nuclei of photoreceptors], OLM = outer limiting membrane, ONL = outer nuclear layer, and OPL = outer plexiform layer); **(C)** cross section of the eye of a common snook larva

at 1 dph (scale bar = 10 µm); **(D)** cross section of the eye of a common snook larva at 1 dph (scale bar = 5 µm; PE = pigment epithelium); **(E)** semithin cross section from the head of a 2-dph common snook larva (scale bar = 90 µm; BC = basihyal cartilage, M = Meckel's cartilage, ON = optic nerve, and T = tongue); **(F)** semithin cross section from the head of a 2-dph common snook larva (scale bar = 40 µm); **(G)** cross section of the a eye of common snook larva at 2 dph (scale bar = 5 µm); **(H)** electron micrograph of a cross section of the eye of a common snook larva at 2 dph (scale bar = 2 µm; C = cones, PEC = pigment epithelium cell granule, and R = rods); **(I)** cross section of the eye of a common snook larva at 2 dph (scale bar = 1 µm; OD = oil droplet, PRES = photoreceptor outer segment); **(J)** cross section of the eye of a common snook larva at 2 dph (scale bar = 10 µm); **(K)** semithin cross section from the head of a 3-dph common snook larva (scale bar = 100 µm); and **(L)** cross section of the eye of a common snook larva at 3 dph (scale bar = 5 µm; B = bodies of photoreceptors and IGL = inner ganglion layer).

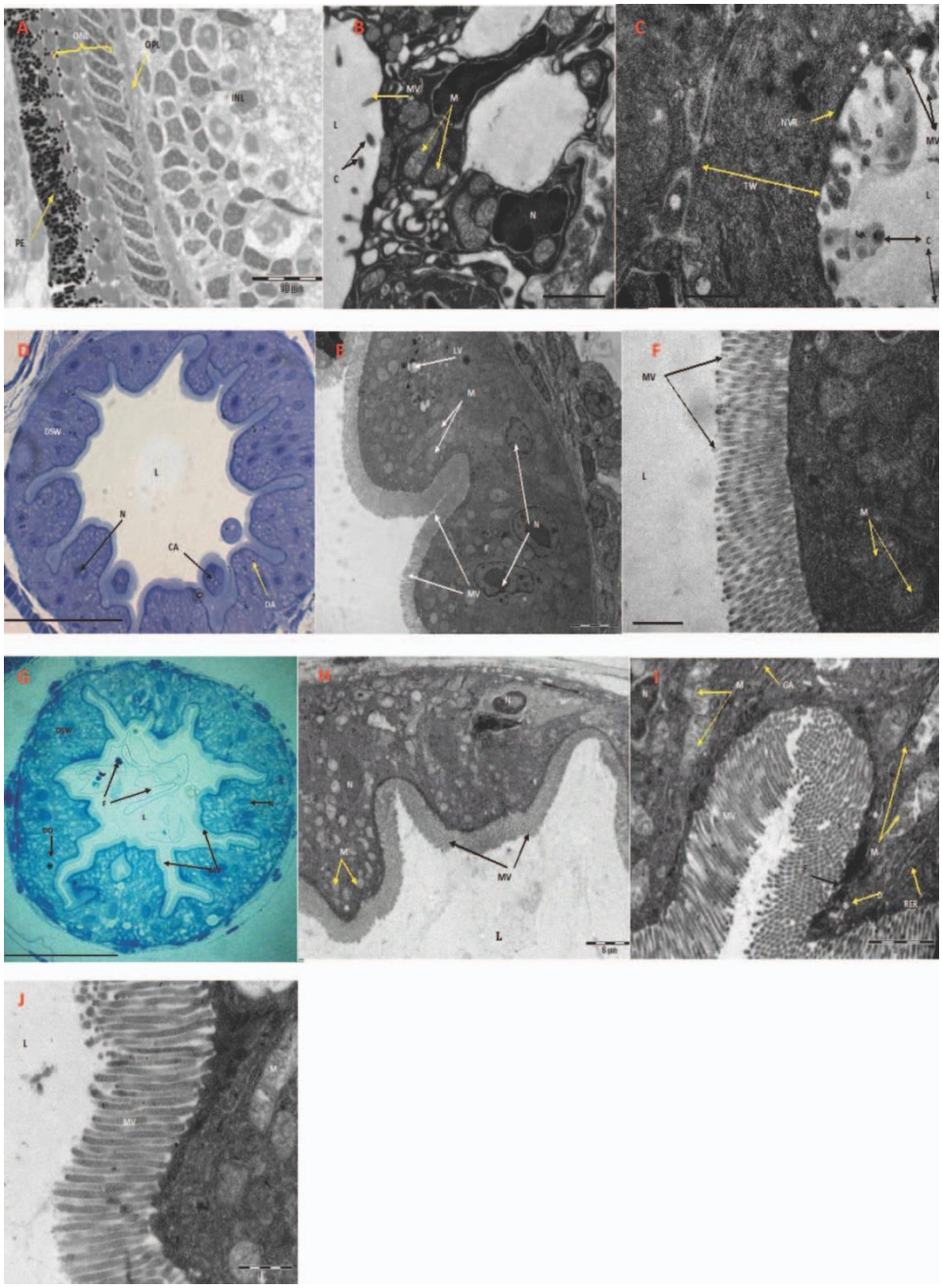

Figure 6. Optical SEM and TEM cross sections of the eye and digestive system of common snook larva from 1 to 3 dph (see Figure 5 for abbreviations not defined here): **(A)** cross section of the eye of a common snook larva at 3 dph (scale = 10 µm); **(B)** cross section of the antero-medial intestine of a common snook at 1 dph (scale bar = 2 µm; C = cilia, L = lumen, M = mitochondrion, MV = microvilli, and N = nucleus); **(C)** cross section of the rectal area of a common snook at 1 dph (scale bar = 2 µm; NVR = nonvillous region and TW = terminal web); **(D)** semithin cross section of the antero-medial intestine of a common snook larva at 2 dph (scale bar = 70 µm;

CA = clear apical cell in epithelium, DA = dark apical cell in epithelial, DSW = digestive system walls, and N = nucleus of columnar cell); **(E)** cross section of the antero-medial intestine of a common snook larva at 2 dph (scale bar = 5 µm; LV = light vesicle); **(F)** cross section of the rectal area of a common snook larva at 2 dph (scale bar = 1 µm); **(G)** cross section of the antero-medial intestine of a common snook larva at 3 dph (scale bar = 100 µm; DD = dark droplet, F = food particles, and N = nucleus of enterocyte); **(H)** cross section of the antero-medial intestine of a common snook larva at 3 dph (scale bar = 5 µm); **(I)** cross section of the rectal area of a common snook at 3 dph (scale bar = 2 µm; D = desmosome of the apical junction, GA = Golgi apparatus, and RER = rough endoplasmic reticulum); and **(J)** cross section of the antero-medial intestine of a common snook larva at 3 dph (scale bar = 1 µm).

3.4 Preflexion stage

Day 4 larvae (Figures 3 and 4, item d) had mean SL of 2.35 mm, ranging from 2.22 to 3.23 mm, and a mean M_H of 0.211 mm, ranging from 0.20 to 0.28 mm (n = 35). By day 4, the yolk sac had been totally absorbed and the oil droplet was not present anymore (Figure 3 and 4, item d). The medulla oblongata could be observed as well as the cerebellum, which was fully formed. The eyes were fully pigmented, the mouth was fully functional with more jaw development, and teeth formation could also be observed. The swim bladder, positioned posterior to the pectoral fin base and above the stomach, was developed and inflated. The gut gained in thickness and became partitioned. Rotifers were observed in the gut.

At this stage larvae were scattered throughout the water column, although no larvae could be seen on the tank bottom. There were many active swimmers, which spent most of the time seeking prey.

On day 6 (Figures 3 and 4, item e), the mean SL of snook larvae was 2.51 mm, ranging from 2.1 to 3.41 mm, with a mean M_H of 0.245 mm, ranging from 0.214 to 0.351 mm (n = 35). Rows of teeth were present at the same time that mouth gap had increased and the gas bladder was inflated. The gut was well partitioned and food was commonly observed inside with 90% of larvae observed having full stomachs. The fin fold around the larvae was no longer present and the fins were starting to take shape, especially the caudal and dorsal fins, and the pectoral fins were fully functional but still developing.

At day 8, snook larvae (Figures 3 and 4, item f) had a mean SL of 2.55 mm, ranging from 2.41 to 3.7 mm, with a mean M_H of 0.276 mm, ranging from 0.21 to 0.34 mm (n = 35). At this stage, the snook larvae had increased body depth and increased head size in relation to eye size. The notochord was fully formed and ended at the caudal fin. There was an increase in volume of the digestive system, and definition of the different organs was more apparent. The caudal fin started developing into the shape of an adult caudal fin, going from a more rounded initial shape towards a forked fin shape.

3.5 Flexion stage

Ten days after hatching (Figures 3 and 4, item g) common snook larvae had a mean SL of 3.136 mm, ranging from 3 to 4.2 mm, and the mean M_H was 0.381 mm, ranging from 0.291 to 0.41 mm (n = 35). At this stage, notochord flexion had started, larvae had increased head size, and both maxillar and premaxillar bones were gaining in thickness and strength. Teeth were present now in both jaw bones. The digestive

system was well differentiated, with all the organs gaining in volume and structure. Also, 90% of the observed stomachs were full, rotifers were observed all along the digestive system. Fin shape definition continued to develop, especially the dorsal and caudal fins (Figures 3, 4g).

Common snook larvae at day 12 (Figures 3 and 4, item h) had a mean SL of 3.57 mm, ranging from 3.41 to 3.84 mm, and an average M_H of 0.401 mm, ranging from 0.266 to 0.467 mm (n = 35). The development of the dorsal and anal fin bases could be observed. At the same time, notochord flexion was nearly complete. Larval body depth continued to increase and the head size was still proportionally larger in width than the rest of the body.

By day 14, common snook larvae (Figures 3 and 4, item i) had a mean SL of 4.43 mm, ranging from 3.57 to 5.71 mm, and their mean M_H was 0.41 mm, ranging from 0.36 to 0.46 mm (n = 35). By this stage, the anterior dorsal fin was developing faster than the posterior one and nine rays could be observed. Similar development happened to the anal fin, although rays were not as developed and notochord flexion was completed.

During the first 14 d after hatching, development of common snook was rapid in terms of SL (Figure 2). We observed three stages: the first stage or the yolk sac stage occurred over a 2-d period where the SL increased from 1.78 to 2.311 mm. The next stage was the preflexion stage, which occurred over a 4-d period, where the increase in SL was less than in the yolk sac stage, going from an average SL of 2.26–2.56 mm (Figure 2). The last stage or the flexion stage occurred over a 6-d period and the average

SL went from 2.56 mm to 4.43 mm, increasing exponentially each day. The mean SL and M_H measurements during the first 14 d of growth can be seen in Table 1.

4. Discussion

4.1 Common snook embryonic development

The developmental features of common snook described in this paper are typical of most teleost species with planktonic eggs. No common snook eggs have been described from the wild, although it is known that, immediately after spawning, eggs are taken towards the open sea by the outgoing tide and it is assumed that the following incoming tide brings them back into the estuarine and mangrove environment. Sampling efforts to find wild common snook eggs have been carried out, but the quest has been so far unsuccessful. Many factors may have resulted in this unsuccessful outcome, such as sampling gear, location, time, and the inability to recognize common snook eggs once collected.

The search for buoyant planktonic embryos in the wild will provide useful information for the development of incubation protocols in the laboratory. This is important since the influence of the environmental conditions influences early development and physiology of the offspring (Blaxter, 1992).

The processes of cleavage, formation of layer, and morphogenesis of teleost eggs during incubation have been described in a number of standard textbooks (such as Rudnick, 1955; Waddington, 1956; and Smith, 1957), with Oppenheimer (1947) and Devillers (1961) stressing structural changes from the viewpoint of experimental

embryology. Most of those descriptions were done for freshwater species, although extensive work has been done for some marine species including silver warehou *Seriolella punctata* (Grimes and Robertson, 1981), gilthead bream *Sparus aurata*, European flounder *Platichthys flesus*, dab *Limanda limanda*, Atlantic herring *Clupea harengus*, Atlantic cod *Gadus morhua*, plaice, and Atlantic salmon *Salmo salar*.

Common snook eggs are similar to those of many other fish species in their round shape, although in the anchovy *Engraulis* spp. and bitterling *Rhodeus sericeus* the eggs are ovoid and in certain gobies (family Gobiidae) the eggs are pear shaped. At the same time, the common snook embryo has only one oil globule, whereas more than one can be found in other marine species (Simpson, 1956) and in most fish species that have telolecithal eggs with the yolk more concentrated at the vegetative pole. As with hagfish (family Myxinidae) and elasmobranches, common snook exhibits meroblastic cleavage, even though snook are teleosts. They do not have the holoblastic cleavage that characterizes lampreys (family Petromyzontidae). Other groups, such as bowfin *Amia* spp., gar *Lepisosteus* spp., and sturgeon *Acipenser* spp., have intermediate features. All the above embryo characteristics, plus the melanophores pattern, egg size, and oil droplet size described in the results, will make the recognition of common snook embryos more accurate and provide a tool for egg quality evaluation in common snook culture.

4.2 Common snook morphological development

Common snook larval development was described by Lau and Shafland (1982). They primarily focused on larvae from 14 d old and beyond, looking at osteological, cephalic, and fin development. Wittenrich et al. (2009) described the osteological development of the feeding apparatus with feeding performance, but other than this no reported common snook development studies have been carried out. Data presented in this study is the first to document the early ontogentic growth of common snook and provides valuable information on development of the visual and digestive system that can be used to develop larval culture technologies for use in aquaculture.

4.3 Eye development

At hatching, common snook larvae have an unpigmented and nonfunctional visual system; many other species are similar in this respect (e.g., sole, Atlantic mackerel *Scomber scombrus*, whiting *Merlangius merlangus*, European pilchard *Sardina pilchardus*, and Pacific sardine *Sardinops sagax caeruleus*), while some others have pigmented eyes (e.g., plaice, Atlantic cod, Atlantic herring and salmonids) (Blaxter and Staines,1970). Blaxter (1986) described the important role that vision has on fish larvae orientation, as a consequence of them being visual feeders. Fish species with a relatively small yolk sac need to develop fast in order to survive. In common snook larvae, just like in sparids such as madai *Pagrus major* (Kawamura, 1984) and New Zealand snapper *Pagrus auratus* (Pankhurst, 1996), the most important changes in the eye structure occur in the lecitotrophic larvae as a preparation for prey capture.

One-day-old common snook larvae had all the basic structural elements necessary for visual function, but most of them were incomplete. This was an indication that the eye was about to become functional. Kawamura (1984) found that the visual system of madai is functional at 36 h posthatch when visual cells and pigments are present and nerve optic fibers connect with the optic tectum. In common snook, the visual system could not be functional at day 1 posthatch, principally because the pigmentation pattern, responsible for photon absorption, was very sparse at this stage. Retinas of most fish larvae mainly have green- sensitive single cones (Evans and Browman, 2004). This is the case of 2-d-old common snook larvae: although the retina was not as fully developed as in the adult stage, all the retina structural layers were complete but not fully functional. Histological observations support the partial functionality of the eye; by this time the pigment cell layer was present and the optic nerves were connected to the optic tectum. Thus, the visual system was completely ready for prey capture.

The pure cone retina has been found in the earlier stages of many teleost larvae such as Pacific salmon *Oncorhynchus* spp. (Ali, 1959), Atlantic herring (Blaxter and Jones, 1967) and plaice (Blaxter, 1968). However, at first feeding common snook are only equipped with simple cones, as with madai (Kawamura, 1984) and New Zealand snapper (Pankhurst, 1996), and rods and twin cones appear at metamorphosis (Blaxter and Staines, 1970). At day 3 the common snook larval retina has well-developed presumptive cone receptors, the pigmentation layer is fully complete, the structural layers are fully functional and differentiated, and rod precursors can be spotted, although they are scarce.

Overall, by day 2 posthatching the visual system of common snook larvae is developed sufficiently to locate and capture prey, although due to the underdeveloped stage of the rods (which provide better vision in low light (O'Connell, 1981; Kawamura, 1984; Pankhurst, 1996) adequate light conditions are necessary to optimize their ability to capture prey (Huse, 1993). Taking the rod development into consideration, light intensity during larval development should be altered accordingly, and to investigate this matter more samples of older larvae should be examined using histology.

4.4 Digestive system development

At hatching, common snook larvae had a simple undifferentiated straight gut linked to an unstructured mouth and anus, as described in other teleost species (Stroband and Dabrowski, 1979; Govoni, 1980; Cousin and Baudin-Laurencin, 1985; Govoni et al., 1986; Boulhic and Gabaudan, 1992; Bisbal and Bengston, 1995; Roo et al., 1999; Peña et al., 2004). It is generally assumed that lipid absorption takes place in the anterior intestine, and based on this assumption and the importance that lipids have over the larval development, all the histological work done in this study was based on the anterior intestine development.

During the first 2 d, the common snook larval digestive system undergoes major changes. The anus and the mouth open, gut cells undergo significant growth, development of organelles is increased, and the intestinal valve is formed. On day 1, the alimentary canal differentiation is starting to appear, the mouth is starting to be formed, but jaw cartilages are still developing. At the cell level, organelles can be

observed but their underdeveloped stage and low numbers render the digestive system unable to function. The alimentary canal epithelium at some parts of the luminal surface showed the presence of microvilli, although in low numbers and underdeveloped, at the same time ciliated cells were present in the lumen. Such ciliated cells were not found after day 1, and similar findings were observed in other species (Govoni et al., 1986; Loewe and Eckman, 1988; Calzada et al., 1998). The presence of these ciliated cells in larvae (which did not have any peristaltic movements) was reported by Iwai (1964) and contributes to the circulation of the intestinal contents (mainly yolk). Common snook larvae by day 2 posthatch had absorbed the yolk sac, which was also observed in goldlined seabream *Rhabosargus sarba* (Ibrahim, 2004) and in sea bass *Lates calcifer* (Walford and Lam, 1993). Other species, such as Atlantic cod and sheephead seabream *Archosargus probatocephalus* (John and Tucker, 1987; Kjørsvik et al., 1991), exhausted their yolk sac at 4 d after hatching. The mouth was open and the main jaw cartilages such as the Meckel's cartilage or the hyomandibular cartilage were present. These results were also seen in other species, such as rainbow darter *Etheostoma caeruleum*, white sucker *Catostomus commersonii*, logperch *Percina caprodes* and Atlantic cod (McElman and Balon, 1981; Paine and Balon, 1985; Kjørsvik et al., 1991). The dermal bones, such as the maxillary and the premaxillary are formed later on; however, food was observed in the gut.

Morphological and histological observations suggest that 2 d after hatching common snook larvae possess digestive organs enabling digestion, absorption, and metabolization of endogenous food. At the onset of day 2 posthatch, enterocytes are morphologically developed, yet as with cod larvae (Kjørsvik et al., 1991), the digestive mechanisms are immature and their functionality relies on lipid absorption and

possibly temporary lipid storage in the anterior part of the gut (Tanaka, 1972a; Stroband and Dabrowski, 1979). Although no histology of the rectal area was done, the epithelial cells are probably responsible for food protein ingestion and intracellular digestion (Iwai and Tanaka, 1968; Tanaka, 1972b; Watanabe, 1982a, b).

Day 3 common snook larvae had continued to develop. Mouth jaw cartilages were gaining in definition and speeding their functionality. The alimentary canal was now more structured with a clear differentiation between the different gut parts. Organelles, such as mitochondrion, rough endoplasmatic reticule, and the golghi apparatus, increased in numbers. The microvilli layer had increased in length and consistency.

In conclusion, the alimentary canal of common snook larvae develops from a undifferentiated tube at hatching to a complex tract before the onset of day 2 after hatching (when the yolk sac is exhausted). This fast development is parallel to that of the visual system; synchronization of the formation of these two systems is important for prey capture and predator avoidance. Together with the digestive and visual system development, common snook larvae have a partially developed fin structure that allows them to move in the water column and approach prey.

Like their feeding habits and behavior, the location of common snook larvae in the planktonic column is unknown. This paper has described the common snook larval development during the first 14 d in laboratory conditions in order to provide information for future studies on wild common snook larvae and for aquaculture purposes. Although there are many theories regarding the diet of common snook larvae, there are no reported studies on this topic; common snook larval diets are one of

the major bottlenecks in snook aquaculture. In addition, issues such as prey type and size during the first 5–7 d are still poorly understood. Therefore, the collection of wild larvae will provide useful information to identify the optimal prey, improve the rearing protocol, and increase knowledge of the wild common snook larvae ecology.

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CHAPTER 7

SUMMARY OF FINDINGS

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In this section, the main findings of each research chapter are summarized:

Chapter 2. *Assessing reproductive condition in captive and wild common snook stocks: A comparison between the wet mount technique and histological preparations***.**

- Using a tiered and adaptable staging, the wet mount technique was successfully validated and shown to be a rapid, yet accurate, low-cost alternative to histology for assessing reproductive condition in wild common snook.
- When used in aquaculture applications, the technique provided a precise and noninvasive method for determining whether female broodstock were candidates for hormone induction.

Chapter 3. *Spawning performance and endocrine profiles of captive common snook Centropomus undecimalis broodstock treated with different sustained release GnRHa-delivery systems***.**

- A single treatment of regular release GnRHa, administered at a minimum stage of oogenesis (late secondary growth-SGl), induced and synchronized ovulation and spontaneous spawning in captive females for up to 72 hours post-implantation.
- No effects on the quality or volume of milt produced by male broodstock were observed when treated with different GnRHa delivery systems nor with GnRHa alone or in combination with a dopamine antagonist (pimozide).

Chapter 4. *Parental contribution and spawning performance in captive common snook Centropomus undecimalis broodstock***.**

- Microsatellite markers were used successfully to monitor mating outcomes in snook where males and females were found to be capable of spawning several times per year, including outside their natural spawning season.
- Frequent and repeated handling of captive broodstock was found to have an impact on maturation and spawning performance in both male and female common snook.

Chapter 5. *Influence of tidal cycles on the neuroendocrine control of reproductive activity in common snook* (*Centropomus undecimalis*).

- Common snook collected on the Gulf coast of Florida had a seasonal pattern of gonadal growth and a significant link was found between tide and follicular development.
- In females, genes encoding pituitary mRNA expression of *fshβ* and *lhβ* gradually increased during vitellogenesis and peaked at ovulation whereas in males, *lhβ* mRNA expression peaked at the mid germinal epithelium stage while *fshβ* expression peaked at late germinal epithelium stage.
- LH plasma concentrations increased throughout oogenesis and spermatogenesis, peaking at ovulation and spermiation thus, supporting the already suggested role of LH in the regulation of the later steps of oocyte maturation and spermiation.
- Fluctuations in plasma sex steroid levels corresponded to their known roles in female and male gonadal maturation and found to be similar to that of other teleosts that are batch spawners.

- Data presented represents the first on GTH cloning and characterization in common snook.

Chapter 6. *Embryonic and early larval development in hatchery-reared common snook*

- Common snook embryo development took 15 h at 28°C from the time of fertilization to hatching.
- Gross morphological and histological examinations of larvae showed that at 2 days post-hatch the mouth was open, the eyes were fully pigmented and cellular structures indicative of a functional gut were observed.
- Date presented represents the first description of embryonic and larval development in common snook from fertilization until 14 days post hatch.

CHAPTER 8

GENERAL DISCUSSION

CHAPTER 8. GENERAL DISCUSSION

The overall objective of this thesis was to develop and improve broodstock management strategies and spawning of common snook *Centropomus undecimalis* with the aim to better control spawning under captive conditions. Both applied and basic research was conducted to 1) develop tools to study, monitor and understand broodfish requirements in tank systems and to 2) identify the culture technologies required to increase production of high-quality eggs/larvae. This general discussion will expand on the wider implications of the main findings from the thesis, discuss the limitations of the experimental studies performed and identify current research priorities for future development in these fields of investigation.

1. Pattern of ovarian development in common snook

In order to carry out the work presented in this thesis a rapid, yet accurate and noninvasive method had to be identified and validated for assessing reproductive condition in both captive and wild common snook (Chapter 2). Historically, snook have been defined as having group-synchronous ovarian development, but advances in our understanding of oocyte histo-morphology suggest they may have asynchronous development. Wallace and Selman (1981) define fish with group-synchronous development as having “at least two populations of oocytes that can be distinguished at the same time, a fairly synchronous population of larger oocytes (defined as a "clutch" or “leading cohort”) and a more heterogeneous population of smaller oocytes from which the clutch is recruited”. Wallace and Selman (1981) describe fishes with asynchronous development as having “oocytes of all stages are present without

dominant populations". The determination of spawning is made by examining stages/steps of oocyte development. In group-synchronous spawning fish there should be distinctive populations of developing oocytes and not a continuum. Histological preparations of ovarian biopsies obtained from wild common snook following ovulation revealed that all stages and steps of oocyte development appear to be present (a continuum) and not just several distinctive populations. Studies should be designed to look at oocyte recruitment over a 24 hours cycle in order to fully investigate this hypothesis. Further, the classification of common snook as a group-synchronous spawner needs to be considered carefully in light of more refined and adaptable descriptions of oocyte development which are now available (Grier et al., 2009; Brown-Peterson et al., 2011; Grier et al., 2013).

2. Experimental challenges

In this thesis I conducted a series of studies designed with an overall goal to improve management strategies and spawning of captive common snook *Centropomus undecimalis* broodstock. The biology of common snook has been studied in the wild for a number of years however, most work focused on collecting ecological and fisheries related data designed to provide scientific information for species management (Fore and Schmidt, 1973; Peters et al., 1998). In spite of the technological advances made in tagging and monitoring, having large numbers of wild fish experimentally situated is difficult to accomplish thus making it challenging to document certain behaviors and important life history characteristics (Young et al, 2014). Breakthroughs in the ability to spawn captive snook broodstock using hormonal manipulation were first reported in

2006 (Main et al., 2007) and later in 2011 (Ibarra-Castro et al., 2011), but only in the last 5 years have we begun to study some of these behaviors in a controlled setting and to identify the reproductive bottlenecks that limit production of high quality seeds. In summary, working with a new aquaculture species has not been without its challenges.

2.1 Harvest restrictions on a protected game fish

In Florida, snook are part of an economically important sport fishery that was valued at over $4.9 billion in 2011 (American Sport Fishing Association, 2013). Habitat loss, overfishing and environmental impacts, such as periodic cold kills and red tide events, have historically put pressure on the fishery and a general decline in recruitment has been observed. Today, common snook are a carefully managed game fish (Muller and Taylor 2012). Their importance to the angling community and, various other stakeholders, has led to harvest restrictions and periodic closure of the fishery, like the one imposed from 2009-2013. These harvest restrictions limited the number of wild fish that could be captured as broodstock and in turn the number of tank populations that could be maintained, as well the number of fish sacrificed for experimental purposes. In studies that required the sacrifice of fish, an effort to reduce the impact on wild populations was made by collaborating with researchers at Florida Wildlife Research Institute (FWRI) and the University of South Florida (USF). For example, fish that had their otoliths removed for elemental analysis (Rolls, 2014) and ovaries for batch fecundity studies (Hayslip, 2014) were the same fish used for the work described in Chapter 5, where pituitaries were collected for gene expression profiling. Despite the challenges involved in working with a restricted numbers of

samples, care was taken to maximize the amount of data obtained while minimizing the impact on the fishery.

2.2 Site-specific challenges

All experiments using captive common snook broodstock were conducted at Mote Aquaculture Research Park (MARP) located on the west coast of Florida in Sarasota County (USA). To benefit from lower land cost and protection from hurricane surges, MARP was established 27.3 km (17 miles) inland, with state-of-the-art saltwater filtration system designed for use with zero-discharge recirculating systems. Each broodstock tank had a total working volume measuring between 28 and 48 m^3. Due to the time involved and cost incurred from the feed and maintenance required for each system, a limited number of common snook populations could be held for research purposes. Additionally, one of challenges with working in such large saltwater systems so far away from the coast involved the need for several levels of filtration to maintain water quality. A filtration system composed of a solids filter, a moving bed biofilter, a foam fractionator, a denitrification reactor, UV lamps, ozone and carbon filtration was designed and utilized. All water quality parameters were monitored regularly but particular care was taken to track and maintain low levels of nitrate (< 50 mg/L nitrate-N) as nitrate can act as an endocrine-disrupting contaminant (EDC) and shown to have the ability to alter endocrine function (Guillette and Edwards, 2005). One example was seen in Siberian sturgeon reared in freshwater recirculating systems (Hamlin et al., 2008). Although the acutely toxicological effects of nitrate have long been known, few studies have been conducted in fish and therefore, additional research

is needed to test and define the impact of nitrate (and other ions) on saltwater species reared in recirculating systems.

2.3 Snook biology – considerations for aquaculture

Wild snook exhibit protandric sex reversal (Taylor et al., 2000). Although this was not observed among males in any of the trials, this reproductive trait may have implication for commercial aquaculture operations and should be considered when establishing captive broodstock populations. Other considerations for future studies include the potential impact of handling on captive broodfish. This was highlighted by limited spawning from captive broodstock that were subjected to rigorous (daily/monthly) sampling routines involving repeated anesthesia, gonadal biopsies and blood withdraws (Chapters 3 and 4).

Reduced milt production in captive male broodstock can also be problematic. This has also been seen in common snook and other multiple batch spawner species, such as barramundi *Lates calcifer*, where inadequate milt production was shown to significantly limit fertilization success directly impacting on the production of fry (Loughnan et al, 2013). To address this problem in common snook and to address the potential benefit from short or long term milt storage, several trials were planned but due to time constraints only one was carried out. Milt stripped from wild males was pooled and stored in an extender and effects of short term storage at three different temperatures (4, 10 and 28°C) were recorded over time. Results showed that an increase in incubation temperature resulted in a decrease in the amount of time the milt can be stored successfully. Milt storage at 4°C was viable for seven days although the

percent motility and duration of motility decreased significantly after 24 hours and again at 72 hours (data not shown). More trials would need to be performed to evaluate the feasibility and effectiveness of using different sperm storage methods in common snook aquaculture production.

3. Development of molecular tools used for gene expression profiling

One of the objectives of this PhD work was to confirm the role of tidal pattern on the coordination of oocyte maturation and spawning in common snook which so far was mainly based on empirical data, and to better understand the link between environmental and endocrine control of reproduction in this tropical, seasonal, batch spawning species. To do so, we studied gonadal development during the reproductive season in both males and females by means of gonad histology and levels of major endocrine control components along the pituitary-gonadal axis. For this purpose, a range of assays were validated in the species and used: radioimmunoassay for plasma steroid profiling, ELISA for plasma gonadotropin assessment and quantitative RT-PCR (QPCR) assays for gonadotropin gene mRNA expression. These assays were developed based on existing knowledge and in silico analyses. Several bioinformatics tools were used including genetic and genomic databases (GenBank in NCBI, ENSAEMBL), PrimerSelect, BLAST and ClustalW. In addition to sequence alignments, phylogenetic relationships were reconstructed using ClustalW, Bioedit and MEGA. Besides these techniques and protocols, during the course of the experiments, general fish experimental techniques have been used (PIT-tagging, anaesthesia, handling, blood sampling, dissection of targeted organs and general fish husbandry).

An additional component of this doctoral research required gene expression analysis where the expression of two gonadotropins (*fshβ and lhβ*) was studied in common snook throughout a spawning season. Both real-time PCR methods can be performed using different chemicals, the most common being SYBR Green or probe based assays e.g. TaqMan. SYBR green is an easy method that requires a double-stranded DNA dye in the PCR reaction, which binds to the newly synthesized DNA, and detection of fluorescent signal occurs during the PCR cycle. Since the aim was to study the variation in mRNA expression levels of candidate genes for different developmental stages, absolute QPCR was performed using SYBR Green, which is easy to use with low reaction cost compared to other fluorescent probes. One of the key points in the QPCR quantification is the choice of the reference gene (house-keeping gene); usually the reference genes are selected due to their uniform expression across the sample set. In the present studies we designed primers for two housekeeping genes, *β-actin* and elongation factor 1 alpha (*ef1*) and tested them by RT-PCR, to confirm that they were suitable to use. However, *β-actin* was selected for normalization because *ef1* did not work. Had time and resources allowed it would have been preferable to confirm this selection of the house-keeping gene in the present sample sets by comparing the stability of a suite of potential genes using the geNorm methodology (Vandesompele et al., 2002). Where possible I have tried to comply with the MIQE guidelines for QPCR data (Bustin et al., 2009), with my greatest challenge being the confirmation of RNA quality. As I did not have access to an Agilent Bioanalyzer, RNA quality had to be subjectively assessed by gel electrophoresis however, in all cases this indicated ribosomal RNA of good quality thus it is fair to assume the integrity of the samples used was not compromised.

4. Importance of the environmental and neuroendocrine control of reproduction for aquaculture

An understanding of the underlying control of reproduction is of key importance in aquaculture for commercial production (Zohar et al., 2010). The importance of lunar cycle, in particular, on fish reproduction has been reported in a range of fish species, although, to date, few physiological studies have investigated how changes in tidal cycle can act as an ultimate factor that entrains and synchronizes reproductive functions (Takemura et al., 2004; Ando et al., 2013). One of the interesting findings during this work involved the significant link that was found between tide and follicular development where, female snook sampled on the rising tide were all found to have oocytes in the secondary growth stage whereas females sampled at high tide or on the falling tide had oocytes in the later stages of maturation and ovulation (Chapter 5). This information could have broad implications for other species reared in captivity which rely on this cue to spawn. Female snook failed to ovulate in the absence of hormonal manipulation during the trials and this may be linked to one or more missing environmental cues such as suggested by the present findings. To study this hypothesis under controlled conditions, a system has been designed to simulate changes in directional tidal flow and trials are currently underway with captive broodstock.

5. Conclusions

This thesis presents novel research investigating the reproductive biology and spawning capabilities in common snook and provides a better understanding of the environmental control of reproduction that in aquaculture can assist with the mitigation

of reproductive bottlenecks, such as a lack of spontaneous spawning in captive broodstocks. Furthermore, the current work provides the first empirical evidence of a circa-tidal control of the later phases of the gametogenic cycle in common snook and offers new insight into wild population reproductive strategy as well as how reproduction is entrained through environmental cues and the pathways leading to oocyte recruitment and maturation. It may also be considered in a broader sense for the control of reproduction in other tropical fish species. Lastly, the work conducted for this thesis describes, for the first time, early embryonic and larval development in common snook. As a whole, information obtained from this research will be critical for determining culture technologies which optimize broodstock management ultimately maximize the production of fingerlings either for fisheries enhancement or commercial-scale food fish production.

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PUBLICATIONS AND CONFERENCES ATTENDED

PUBLICATIONS

Rhody, N.R., Davie, A., Zmora, N., Zohar, Y., Main, K.L., Migaud, H. Influence of tidal cycles on the endocrine control of reproductive activity in common snook (*Centropomus undecimalis*). **In Review.**

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