CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Aquaculture in Australia

Australia's commercial catch ranks 52nd in the world representing only 0.2 % of world tonnage (ABARE, 2004). This is despite the fact that Australia has the world's third largest fishing zone, covering approximately 11 million km^2. Many of Australia's wild fish stocks are already fully or over exploited (Kailola *et al.*, 1993) and demand for seafood continues to grow. Consequently Australia imports more than 60% of the seafood it consumes (Yearsley *et al.*, 2003; Love *et al.*, 2004). Aquaculture is increasingly seen as an alternative to harvesting from wild stocks (Love and Langenkamp, 2003). In Australia, the gross production value of aquaculture has nearly trebled over the past decade (O'Sullivan and Dobson, 2002; Love and Langenkamp, 2003) In 2002-03, aquaculture represented 32% of Australia's gross fisheries value of approximately $743 million (ABARE, 2004). With the exception of cultured pearls, the bulk of this value arises from farmed finfish. Southern bluefin tuna (*Thunnus maccoyii*), Atlantic salmon (*Salmo salar*), and to a lesser extent, Barramundi (*Lates calcarifer*) dominate the marine farming industry. Trout (*Oncorhynchus mykiss*) and silver perch (*Bidyanus bidyanus*) dominate the freshwater industry (O'Sullivan and Dobson, 2002; ABARE, 2004). With the exception of the Southern bluefin tuna industry (which source juveniles from wild populations under strict quotas), the success of these industries can be attributed to well developed and established hatchery propagation techniques (Rowland, 1984a; Ruangpanit, 1987; Garratt and Connell, 1991; Rimmer and Rutledge, 1991; Billard, 1992; Bromage *et al.*, 1992; Garratt and O'Brien, 1994; Thurstan and Rowland, 1994). In relation to the tuna farming, research efforts are directed towards the development of captive brood fish breeding techniques to allow for further expansion of the industry independent of wild socks (FRDC, 2001; Montague, 2002).

1.2 Reproduction in Aquaculture

The fundamental requirement of any aquaculture grow-out operation is the supply of juveniles. Although some aquaculture operations rely on the collection of juveniles from the wild, the preferred source within Australia is from hatcheries that have captive brood fish populations maintained in isolation from wild stocks (Rowland, 1983a, 1984a, 1989, 1989; Thurstan and Rowland, 1994; Quartararo, 1996; Partridge

et al., 2003). The primary objective of hatchery operators is to obtain high quality gametes (ova and sperm) from captive brood fish in a cost effective and timely manner. For many species, the major constraint in achieving this objective is an inhibition of the fish to spawn naturally in captivity (Clemens, 1968; Fontaine, 1976; Lam, 1982; Shelton, 1989; Pankhurst, 1998). While some species will spontaneously spawn in captivity in response to controlled environmental stimuli, the time of the spawning(s), the numbers of participants, and therefore, the supply of fertilized eggs, are often erratic and difficult to predict. Furthermore, naturally spawning brood fish do not facilitate the timely preparation of larval rearing facilities, ponds and cultures (Bromage and Roberts, 1995; Peter and Yu, 1997). Therefore, it can be difficult to maintain a commercially viable hatchery that is reliant on naturally spawning populations of fish (Fukusho *et al.*, 1986; Bromage and Roberts, 1995). To overcome this problem manipulation of the fishes' endocrine system by the injection of hormones to induce maturation and spawning is often the method employed by hatchery operators to facilitate timely spawning (Lam, 1982; Pankhurst, 1998).

1.3 Hormone Induced Spawning

Induced spawning involves the injection or treatment of fish that have reproductively advanced gonads to bring about final gamete maturation (ovulation and spermiation) and spawning. Historically, this involved the injection of carp pituitary extracts (Lam, 1982). Unlike pituitary extracts, the use of human chorionic gonadotropin (hCG) has allowed hormone dose to be standardized (Sneed and Clemens, 1959; Clemens and Sneed, 1962; Hodgen, 1981; Lam, 1982; Rowland, 1983b). More recently, the use of synthetic analogues of gonadotropin releasing hormone (GnRHa) coupled with dopamine (DA) blocking drugs and improved delivery systems (such as slow release pellet implants) has facilitated a greater degree of success in spawning a wide variety of fish (Lin and Peter, 1986; Crim *et al.*, 1987; Sherwood, 1987; Peter *et al.*, 1988; Peter and Yu, 1997). Despite these advances it is well known that many commercially important species still suffer from low egg fertilization and hatch rates (Kjorsvik *et al.*, 1990; Lam, 1994, 1995; Brooks *et al.*, 1997; Coward *et al.*, 2002). Although many factors have been demonstrated to affect egg quality in fishes, of particular importance to the hatchery operator is the hormone type, dose and method of delivery.

Table 1.1. Summary of hormone dosages and delivery methods used to induce spawning and respective spawning response in some important members of the Sparidae family. Intramuscular and intraperitoneal injections abbreviated as i.m. and i.p., respectively.

Species	Spawning Method	Hormone Type	Dose / kg	Delivery / solvent	Injection	Response	Males treated	Reference
A. australis	Hormone induced	LHRHa	40 µg	Aqueous injection	i.m.	Variable	No	Cowden (1995)
		LHRHa	15-25 µg	Pellet implant	i.m.	Variable	No	
		Ovaprim	0.5 ml	Propylene glycol	i.m.	Variable-poor	No	
		hCG	250-1500 IU	Aqueous injection	i.p.	Variable	Yes - 500 IU	Black (2000)
A. latus	Hormone induced	hCG	1000 IU	Aqueous injection	i.p.	Poor	No	Leu & Chou (1996)
		hCG + Ovaprim	500 IU + 0.25 ml	Aqueous injection	i.p.	Variable	No	
		Ovaprim	0.5 ml	Propylene glycol	i.p.	Good	No	
A. berda	Hormone induced	hCG	500-1500 IU	Aqueous injection	i.m.	Variable-poor	No	Mok (1985)
		Synahorin	50-150 RU	Aqueous injection	i.m.	Variable-poor	No	
	Natural spawning	-				Variable-poor		
A. butcheri	Hormone induced	LHRHa	50 µg	Aqueous injection	i.p.	Variable	No	Haddy & Pankhurst (2000)
		hCG	1000 IU	Aqueous injection	i.p.	Variable	No	
		Ovaprim	0.5 ml	Propylene glycol	i.m.	Unknown	No	Doupé *et al.* (2005)
	Natural spawning	-				Good		Partridge *et al.* (2003)
								Partridge *et al.* (2004)
A. cuvieri	Natural spawning					Variable		Hussain *et al.* (1981)
A. schlegeli	Natural spawning					Good		Lue (1997)
Pagrus auratus	Hormone induced	HCG	1000 IU	Aqueous injection	Unknown	Variable-poor	Yes - 1000 IU	Battaglene & Talbot (1992)
		LHRHa	120-207 µg	Pellet implant	i.m.	Unknown	No	Fielder *et al.* (2002)
		Ovaprim	0.5 ml	Propylene glycol	i.p.	Poor		Partridge *et al.* (2003)
	Natural spawning					Variable		Fukusho *et al.* (1986)
Sparus aurata	Hormone induced	GnRHa	20-80 µg	Pellet implant	i.m.	Variable-poor	No	Barbaro *et a.,* (1997)
	Natural spawning					Variable-poor		
		LHRHa	1-25 µg	Aqueous injection	i.m.	Good-variable	Yes 50% 5 µg	Colombo *et al.* (1989)
		hCG	250 IU	Aqueous injection	i.m.	Variable	Yes 50% 1 µg	
	Natural spawning					Variable-poor		
	Hormone induced	LHRHa	5-20 µg	Aqueous injection	i.m.	Variable	No	Zohar *et al.* (1989)
		hCG	150-200 IU	Aqueous injection	i.m.	Variable	No	

Studies have shown that these factors can greatly affect spawning success and egg quality in domesticated brood fish (Rowland, 1983b, 1984a; Mok, 1985; Ramos, 1986; Rowland, 1988; Colombo *et al.*, 1989; Zohar *et al.*, 1989; Bromage and Roberts, 1995; Battaglene and Selosse, 1996; Berlinsky *et al.*, 1996; Leu and Chou, 1996; Barbaro *et al.*, 1997; Smith *et al.*, 1999). Failure to obtain viable gametes (those that can result in health first feeding larvae) may arise from incorrect hormone use. If the hormone dose is too low, the brood fish can fail to ovulate. There may be partial ovulation, where only a proportion of the oocytes (those most mature) are ovulated which can result in a failure to spawn, necessitating manual collection or stripping of the eggs. Excessively high doses can produce overripe or poor quality eggs due to the rapid maturation of immature oocytes. It can be seen in Table 1.1 that hormone types, dosages and methods of hormone administration can vary greatly. A variable response to hormone treatment is not conducive to the reliable supply of juveniles. There is a clear need to standardize hormone dose and delivery methods for individual species, thereby developing protocols that give the greatest chance of repeated and consistent spawning success. Critical to this is an understanding of the reproductive physiology and endocrinology control of gamete maturation of teleost fish.

1.4 Endocrine Control of Reproduction in Teleosts

Reproduction in teleosts usually occurs annually and represents substantial energy investment in the form of proteins and lipids stored in the gonads (Dodd and Sumpter, 1984; Tyler and Sumpter, 1996). Gonadal development is triggered by external environmental, behavioural and pheromonal cues (Peter, 1982; Pankhurst, 1998; Kobayashi *et al.*, 2002). These cues are received by the central nervous system via the sensory organs and are interpreted by the preoptic regions of the brain where they are converted from electrical to neuroendocrine signals (Peter and Yu, 1997; Weltzien *et al.*, 2004). The preoptic region of the teleost brain provides central neural and hormonal control of many functions, one of which is the production and secretion of gonadotropin releasing hormone (GnRH). GnRH stimulates the pituitary gland to start producing and releasing two forms of the reproductive hormone gonadotropin (GtH) (Fig.1.1).

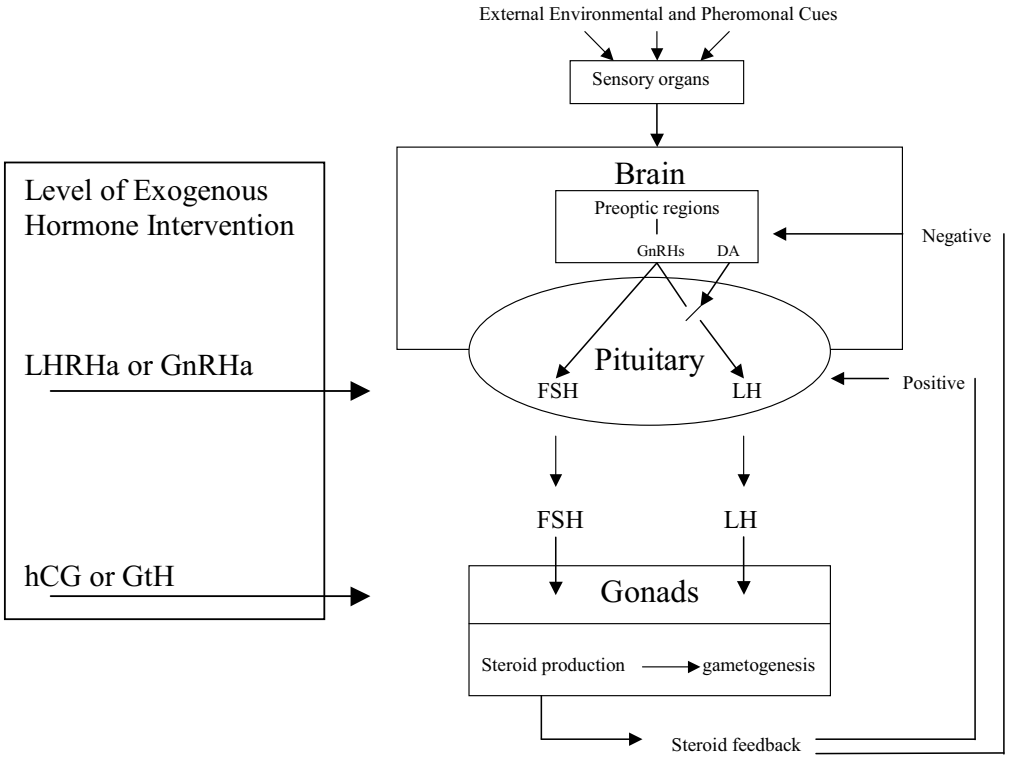


Fig.1.1. Simplified schematic representation of the endocrine regulation of gonadotropins (FSH and LH) release in teleosts. Adapted from Donaldson and Hunter, 1983; Trudeau, 1997; Yaron *et al.*, 2003). Also depicted levels of exogenous hormone intervention used to induce gamete maturation.

FSH and LH have temporally separated actions and stimulate the biosynthesis of steroids that ultimately guide gametogenesis and gamete maturation. As with all vertebrates, the teleost brain contains at least two GnRH variants (Lethimonier *et al.*, 2004). However, there are a number of teleost species in which three forms of GnRH have been identified, suggesting multiple mechanisms of gonadotropin release (Trudeau, 1997; Holland *et al.*, 1998; Adams *et al.*, 2002). The functional significance of these multiple forms is unclear (Habibi *et al.*, 2001). Most of the native forms of GnRH that have been studied have GtH releasing activity (Peter *et al.*, 1985; Zohar *et al.*, 1990; Ngamvongchon *et al.*, 1992; Zohar *et al.*, 1995). However, their relative potencies to stimulate LH release, their abundance in the pituitary, the location of the separate GnRH producing cells and their receptors suggest that they may not all be endogenous regulators of GtH synthesis and release (Peter and Yu, 1997; Holland *et al.*, 1998; Parhar, 2003). The neurohormone dopamine (DA) acts as a LH release inhibitor in salmonoids, cyprinids and catfish, although there appears to be a lack of evidence of its inhibitory function in some sciaenid and sparid species (Peter *et al.*, 1988; Copland and Thomas, 1989; Lin *et al.*, 1991; Zohar *et al.*, 1995; Trudeau, 1997;

Saligaut *et al.*, 1999; Haddy, 2000; Joy and Chaube, 2003). It remains unclear whether the inhibitory effects of DA on LH release can be generalized or if its effect is restricted to certain groups of fish (Pankhurst, 1998). Although a number of other neurohormones play more subtle roles, GnRH and DA appear to be the principal stimulatory and inhibitory hormones regulating LH release in the majority of species studied (Zheng and Stacey, 1996; Trudeau, 1997; Weltzien *et al.*, 2004).

1.5 Physiology of Gametogenesis and Its Endocrine Regulation

The reproductive patterns and strategies of teleost fishes are diverse. Reproduction usually occurs dioeciously; however, hermaphroditism is not uncommon, especially among marine species and gynogenesis also occurs (Nagahama, 1983; Buxton and Garratt, 1990; Tyler and Sumpter, 1996; Rhen and Crews, 2002; Murua and Saborido-Rey, 2003). There are three recognized patterns of ovarian development: those that produce a single clutch (synchronous), those that typically spawn once per season (group synchronous), or those that produce more than one developing clutch of oocytes within a single season (multiple group synchrony) (Nagahama, 1983; Tyler and Sumpter, 1996; Pankhurst, 1998). Despite this complexity, the fundamental structures and morphological characteristics of reproduction are similar (Nagahama, 1983; Brooks *et al.*, 1997).

1.5.1 Physiology of ovarian development

Patino and Sullivan (2002) describe six major steps that occur during oogenesis: (1) formation of primordial germ cells (PGC), (2) transformation of PGCs into oogonia (3) transformation of oogonia into oocytes (folliculogenesis; onset of meiosis), (4) growth of oocytes (vitellogenesis, while under meiotic arrest), (5) maturation (resumption of meiosis) and, (6) expulsion of the ovum from its follicle (ovulation).

Primordial germ cells in the early embryo ultimately give rise to germinal cells following sexual differentiation (Strüssmann and Nakamura, 2002; Yoshizaki *et al.*, 2002). However, unlike the situation in all other vertebrates, dividing oogonia cells persist in the adult teleost ovary, yielding potentially an unlimited number of oogonia during the lifetime of the female. These oogonia may be found individually or may be found grouped as “nests” (Tyler and Sumpter, 1996). Oogonia committed to the process of meiotic transformation and not to a self-renewal pathway migrate away from the area of mitotic proliferation and begin to grow and develop into primary

oocytes, a process known as folliculogenesis (Dodd and Sumpter, 1984). During folliculogenesis a supporting layer of follicle cells surrounds the oocyte. As the oocyte develops these cells multiply and form a surrounding monolayer known as the granulosa. Simultaneously, the surrounding stromal connective tissue also becomes organized forming the outer thecal layer that is separated from the inner granulosa by a basement membrane (Nagahama, 1983; Dodd and Sumpter, 1984; Patiño and Sullivan, 2002). The outer margins of the granulosa are in contact with a complex network of blood vessels that supply nutrients to the developing oocyte. Both the granulosa and thecal layers contain special steroid producing cells that mediate aspects of oocyte growth and maturation.

This structure remains essentially unchanged throughout the process of oocyte growth and is usually complete by the time the oocyte reaches the late pachytene or early diplotene stages of chromosomal development. With chromosomal development arrested at the diplotene stage (Prophase I) the oocyte enters into a previtellogenic growth phase. During this phase the oocyte lies in close contact with the inner surface of the granulosa. Microvilli extend from the oocytes surface towards the granulosa. These microvilli secrete proteins that are associated with formation of the vitellin envelope (zona radiata or egg shell) (Dodd and Sumpter, 1984). Glycoproteins are also synthesised by the oocyte and incorporated into alveoli at the oocytes periphery. The contents of these alveoli build during the growth of the oocyte and are released into the perivitelline space in response to cortical reaction to fertilization, blocking further sperm entry (polyspermy) and contributing to the structural integrity of the vitellin envelope (Iwamatsu *et al.*, 1995; Tyler and Sumpter, 1996; Patiño and Sullivan, 2002).

At the completion of folliculogenesis the oocyte starts to sequester the yolk precursor glycophospholipoprotein, known as vitellogenin (VTG). VTG is produced by the liver and transported via the blood stream to developing oocytes (Mommsen and Walsh, 1988; Specker and Sullivan, 1994; Tyler and Sumpter, 1996; Patiño and Sullivan, 2002). Yolk proteins are stored as yolk globules or platelets through out the ooplasm. The timing of this growth coincides with the formation of extracellular channels in the wall of the follicle that allow the passage of VTG to the oocyte's surface (Tyler and Sumpter, 1996). This process is know as vitellogenesis and is responsible for the majority of oocyte and consequently ovarian growth during the

reproductive cycle. These lipids and essential fatty acids support embryogenesis. By the completion of the growth phase the oocyte contains a large nucleus known as the germinal vesicle that is in meiotic prophase. The germinal vesicle at this stage is generally located centrally or halfway between the centre and the oocyte periphery (Nagahama, 1983).

Shortly after the completion of vitellogenic growth, chromosomal development again becomes arrested this time at Metaphase II. During maturation structural and biochemical changes occur simultaneously within the oocyte. These changes are necessary to allow fertilization to occur. The germinal vesicle migrates to the animal pole where the micropyle is located. Endocrine factors trigger the resumption of meiosis. The germinal vesicle breaks down resulting in the release of the genetic material into the cytoplasm (Nagahama, 1983; Patiño *et al.*, 2001). Cytoplasmic changes also occur; there is an increase in the translucency of the oocyte as a result of the coalescence of lipid droplets and yolk globules. There is also a further increase in oocyte size due to hydration (Goetz, 1983). In marine species with pelagic eggs, hydration my be accompanied by the formation of one or more oil droplets(Pankhurst, 1998). Fully mature oocytes (now ova) are released from the follicular envelope into the ovarian cavity, a process known as ovulation. At this stage the oocytes are capable of being fertilized (Nagahama, 1983; Pankhurst, 1998). Sperm contact with the egg surface induces completion of the second meiotic division and the expulsion of the second polar body (redundant haploid cell) (Swanson *et al.*, 1981; Patiño and Sullivan, 2002).

1.5.2 Regulation of oocyte maturation and ovulation

Little is known about the mechanisms controlling oogonia proliferation or entry into folliculogenesis (Dodd and Sumpter, 1984; Patiño and Sullivan, 2002). At the completion of folliculogenesis, primary oocytes become sensitive to GtH (Fig.1.2). In salmonoids, FSH stimulates the thecal layer to produce testosterone, which is converted to estradiol-17β (E2) in the granulosa (Kagawa *et al.*, 1982; Specker and Sullivan, 1994; Nagahama, 1997; Patiño and Sullivan, 2002). E2 is released into the blood stream and stimulates hepatic production and uptake of VTG and vitelline envelope proteins (Dodd and Sumpter, 1984; Nagahama, 1994; Tyler and Sumpter, 1996; Patiño and Sullivan, 2002).

In the red sea bream (*Pagrus major*), ovarian production of E2 appears to be predominately regulated by LH (Gen *et al.*, 2003). Variations on the salmonid model may also exist in the New Zealand eel (*Anguilla dieffenbachia*) follicle (Lokman and Young, 1995). At the completion of vitellogenic growth the follicle cells become responsive to LH developing oocyte maturational competence (OMC) (Patiño *et al.*, 2001). LH binds to its receptors on the thecal layer stimulating the production of 17α-Hydroxyprogesterone which is in turn converted to maturation inducing hormone (MIH) by interaction of the two follicle cell layers (Nagahama, 1997). In many species the MIH has been identified as either a di- or trihydroxy derivative of progesterone (17α, 20β-dihydroxy-4-pregen-3-one or 17α, 20β, 21-trihydroxy-4-pregnen-3-one) (Goetz, 1983; Nagahama, 1997; Yueh and Chang, 2002). MIH binds to receptors on the oocyte surface stimulating resumption of meiosis and the formation of maturation promoting factor (MPF) in the cytoplasm (Nagahama, 1997; Thomas, 2003). MPF stimulates germinal vesicle breakdown via enzymatic pathways. MIH may also trigger ovulation alone or in conjunction with F series prostaglandins (PGF) (Pankhurst, 2008). PGF most likely stimulate smooth muscle like contraction in specialized cells of the follicular envelope causing it to rupture releasing the ova into the ovarian cavity (Goetz, 1983; Dodd and Sumpter, 1984).

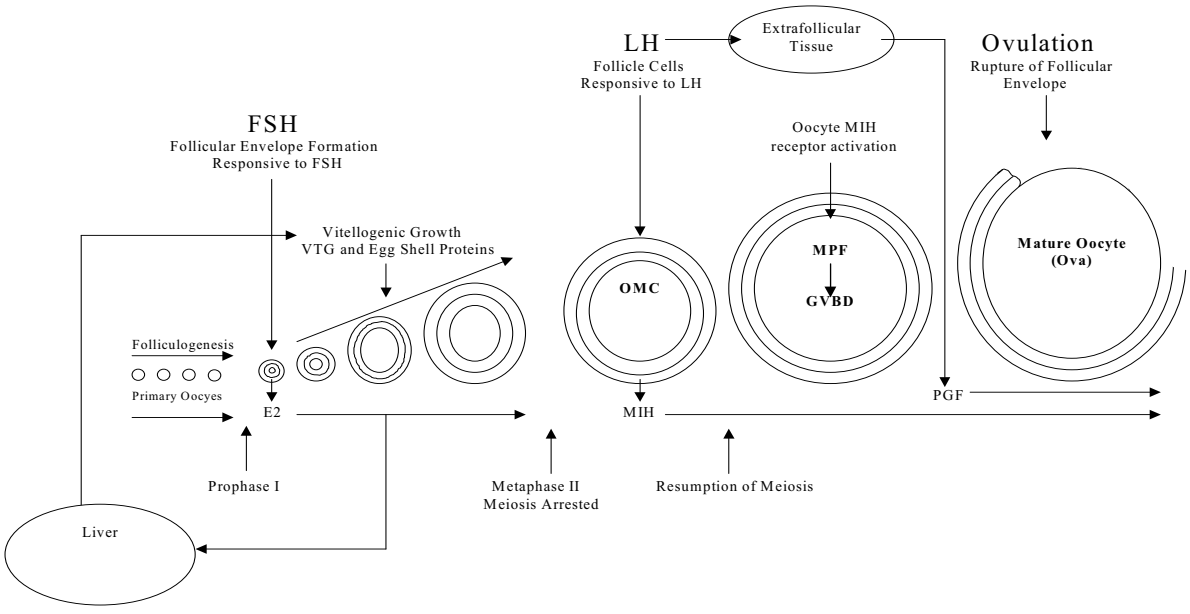


Fig.1.2. Schematic representation of endocrine control of oocyte maturation and ovulation in teleost. Adapted from Brooks *et al*., 1997; Habibi *et al*., 2001; Yaron *et al*., 2003. See text for explanation of abbreviations.

1.5.3 Physiology of testicular development

Spermatogonia ultimately give rise to spermatozoa following a number of meiotic cycles. Spermatogonia that are committed to the process of mitotic proliferation and not to the process of self-renewal enter into a meiotic phase. DNA is duplicated and recombined to form primary spermatocytes. These short-lived primary spermatocytes then divide to produce secondary spermatocytes. The secondary spermatocytes divide again without DNA replication to form haploid spermatids that undergo restructuring to flagellated spermatozoa (Nagahama, 1983; Schulz and Miura, 2002). The transformation of spermatogonia into spermatids is termed spermatogenesis, while the maturation of the haploid spermatids into spermatozoa is termed spermiogenesis (Nagahama, 1983; Schulz and Miura, 2002). Spermatogenesis and spermiogenesis occurs entirely within specialised spermatogenic cysts that are surrounded by a supporting Sertoli cell creating a microenvironment suited to the needs of the developing spermatogonia. Sertoli cells are located within the periphery of lobular or less frequently tubular structures consisting of a basement membrane that separates it from the surrounding interstitium. As spermatogenesis progresses, the mature spermatozoa are released when the wall of the cyst opens to the lobular lumen or efferent ducts in the case of tubular type. The release of matured spermatozoa into the central lumen and consequently into the sperm ducts is termed spermiation (Billard *et al.*, 1982; Pankhurst, 1998).

1.5.4 Regulation of spermatogenesis

Spermatogenesis is primarily governed by the gonadotropins (Billard *et al.*, 1982; Nagahama, 1994; Schulz *et al.*, 2001; Schulz and Miura, 2002). However, the precise nature of their coordinated control is not fully understood (Fig.1.3). In mammals, LH and FSH develop biological activity by virtue of their interaction with specific receptors in the Leydig and Sertoli cells, respectively. However, in fish these receptors appear to be less discriminatory when compared to mammals and there may be some overlap of their biological activity (Fostier *et al.*, 1983; Fostier *et al.*, 1987; Ikeuchi *et al.*, 2001; Schulz *et al.*, 2001). The main biologically active site of LH is the Leydig cells(Fostier *et al.*, 1983). While the Sertoli cells express receptors for FSH. Both FSH and LH stimulate testicular production of 11-KT. However, the potency of these GtHs appears to be dependent upon the stage of spermatogenesis

(Swanson *et al.*, 2003). FSH appears to be most potent in early spermatogenesis while LH develops potency later during spermatid maturation to flagellated spermatozoa (spermiogenesis) (Swanson *et al.*, 2003). FSH appears to be responsible for the production of 11-KT which drives spermatogenesis up until the formation of post-meiotic haploid spermatids (Swanson *et al.*, 2003).

Spermatogonia germ cell renewal is regulated by E2 and a Sertoli cell dependant stem cell renewal factor (Miura and Miura, 2003). 11-KT switches spermatogonia germ cell renewal to the meiosis pathway. This switch is most likely controlled by an 11-KT dependent growth factor produced by the Sertoli cells. The main biological action of LH appears to be for the most part restricted to the interstitial Leydig cells, where it stimulates the production of 11-KT and MIH. In the Japanese eel (*Anguilla japonica*), exogenous 11-KT alone can induce all stages of spermatogenesis (Miura *et al.*, 1991). MIH stimulates the release of spermatozoa to the sperm ducts (spermiation), and milt hydration (Pankhurst and Poortenaar, 2000). MIH is thought to act via receptors on the spermatozoon activating enzymes that increase sperm duct pH allowing the acquisition of sperm motility (Ohta *et al.*, 1999; Miura and Miura, 2003).

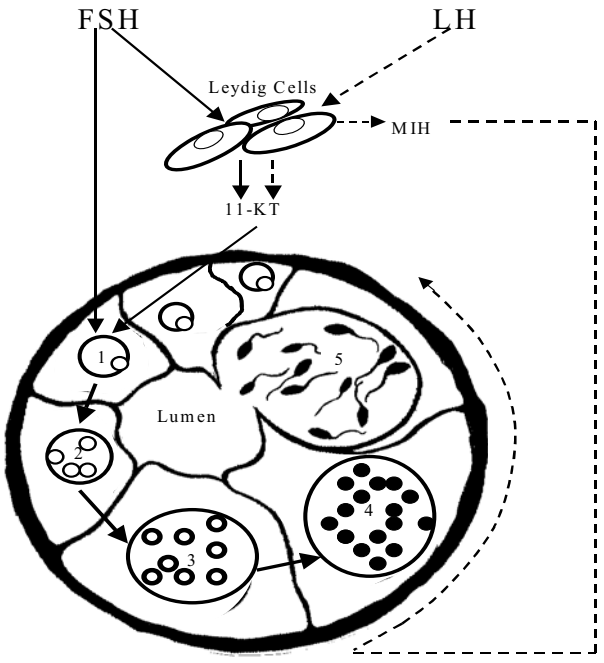


Fig.1.3. Tentative representation of gonadotropin (FSH and LH) control of spermatogenesis. Adapted from Billard *et al.*(1982) and Nagahama (1983)). 1 = Cyst containing spermatogonium committed to meiosis surrounded by supporting Sertoli cell. 2 = primary spermatocytes. 3 = secondary spermatocytes. 4 = spermatids (haploid). 5 = flagellated spermatozoa.

1.5.6 Synchronized sexual maturation

The complex interaction of hormones influencing the behavioural act of spawning is poorly understood in many fish species. In the goldfish (*Carassius auratus*), social and pheromonal interactions play a very important role in synchronizing gamete maturation and spawning (Zheng and Stacey, 1996; Peter and Yu, 1997; Kobayashi *et al.*, 2002). At the completion of vitellogenesis there is a steroidal switch from E2 to testosterone. This switch serves to sensitise the females LH release mechanism. Conducive environmental conditions trigger a surge in LH that favours the production of progestins, including the MIH (17α, 20β-Dihydroxy-4-pregen-3-one). In addition to stimulating final oocyte maturation, MIH is released into the water (together with sulphated progestins and androstenedione) to serve as a pre-ovulatory pheromone. This pheromone is received by the olfactory system of the male and stimulates reproductive behaviour, LH release and spermiation (Zheng and Stacey, 1996; Kobayashi *et al.*, 2002).

1.6 The Yellowfin Bream (*Acanthopagrus australis*)

The Yellowfin bream (*Acanthopagrus australis*) is thought to be endemic to the coastal and estuarine waters of eastern Australia(Munro, 1949; Kailola *et al.*, 1993). However, *A. australis* can be found in the coastal water of Taiwan and there are reports of artificial propagation of the species, however, the details remain unclear (Huang and Chiu, 1997; Liao, 2000; Liao *et al.*, 2001). In addition, there are unconfirmed reports of this species inhabiting the waters of Japan (Huang and Chiu, 1997; Shao, 2005; Torres and Luna, 2005). Within Australia, the yellowfin bream has attracted attention as a candidate for aquaculture because of its market place acceptance, economic value and its ability to withstand wide variations in both salinity and temperature. Consequently, there has been some research directed towards the development of induced spawning protocols. Thorogood (1991) reported that the synthetic hormone D-ala^{6}- LHRHa was capable of advancing ovarian development when injected into the intraperitoneal cavity at doses of 300 or 1000 μg kg^{-1} body weight (bw). Cowden (1996) reported that a minimum aqueous dose of 15-20 μg kg^{-1} bw D-ala^{6}- LHRHa would reliably induce single spontaneous spawning in wild caught females with vitellogenic oocytes having a mean diameter of approximately 450 μm. When this hormone was incorporated into slow release pellet

implants, multiple spawnings were possible. Both of these studies focus on the use of wild caught fish as brood fish. The use of wild caught fish, as brood fish, requires the researcher/hatchery operator to overcome many logistical challenges (Black, 2000). The successful spawning of wild fish is dependent upon the ability to capture sexually mature brood fish (both male and female) with reproductively advanced gonads coincident with the preparation of larval feeds, cultures and rearing ponds. Further more, the timely application of hormones (often in the field prior to transport to the spawning facilities) is often necessary to reduce the detrimental effects of capture stress on ovarian development (Carragher and Pankhurst, 1991; Pankhurst and Sharples, 1992; Haddy and Pankhurst, 2000b). There is also limited control over the reproductive status of the captured fish. Yellowfin bream are multiple group synchronous spawners, spawning many times during a single spawning season. Although ovarian biopsy may reveal oocytes that have reached an appropriate state of maturation at which hormone intervention can induce spawning, there is no guarantee that commercial quantities of eggs will be spawned as the females may have already spawned several times prior to capture. These factors may be responsible for inconsistencies in spawning success reported in studies investigating the effect of hormone dose on wild caught yellowfin bream.

1.7 Scope and Aims of the Study

The study aimed to extend knowledge of the reproductive biology of captive yellowfin bream as it pertains to the aquaculture potential of the species. The major aims were: 1) the development of reliable protocols for the induction of spontaneous spawning through the application of exogenous hormone. 2) determine the feasible salinity range at which eggs can be fertilized and undergo normal embryonic development, hatching, growth and survival until the time of first feed. The study also aimed to further the understanding of the mechanisms controlling spermiation, spontaneous spawning and the effect of salinity on the viability eggs and larvae.

The approach taken was to assess:

a) the dose specific response of male yellowfin bream to GnRHa and hCG treatment in terms of milt characteristics and gonadal steroids.
b) the dose related efficacy of GnRHa and hCG to induce spontaneous spawning in females in terms of the number of spawns, egg numbers

and fertilization rates.

c) the effects of gamete activation salinity on egg fertilization, larval hatching and deformity rates, together with, larval growth and survival until the time of first feed.

The purpose of this research was to develop protocols for the controlled reproduction of the species and to provide insight into the salinity range of water that could potentially be used by the hatchery operator during spawning, egg incubation and early larval development.

Chapters 2 - 4 are presented in format in preparation for publication. Consequently, there is some intended repetition in some sections.

Chapter 2: Black, B.J. and Pankhurst, N.W. (submitted), Effect of gonadotropin releasing-hormone analogue and human chorionic gonadotropin on milt characteristics and gonadal steroids in the yellowfin bream, *Acanthopagrus australis* (Sparidae). New Zealand Journal of Marine and Freshwater research.

Chapter 3: Black, B.J. and Black, M. (in preparation), Efficacy of two exogenous hormones (GnRHa and hCG) for induction of spawning captive yellowfin bream, *Acanthopagrus australis* (Sparidae).

Chapter 4: Black, B.J. and Black, M. (in preparation), Effect of salinity on fertilization, hatch, growth and survival of yellowfin bream (*Acanthopagrus australis*) eggs and yolk-sac larvae.

The University of New England Animal Ethics Committee authorized this research under animal research permits AEC03/066 and AEC04/071.

CHAPTER 2

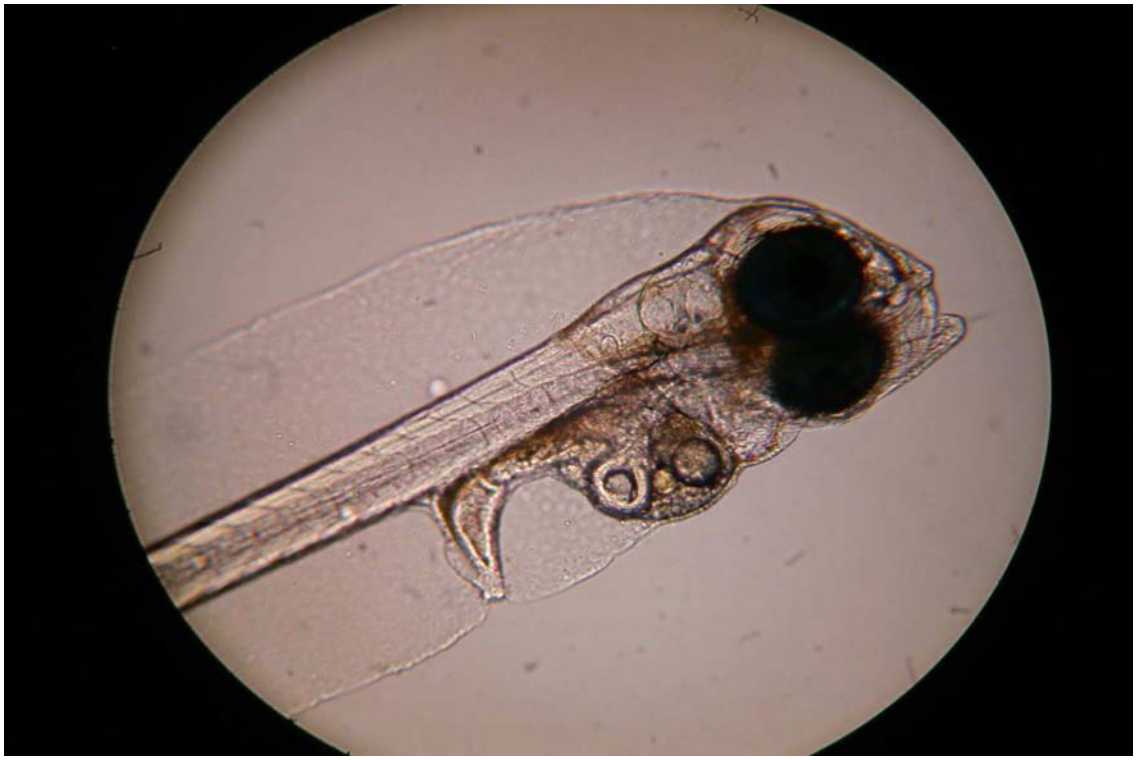
EFFECT OF GONADOTROPIN RELEASING-HORMONE ANALOGUE AND HUMAN CHORIONIC GONADOTROPIN ON MILT CHARACTERISTICS AND GONADAL STEROIDS IN THE YELLOWFIN BREAM, *ACANTHOPAGRUS AUSTRALIS* (SPARIDAE)

CHAPTER 3

EFFICACY OF TWO EXOGENOUS HORMONES (GnRHa AND hCG) FOR INDUCTION OF SPAWNING IN CAPTIVE FEMALE YELLOWFIN BREAM, *ACANTHOPAGRUS AUSTRALIS* (SPARIDAE)

CHAPTER 4

EFFECT OF SALINITY ON FERTILIZATION, HATCH, GROWTH AND SURVIVAL OF YELLOWFIN BREAM (*ACANTHOPAGRUS AUSTRALIS*) EGGS AND YOLK-SAC LARVAE



CHAPTER 5

GENERAL DISCUSSION

5. General discussion

5.1 Response of brood fish to captivity

Successful induced spawning is a product of both male and female contribution and relies upon having sexually mature brood fish of both sexes. Species that are closely related to the yellowfin bream (*Acanthopagrus australis*) (such as the black porgy (*Acanthopagrus schlegeli*), yellowfin seabream (*Acanthopagrus latus*) and black bream (*Acanthopagrus butcheri*) are known to undergo gametogenesis in captivity. However, despite anecdotal reports that yellowfin bream undergo gametogenesis in large public aquaria and earthen ponds, it was not known whether wild caught yellowfin bream would undergo gametogenesis following a short period of acclimation in relatively small tanks (3.2 m^3).

Most wild fish are highly sensitive to the stress of capture and confinement that can compromise reproductive development and the immune system (Pankhurst and Sharples, 1992; Pankhurst and Van Der Kraak, 1997; Haddy, 2000). However, adult yellowfin bream quickly became accustomed to the tank environment and by the onset of the natural spawning season had undergone gametogenesis. Several factors relating to brood fish management were thought to be important in achieving this outcome. However, brood fish stocking density was thought to be of critical factor. When stocking density was less than approximately 20 fish per tank (such as during the stocking of the tanks or after transfer to separate holding tanks at the completion of experimentation) the brood fish showed a loss of colour, became timid and would gather around the central standpipe in an apparent attempt to hide. The brood fish were also reluctant to feed from the surface. When stocking density was above 20 fish per tank the brood fish tended to uniformly spread throughout the tanks and responded well to the artificial pellet feed. These observations suggest that the these negative behavioural responses can somewhat be mitigated for wild yellowfin bream by maintaining an adequate stocking density. However, even when fish were stocked at a density greater than 20 fish per tank they did not respond well to loud noises or movement over the tops of the tanks.

Large mortalities (about 35 fish) occurred when stocking the tanks during the second spawning season. This was most likely a result of the ongoing stress associated with low stocking density. During the first spawning season, three holding tanks were stocked relatively quickly (93 fish in 17 days). However, stocking of four tanks took longer during the second spawning season (approximately 129 fish in 71

days). After capture, fish were transferred into the holding tanks in such a way as to keep the stocking of the tanks random and uniform over time. However, following mortalities in the four holding tanks, the remaining fish were consolidated into three tanks to keep stocking density high.

These mortalities were preceded by a loss of appetite, and some fish lost the ability to maintain their position in the water column. The affected fish would "crest" or rise to the surface. Histological examination of a symptomatic fish revealed severe, diffuse bronchitis and the fusion of the gill lamellae caused by protozoan infestation, most likely *Cryptocaryon irritans*. These symptoms are similar to those reported for the gilt head sea bream (*Sparus aurata*) suffering from *Cryptocaryon irritans* (Kissil *et al.*, 2000). Yellowfin bream are susceptible to a wide variety of external parasites (Roubal, 1981), and rapid mortalities of a similar nature to those have been reported elsewhere under experimental conditions (Broadhurst *et al.*, 1999). In the current study, following the death of these brood fish, despite maintaining stocking density above 20 fish per tank, there was occasionally a small number of further mortalities. However, short periods of hyposalinity (< 2 ‰), by means of a 3-4 h freshwater bath at the onset of behavioural signs of infestation proved to be an effective treatment (Partridge *et al.*, 2003) and most brood fish generally began feeding again within 48 h.

5.2 Response to hormone treatments

Although male fish were naturally spermiating, the volume of milt and the number of sperm available from saline treated (control) males held under experimental conditions remained low. Treatment with hCG up to a maximum scheduled dose of 1000 IU kg^{-1} bw did not increase milt volume or the number of sperm produced. Whereas, GnRHa at a dose of 100 µg kg^{-1} bw increased the volume of milt over saline treated males at 48 h p.i. and the cumulative number of sperm produced over the 48h sample period (6-48 h). Endocrine response to GnRHa treatment indicated that 11-KT (but not T) was associated with the maintenance of spermatogenesis but not short-term elevations in milt volume. Haddy and Pankhurst (2000b) found that in the closely related black bream, plasma levels of key sex steroids were elevated during the spawning season but were quickly suppressed following the stress of capture and confinement. In the current study, plasma levels of 11-KT and T were not elevated in naturally sperimating males. However, the proportion of males with levels of 11-KT above detection limits (0.15 ng ml^{-1}) increased with the progression of the spawning season. Given the timing of the study (early spawning season) and that the natural

process of spermiation is highly efficacious, suppression of endocrine mechanisms towards spermiation following handling and confinement during experimental procedures is the most likely cause of low milt volume and the reduction sperm number in untreated males. Prior to this study there was no information available on the dose related effects of hCG or GnRHa for enhancement of spermiation in the yellowfin bream. Future research should examine the possible effect of social and behavioural cues such as the presence of ovulating females and a competitive spawning environment (multiple males) on milt characteristics and the accompanying steroid profiles to further elucidate their roles in the regulation of spermiation and milt volume in the yellowfin bream.

The extent of the natural spawning season of the yellowfin bream in the Coffs Harbour area is not known. During this study, the pre-spawning reproductive status of both male and females was more advanced in June than May. Males produced larger volumes of milt and sperm while females had larger oocytes. This finding supports anecdotal reports from local fishermen that yellowfin bream start to spawn on or around the full moon in June in the Coffs Harbour area. This finding agrees with the peak spawning season identified by Thorogood (1991) and Dredge (1976) for Moreton Bay QLD, but is somewhat earlier than that (July and August) reported by Pollock (1982b) for the same location.

Female fish also appeared to be more responsive to hormone treatments later in the spawning season. This finding is most likely due to a larger portion of oocytes being in a later state of maturation. In captive gilthead seabream, LH is stored in the pituitary but is not released to the blood stream in sufficient amounts to induce ovulation (Zohar *et al.*, 1995; Holland *et al.*, 1998). It is possible that a similar process occurs in the yellowfin bream and this may also help to explain the apparent increase in responsiveness to GnRHa with the progression of the spawning season. This finding suggests that hormone dose may be adjusted according to the reproductive status of the individual brood fish and on average this may be assumed to correlate with the time of year. However, the reproductive status of the individual did not always correspond to spawning performance. In addition, reproductive status may vary between seasons and is most likely related to water temperature and photoperiod (Kesteven and Serventy, 1941; Dredge, 1976; Pollock, 1982b; Suparta *et al.*, 1984; Thorogood, 1991; Cowden, 1995). In this study the reproductive status of both males and females was more advance in June than May. The month of June over the three

consecutive spawning seasons corresponded with the shortest day length (photoperiod) and a corresponding fall in water temperature towards annual minimums (Fig. A.1. Appendix –A).

The hormone schedules identified in this study (75 and 100 μg kg^{-1} bw GnRHa for females and males respectively) appear to limit the variability in response from brood fish in which there are large variations in reproductive status, which can be difficult to accurately assess. The presence of multiple males would also appear, at least for some females, to be an important behavioural and/or visual cue that serves to trigger the release of eggs at the correct time. The purpose of this study was to quantify the individual response of brood fish to hormone treatment. However, under hatchery conditions brood fish could be induced to spawn together in a group to produce large batches of eggs and larvae in a timely and efficient manner. This would facilitate large scale larval rearing and minimise duplication of hatchery processes as well as help to maximise genetic diversity of progeny.

In the current study, there were differences in the tolerance of eggs and larvae to salinity when compared to that reported by Cowden (1995) from populations near the northern distribution limit of this species. The current study demonstrated that ova of the yellowfin bream, from the subtropical regions of NSW, can be successfully and consistently fertilized at salinities between 20 and 50 ‰. However, larval hatching rates and normal larval development was best at salinities between 25 and 40 ‰. Eggs sank at salinities below 35 ‰ and successful incubation was dependant upon keeping the eggs suspended off the bottom and exposed to a constant flow of aerated water. This demonstrated that ova from the yellowfin bream could be fertilized using water reduced from oceanic strength and that the hatchery production from fertilization to first feeding larvae would only be restricted on a technical rather than a biological basis provided that salinity was kept between 25 and 40 ‰. Hatching jars or alternative up-welling vessels may be suitable for incubating large numbers eggs at salinities reduced from 35 ‰. Potential sites for the production of yellowfin bream would not be restricted to locations that draw water directly from the marine environment and suitable sites may be located on estuaries where there are fluctuations in salinity. There also may be potential for spawning and larval rearing of yellowfin bream at inland sites that utilize saline ground water, however, the effects of the chemical composition of the water on eggs and larvae would need to be evaluated experimentally.

5.3 Potential of yellowfin bream (*Acanthopagrus australis*) for aquaculture

Of the ten reported species belonging to the genus *Acanthopagrus,* two in particular form the basis of extensive aquaculture overseas (Hussain *et al.*, 1981; Abu-Hakima, 1984; Buxton and Garratt, 1990; Chen, 1990; Nelson, 1994; Leu and Chou, 1996; Torres and Luna, 2005; Iwatsuki *et al.*, 2006). The yellowfin seabream is widely distributed throughout the Indo-west Pacific and is extensively cultured in Taiwan (Gwo, 1994; Leu and Chou, 1996; Huang and Chiu, 1997; Liao *et al.*, 2001). The black porgy forms one of three major species of finfish cultured in the Seto Inland Sea and the Japan sea. Between 1993-97, on average over 10 million juvenile black sea bream were produced for wild stock enhancement (release) and aquaculture (Fushimi, 2001). There is a long history of research and culture of these species dating back at least to the 1950's (Kasahara et al., 1960). This research has been ongoing, with efforts focusing on several key areas including elucidating the role of sex steroids in the regulation of reproduction (including sex inversion), induced spawning, larval rearing and growth maximization (Tang *et al.*, 1979; Hu and Hsu, 1980; Lin *et al.*, 1987; Chang and Yueh, 1990; Yueh *et al.*, 1990; Chang *et al.*, 1991; Chang *et al.*, 1995a, 1995b; Huang and Chiu, 1997; Lau *et al.*, 1997; Leu, 1997; Tsai *et al.*, 1997; Yueh and Chang, 1997; Chang and Lin, 1998; Huang *et al.*, 1999, 2000; Lee *et al.*, 2001; Yen *et al.*, 2002; Yueh and Chang, 2002; Om *et al.,* 2003; Wu *et al.*, 2005). Given the similarities of these species it is likely that much of this information would be transferable to the culture of yellowfin bream in Australia.

In Australia, wild yellowfin seabream reach lengths of 10.7, 19.2, 25.4 cm in their first three years (Hesp, 2003; Hesp *et al.*, 2004). This growth rate is similar to that of wild populations of the same species in Kuwait. Under net cage conditions in Taiwan, when stocked at 5 - 6 cm (10 - 15 g) yellowfin seabream can reportedly reach 600 g in 12 months. However, when this species is grown in ponds they reach an average size of 225 g in 14 months and 350 g in 20 months (Chen, 1990). The former account would appear to be erroneous as another more reliable report suggest that it takes a minimum of two years for *A. latus* to reach a marketable size of 292 –346 g and a length of approximately 21 cm with growth being most rapid in the first year (Tsay and Yu, 1980). The situation is similar for the black porgy in Taiwan. When stocked in net cages at 5-6 cm, fingerlings can reach 300 - 400 g in about 12 months. The same species when grown in ponds from 3 cm (0.4g) can reach 22 cm and 180 g in 12 months and 29 cm and 475 g in 18 months (Chen, 1990). It is apparent that there

are large variations in the reported growth rates of both species, particularly under different culture conditions. It is also difficult to account for the age at stocking to make comparisons more meaningful.

In Australia, the black bream has been the subject of much interest as a potential candidate for inland aquaculture that utilizes salt affected agricultural lands in the Murray –Darling River basin. The growth of wild black bream varies considerably between estuaries. Estimates in South Australia are 10, 17 and 23 cm (FL) in the first three years (Kailola *et al.*, 1993). In Victoria growth rate are slower being 6, 11.5 and 16 cm in the first three years (Kailola *et al.*, 1993). The fastest growing populations of black bream require at least 2 years to reach 20 cm in length and would need to be increased by an estimated 33 % to become economically viable for commercial aquaculture (Doupé *et al.*, 2005).

Ultimately, the potential of yellowfin bream for commercial aquaculture will also be determined by the growth rates of juveniles to a marketable size and by their economic value. The growth of wild yellowfin bream is faster than that reported for wild black bream. There are several studies that make estimates of the growth rate of yellowfin bream in the wild (Munro, 1944; Dredge, 1976; Pollock, 1982b; Henry, 1983). The studies by Pollock (1982b) and Henry (1983) are the most recent and likely to be the most reliable owing to the more reliable methodologies used. Pollock (1982b) found that in Moreton Bay (QLD), wild yellowfin bream reach 14.5cm by the first year, 20.5 cm by the second year and 24.1 cm (fork length) by the third year. Henry (1983) found a slightly slower growth rate for the Tuggerah Lakes, NSW, being 13, 18 and 23 cm in the first three years. This variation in growth rate might be a reflection of differing water temperature between the study locations (Kailola *et al.*, 1993). These studies show that wild yellowfin bream can reach minimum legal total length of 25 cm by the end of their third year and that they may be potential candidates for wild stock enhancement and for recreational fish-out operations where the fish are returned to the ponds after capture.

In Australia, snapper has been the subject of much research as a candidate for marine aquaculture (Quartararo, 1996). This species is relatively slow growing in the wild taking 3-5 years to reach 25 cm fork length. When juveniles were held under culture conditions, they attained 12.1 cm and 50 g at eight months, 24.9 cm (FL) and 403 g by 21 months (Bell *et al.*, 1991). Whether the growth rate of yellowfin bream could be similarly improved under culture conditions to make commercial farming

economically viable remains to be demonstrated. Cowden (1995) reported the first account of the growth of yellowfin bream under culture conditions, with fish reaching 15 cm by 12 months and 22.2 cm and 252 g by 25 months. This growth rate is somewhat faster than that reported by Pollock (1982b) for wild yellowfin bream using tag and recapture analysis. However, Cowden (1995) noted that the escape of all but 22 hatchery reared juveniles after approximately 8 months, the stress of repeated sampling, and less than ideal shared culture environment during the later stages of the trial left scope for improvement in growth rates.

Within NSW, juvenile yellowfin bream are currently produced at Searle Aquaculture, Palmers Island, NSW. Juveniles from this hatchery were stocked into a recirculation tank at the National Fishing Industry Education Centre of TAFE (NATFISH) at Junction Hill, near Grafton NSW at the end of 2002. These fish reaching on average 24.5 cm and approximately 300 g by 27 months (approximately 2.5 years from hatch). Weight at stocking was 2.3 g and temperature throughout the study ranged between 21.6 and 28.2 ^{o}C (unpublished data supplied by Glen Searle, NATFISH). It is expected that water quality in the recirculation tank was less than ideal for optimal growth. Nevertheless, this growth rate is similar to that reported for *A. latus* by Tsay (1980) in Taiwan. Both of these studies show a faster growth rate for yellowfin bream than wild fish. However, these findings are limited by a lack of replication, owing to limited resources. Given the euryhaline nature and thermal tolerance of yellowfin bream, there is potential for commercial aquaculture ventures to be established in a variety of locations. Clearly, replicated grow-out trials to determine growth rates of hatchery reared fingerlings to a marketable size would allow the economics of commercial farming, recreational fish-out operations and the potential for wild stock enhancement to be evaluated more clearly.

5.4 Final summary

- Adult yellowfin bream are amendable to hatchery conditions and underwent gametogenesis in captivity.
- During the spawning season males were naturally sperimating and females had developed vitellogenic oocytes.
- Induction of spermiation was possible by injection of 100 μg kg^{-1} bw GnRHa.
- In contrast, milt volume and the number of sperm produced by control males treated with saline remained low under experimental conditions.
- Elevated levels of 11-KT, but not T, was associated with increases in milt volume and spermatozoan liberation in response to GnRHa treatment.
- At the doses tested hCG was ineffective in stimulating spermiation
- Females could be induced to spawn by injection of either hCG or GnRHa. However, GnRHa at a dose of 75 μg kg^{-1} bw proved to be most efficacious; consistently inducing three or more consecutive spawns and the production of commercial quantities of eggs.
- Delayed spawning was associated with poor quality eggs with low fertilization rates.
- Improvements in eggs quality and fertilization rates were possible by increasing the number of males placed with a single female.
- For some females, the presence of multiple males appears to be a visual or behavioural cue the serves to trigger the release of the eggs (spawning) at the correct time.
- Ova of the yellowfin bream could be fertilized at salinities between 15 and 50 ‰. However, embryonic development and hatching was best at salinities between 25 and 40 ‰. Provided that eggs incubated at salinities < 35 ‰ were kept suspended off the bottom.
- The efficiency of larval yolk utilization was inversely related to salinity level.
- The median survival of times of unfed larvae formed an inverse curvilinear relationship with salinity level.
- Mass production of first feeding larvae of the yellowfin bream should be feasible between salinities of 25 and 40 ‰.

The major aims of this study were: 1) the development of reliable protocols for the induction of spontaneous spawning through the application of exogenous hormone; and 2) determine the feasible salinity range at which eggs can be fertilized and undergo normal embryonic development, hatching, growth and survival until the time of first feed. This research has established induced spawning protocols for captive yellowfin bream. Protocols for inducing consistent multiple spawns and the efficient production of commercial quantities of viable eggs and larvae from captive brood fish are as follows:

1) Induced spawning should commence from June onwards in the Coffs Harbour area as reproductive condition of brood fish is on average more advanced.
2) Females should be injected with 75 μg kg^{-1} bw GnRHa and placed with three males to ensure the timely release of the ova.
3) Males should be injected with 100 μg kg^{-1} bw GnRHa to ensure large volumes of milt for repeat spawning events.
4) During gamete activation and the incubation of eggs and yolk sac larvae until the time of first feed, salinity levels should be kept between 25 and 40 ‰. Eggs incubated at less than 35 ‰ sink and should be kept in suspension and exposed to oxygen rich water.

This research will help to facilitate efficient hatchery production of first feeding larvae of the yellowfin bream, and allow further research into larval rearing and the production of juveniles for experimental grow-out trials to proceed unhindered.

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APPENDIX – A

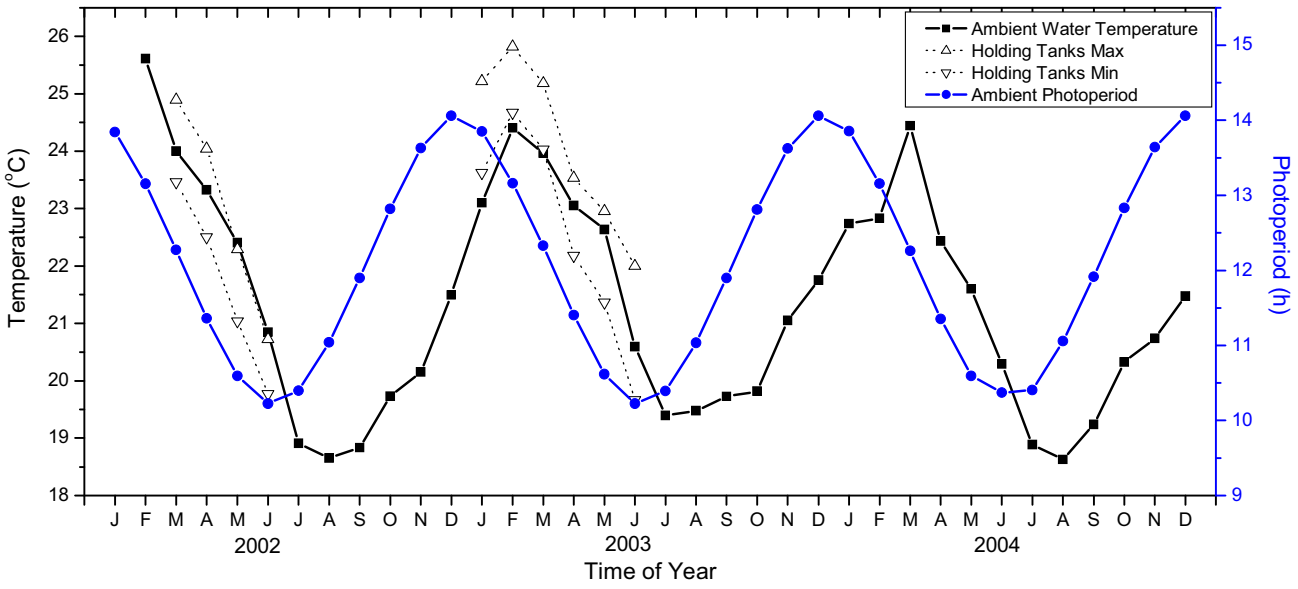

Fig. A.1. Mean monthly ambient water temperatures (°C) and photoperiod (h) for Coffs Harbour, NSW, during 2002, 2003 and 2004. Mean monthly maximum and minium water temperatures of brood fish holding tanks during the approaching 2002 and 2003 spawning seasons are also depicted.

Table A.1. Effect of salinity on fertilization, hatch and deformity rates for eggs incubated with screens. Data represents means ± S.D. Values with dissimilar superscripts within columns indicated significant ($P < 0.01$) differences.

Salinity (‰)	Fertilization (%)	Hatch (%)	Deformity (%)
0	0^{b}		
5	0^{b}		
10	0^{b}		
15	65 ± 44^{a}	8 ± 9^{b}	100 ± 0^{a}
20	93 ± 7^{a}	46 ± 20^{c}	59 ± 21^{b}
25	94 ± 7^{a}	80 ± 14^{a}	19 ± 15^{bc}
30	93 ± 6^{a}	85 ± 9^{a}	11 ± 17^{c}
35	95 ± 5^{a}	85 ± 11^{a}	2 ± 2^{c}
C	94 ± 8^{a}	86 ± 11^{a}	3 ± 4^{c}
40	91 ± 13^{a}	87 ± 12^{a}	5 ± 10^{c}
45	95 ± 8^{a}	85 ± 13^{a}	2 ± 2^{c}
50	93 ± 10^{a}	77 ± 17^{ac}	15 ± 18^{c}

Table A.2. Summary of the effect of salinity on fertilization, hatch and deformity rates for eggs incubated during initial trials with aeration as the only means of keeping eggs in suspension. Data represents means ± S.D. Values with dissimilar superscripts within columns indicated significant ($P < 0.01$) differences.

Salinity (‰)	Fertilization (%)	Hatch (%)	Deformity (%)
0	0^{c}		
5	0^{c}		
10	0^{c}		
15	47 ± 36^{b}	8 ± 10^{c}	100 ± 0^{a}
20	84 ± 18^{ab}	28 ± 12^{bc}	90 ± 10^{a}
25	91 ± 12^{a}	26 ± 23^{c}	67 ± 3^{a}
30	92 ± 105^{a}	11 ± 11^{c}	88 ± 11^{a}
35	94 ± 7^{a}	81 ± 6^{ab}	26 ± 1^{c}
40	94 ± 78^{a}	86 ± 5^{a}	10 ± 1^{c}
45	92 ± 6^{a}	86 ± 1^{a}	14 ± 15^{c}
50	96 ± 5^{a}	30 ± 39^{bc}	84 ± 27^{a}

Table A.3. Effect of salinity on total larval length (µm) at 12 h intervals p.h. Values are means ± S.D. Values with dissimilar superscripts / subscripts are significantly different ($P < 0.01$). Comparisons made at sample times between treatments and within treatment between sample times are denoted by superscripts and subscripts, respectively. Shaded cells indicate where no significant differences were found within sample times.

(‰)	Time (h)						
	0	12	24	36	48	60	72
15	$_{a}1959.4 \pm 57^{a}$	$_{b}2545.9 \pm 76.8^{ab}$	$_{c}2917 \pm 64.4^{ab}$	$_{cd}2997.2 \pm 67^{b}$	$_{c}2962.7 \pm 95.1^{c}$	$_{cd}3061.8 \pm 85.9^{b}$	$_{d}3118.8 \pm 99.7^{ab}$
20	$_{a}2066.7 \pm 44.9^{b}$	$_{b}2641.7 \pm 53.8^{a}$	$_{c}2940.2 \pm 83.9^{ab}$	$_{d}3110.7 \pm 58.6^{a}$	$_{de}3128.4 \pm 70.6^{a}$	$_{ef}3201.1 \pm 74.26^{a}$	$_{f}3221.1 \pm 46.3^{a}$
25	$_{a}2072.4 \pm 33.4^{b}$	$_{b}2565.3 \pm 55.6^{ab}$	$_{c}2927.6 \pm 61^{ab}$	$_{d}3081.7 \pm 59.7^{ab}$	$_{d}3096.3 \pm 74.4^{ab}$	$_{e}3186.9 \pm 55.9^{a}$	$_{e}3207.6 \pm 81.7^{ab}$
30	$_{a}2069.7 \pm 34^{b}$	$_{b}2592 \pm 65.6^{ab}$	$_{c}2881 \pm 78.3^{ab}$	$_{d}3056.6 \pm 72.4^{ab}$	$_{d}3088.7 \pm 58^{ab}$	$_{e}3177 \pm 44^{a}$	$_{e}3187.3 \pm 68^{ab}$
35	$_{a}1985 \pm 47.6^{a}$	$_{b}2610.9 \pm 47.8^{ab}$	$_{c}2874 \pm 60.9^{ab}$	$_{d}3075.6 \pm 36.7^{ab}$	$_{d}3104.4 \pm 58.1^{ab}$	$_{e}3180.6 \pm 43.4^{a}$	$_{e}3218.7 \pm 53.2^{ab}$
C	$_{a}1955.5 \pm 29^{a}$	$_{b}2530.3 \pm 68^{b}$	$_{c}2860.3 \pm 47.4^{ab}$	$_{d}3043.5 \pm 48.8^{ab}$	$_{d}3077.9 \pm 77.4^{ab}$	$_{e}3117.1 \pm 40.3^{ab}$	$_{e}3114.7 \pm 88.6^{b}$
40	$_{a}1984.6 \pm 27.7^{a}$	$_{b}2536.5 \pm 70.7^{b}$	$_{c}2843.5 \pm 64.1^{b}$	$_{d}3006.6 \pm 71.3^{b}$	$_{d}3018.2 \pm 78^{bc}$	$_{e}3148.9 \pm 60.9^{ab}$	$_{e}3181.7 \pm 62.5^{ab}$
45	$_{a}1884.1 \pm 48^{c}$	$_{b}2267.2 \pm 71.8^{c}$	$_{c}2535.8 \pm 97.8^{c}$	$_{c}2647.7 \pm 75.3^{c}$	$_{c}2613.1 \pm 90.5^{d}$	$_{c}2529 \pm 93.9^{c}$	$_{c}2629.8 \pm 155.3^{c}$
50	$_{a}1859.6 \pm 43.6^{c}$	$_{b}2241.5 \pm 64.1^{c}$	$_{c}2552.6 \pm 97.9^{c}$	$_{c}2617.9 \pm 78.4^{c}$	$_{cd}2698 \pm 90.5^{d}$	$_{d}2738.9 \pm 81.6^{d}$	$_{d}2833.6 \pm 99.2^{d}$

Table A.4. Effect of salinity on larval area (μm^2) excluding yolk-sac. Other details as for Table A.3.

(‰)	Time (h)						
	0	12	24	36	48	60	72
15	$_{a}452508.4 \pm 44714.6^{c}$	$_{b}1122050.2 \pm 65718.8^{bc}$	$_{c}1574777.2 \pm 56524.9^{a}$	$_{c}1740949.5 \pm 50852.6^{abc}$	$_{c}1739195.5 \pm 77949.4^{ab}$	$_{c}1647306.2 \pm 114615.1^{b}$	$_{c}1692699.9 \pm 108403.5^{ab}$
20	$_{a}565674.5 \pm 36322.4^{a}$	$_{b}1251922.4 \pm 57111.5^{a}$	$_{c}1579018.8 \pm 57023.2^{a}$	$_{d}1846619.6 \pm 62922.1^{a}$	$_{d}1846617.5 \pm 78999^{a}$	$_{d}1807036.5 \pm 108495.3^{a}$	$_{d}1827049.3 \pm 54543.9^{a}$
25	$_{a}574249.1 \pm 32126.3^{a}$	$_{b}1173584.5 \pm 56094.4^{b}$	$_{c}1531370.2 \pm 40957.1^{ab}$	$_{de}1776673 \pm 82191.5^{ab}$	$_{e}1719844.5 \pm 79830.9^{b}$	$_{d}1840157.6 \pm 102286.7^{a}$	$_{de}1797320.1 \pm 107031.2^{ab}$
30	$_{a}568052.9 \pm 25226.8^{a}$	$_{b}1176309.4 \pm 48704.6^{b}$	$_{c}1468935.5 \pm 48778.5^{b}$	$_{d}1701030.2 \pm 72608^{bc}$	$_{d}1672245.1 \pm 90699.8^{bc}$	$_{e}1805680.8 \pm 73053.6^{a}$	$_{de}1746645.6 \pm 66939.92^{b}$
35	$_{a}533197.8 \pm 43981.8^{ab}$	$_{b}1172639.8 \pm 42074^{b}$	$_{c}1470590.9 \pm 40491.6^{b}$	$_{d}1682386.3 \pm 44383.7^{c}$	$_{d}1654371.9\8 \pm 89389.8^{bc}$	$_{e}1773047.9 \pm 40922.3^{ab}$	$_{de}1709426.8 \pm 62153.4^{b}$
C	$_{a}507817.5 \pm 29292.8^{bc}$	$_{b}1100159.1 \pm 41871.7^{c}$	$_{c}1438477.6 \pm 32513.2^{bc}$	$_{d}1664026.4 \pm 41799.3^{c}$	$_{d}1630998.3 \pm 92246.1^{bc}$	$_{d}1680823.7 \pm 88427.8^{b}$	$_{d}1715178.5 \pm 69795.6^{ab}$
40	$_{a}540006.2 \pm 31304.1^{ab}$	$_{b}1098521.8 \pm 48808^{c}$	$_{c}1390824.9 \pm 27596^{c}$	$_{de}1576603.6 \pm 40173.7^{d}$	$_{e}1586883.3 \pm 81738.7^{c}$	$_{f}1665213.2 \pm 60946.6^{b}$	$_{f}1717742.7 \pm 42753^{b}$
45	$_{a}519975.7 \pm 31356.6^{ab}$	$_{bc}898707.6 \pm 37014.3^{d}$	$_{cd}1014565.5 \pm 65286.5^{d}$	$_{d}1156549.6 \pm 47547.6^{e}$	$_{d}1053688.9 \pm 93925.6^{e}$	$_{b}776596.6 \pm 107845^{d}$	$_{cd}1019477.4 \pm 96930.4^{d}$
50	$_{a}531649.6 \pm 40453.3^{ab}$	$_{b}860920 \pm 32650.8^{d}$	$_{c}1083171.4 \pm 48518.3^{d}$	$_{c}1165685.8 \pm 64915.2^{e}$	$_{d}1330668.7 \pm 85429.7^{d}$	$_{d}1301751.3 \pm 55019.5^{c}$	$_{d}325550 \pm 97242.1.^{c}$

Table A.5. Effect of incubation salinity on larval length (µm) and body area (µm^2). Data represents means ± s.e. (n = 3). Different superscripts indicate significant differences within times.

(‰)	Time (h p.h.)				
	60	72	84	96	108
Length (µm)					
C^F	3117.1± 40.3^a	3114.7 ± 88.6^b	3199.4 ± 41.1	3185.2± 72.9^{ab}	3160.7 ± 107.4^b
C^{SJ}	3161.3 ± 58.36^a	3194.6 ± 31.4^a	3170.8± 36.5^b	3300.3 ± 74.4^a	3227.1± 130.5
45	3060.8 ± 59.32^a	3175.9± 68.2^{ab}	3238.4 ± 31.7^a	3234.1± 59.1^{ab}	3188.4± 68.9^b
50	2922.9± 85.75^b	3020.2 ± 106.5	3082.1 ± 78.6^b	3102.9± 28.3^b	3043.1 ± 56^b
Body (µm^2)					
C^F	1680823.7 ± 88427.8	1715178.5± 69795.6^a	1715762.4± 86032	1626929.3 ± 169228.1^a	1517469.8 ± 161311^{ab}
C^{SJ}	1724565± 183441.9^a	1698241.2 ± 84733.2^{ab}	1815682.7 ± 130883	1735364.9 ± 118833.7	1715662.6 ± 119814.5^a
45	1562994.3± 131020.5^b	1640307.5± 79701.6^{ab}	1706748.1± 118838.7^a	1637374.6 ± 167208.2	1582763.1 ± 56419.3^b
50	1422084.8± 80815.8	1514431.9 ± 121071.9^b	1640681.3± 166368.1^a	1574781.8 ± 67913.6^a	1464066.2 ± 90854.4

Table A.6. Effect of salinity on yolk and globule oil area (µm^2) at 12 h intervals p.h.. Data represents means ± s.e. (n = 3). Different superscripts indicate significant differences within times.

(‰)	Time (h p.h.)		
	60	72	84
Yolk			
C^F	18296.8 ± 2574.2	8906± 1045.4	5903.4 ± 2506.7
C^{SJ}	11783.6± 1788.1^b	7016.7 ± 1645.6^c	5730.6 ± 408.2^a
45	15083.4± 3317.7^b	5457.2± 1313.2	3800.2 ± 1805.5
50	18115.7± 3468.4	19178.3 ± 2917.4^a	3241.1 ± 1604.4^a
Oil globule			
C^F	9744.8± 1075.3^a	8742.6 ± 849.6^a	5928± 762.6^a
C^{SJ}	9004.3± 1648.3^a	7560.3 ± 1027.9^a	4827.7 ± 424.8^b
45	8768± 2897.8^a	6958.9± 1448.4	3956.9 ± 762.7
50	9451.4± 946.8^a	7486.2 ± 911.9^a	5081.7 ± 1033.9^b

Table A.7. Effect of salinity on yolk-sac area (μm^2) 12 h intervals p.h. Other details as for Table A.3.

‰	Time (h)						
	0	12	24	36	48	60	72
15	631724.3 ± 42705^{a}	352383.6 ± 26775.6^{a}	197536.7 ± 26324.1^{a}	110286.3 ± 7613.9^{a}	42302.9 ± 3952^{c}	21825.4 ± 2557.4^{bc}	12814.2 ± 2710^{c}
20	516197.3 ± 19241.7^{b}	316841.9 ± 31767.3^{ab}	181389.3 ± 17910.3^{ab}	91514.8 ± 10361.8^{b}	31297.1 ± 4724.7^{d}	16001.3 ± 2743.7^{d}	6695.2 ± 1736.3^{de}
25	526055 ± 35945.9^{b}	305325.8 ± 30807.5^{bc}	175906.3 ± 22369.0^{ab}	83651.6 ± 13398.1^{bc}	29892.6 ± 3500.7^{d}	17744 ± 2549.5^{d}	5630.6 ± 1527.2^{e}
30	513439.6 ± 22695.5^{b}	285097.7 ± 27564.9^{bc}	179431.4 ± 21498.7^{ab}	75520.4 ± 13918.4^{c}	30768 ± 3836^{d}	17672.6 ± 2529.2^{d}	5060.7 ± 2175.8^{e}
35	515855.7 ± 44900.7^{b}	271630.7 ± 29969.7^{cd}	161633.3 ± 21165.9^{abc}	74025.6 ± 6437.3^{c}	31375.6 ± 3312.6^{d}	18390.9 ± 1921^{cd}	5748.8 ± 1690^{e}
C	526354.8 ± 40375.4^{b}	305511.4 ± 21119.5^{bc}	153260.8 ± 22218.2^{bc}	75535.6 ± 8271.7^{c}	30690.8 ± 3611^{d}	18296.8 ± 2574.2^{cd}	8906 ± 1045.4^{d}
40	519985.4 ± 44868.2^{b}	281757.9 ± 23139.9^{bc}	155194.6 ± 21428.5^{bc}	85784.4 ± 11876.6^{bc}	34356.6 ± 4349.6^{d}	18398 ± 2810.3^{cd}	7590.3 ± 1417.4^{de}
45	434871.5 ± 30431.9^{c}	268385.7 ± 41157.4^{bcd}	151778 ± 18879.5^{bc}	93271.9 ± 12308.1^{ab}	60730.3 ± 5757.8^{a}	27865.4 ± 2488^{a}	24925.7 ± 2471.4^{a}
50	380876.2 ± 43211.7^{c}	235048 ± 23589.5^{d}	135334.4 ± 22443^{c}	73241.5 ± 8690.4^{c}	53333.8 ± 5626.8^{b}	23957.1 ± 2760.6^{ab}	15453.3 ± 2844.5^{bc}

Table A.8. Effect of salinity on oil globule area (μm^2) at 12 h intervals p.h. Other details as for Table A.3.

(‰)	Time (h)						
	0	12	24	36	48	60	72
15	35408.76 ± 1905.11^{a}	29575.08 ± 1479.34^{a}	24284.99 ± 759.2^{a}	20185.66 ± 1154.63^{a}	14663.01 ± 1033.34^{a}	10674.07 ± 1021.77^{a}	7754.85 ± 574.7^{cd}
20	34621.86 ± 1545.93^{a}	26020.05 ± 979.72^{b}	22074.07 ± 1072.73^{ab}	18695.79 ± 1383.55ab	12841.54 ± 1093.11^{bc}	9778.86 ± 886.68^{a}	7288.28 ± 939.7^{d}
25	33547.87 ± 1586.49^{a}	27097.49 ± 1085.29^{ab}	21931.39 ± 1004.57^{b}	18834.58 ± 1471.95ab	13541.67 ± 1289.19^{ab}	10372.51 ± 895.71^{a}	7499.87 ± 546.64^{d}
30	34619.2 ± 1013.11^{a}	26488.24 ± 1767.58^{b}	22089.08 ± 1176.12^{ab}	18030.17 ± 1339.84bc	12536.93 ± 1061.38^{b}	10929.48 ± 854.73^{a}	7968.24 ± 904.91^{cd}
35	35039.6 ± 1550.36^{a}	25891.5 ± 718.61^{b}	21551.22 ± 1380.13^{a}	18351.41 ± 1045.08abc	12710.21 ± 1163.61^{b}	10728.49 ± 968.39^{a}	7733.22 ± 636.09^{cd}
C	33818.46 ± 1528.99^{a}	27741.86 ± 666.6^{ab}	22034.59 ± 1035.79^{ab}	20010.46 ± 1297.43a	13805.71 ± 754.46^{ab}	9744.75 ± 1075.33^{a}	8742.61 ± 849.64^{bc}
40	35762.17 ± 1982.13^{a}	27558.99 ± 734.75^{ab}	23014.89 ± 1232.89^{ab}	18622.02 ± 1597.22ab	12816.68 ± 990.41^{bc}	10781.73 ± 976.58^{a}	9106.65 ± 779.82^{b}
45	34609.82 ± 1090.7^{a}	28709.84 ± 1761.36^{a}	23668.66 ± 784.16^{ab}	18740.11 ± 1185.12ab	14764.74 ± 988.02^{a}	12638.07 ± 1348.01^{b}	11138.52 ± 884.51^{a}
50	35451.18 ± 951.81^{a}	27762.25 ± 1223.82^{ab}	22423.31 ± 1436.33^{ab}	16556.85 ± 1005.11c	14443.49 ± 1037.88^{ac}	10805.37 ± 941.45^{a}	9859.63 ± 823.66^{ab}

Table A.9. Parameter values for linear regression of Log $_{(10)}$ transformed yolk and refected Log $_{(10)}$ transformed larval body area and length data over time at different at salinity levels, n = number of samples.

Linear regression y = a –bx		Salinity (‰)								
		15	20	25	30	35	C	40	45	50
	Parameter									
Yolk area										
	a	5.79914	5.72769	5.73651	5.72828	5.71614	5.73876	5.71225	5.63967	5.59312
	b	-0.02107	-0.02081	-0.02195	-0.02279	-0.023	-0.02359	-0.02178	-0.01877	-0.02
	r^2	0.98547	0.97929	0.96582	0.95836	0.97544	0.9792	0.97744	0.96623	0.96309
	n	36	51	60	61	56	54	60	33	49
Body area										
	a	6.1925	6.1859	6.17682	6.1532	6.15442	6.17305	6.14944	6.14983	6.15036
	b	-0.02217	-0.02693	-0.02292	-0.01893	-0.01831	-0.01798	-0.01482	-0.00649	-0.00687
	r^2	0.97419	0.93907	0.91848	0.94704	0.97627	0.98327	0.97831	0.92362	0.91953
	n	28	51	54	54	50	49	58	29	42
Length										
	a	3.08012	3.09784	3.09496	3.08091	3.1013	3.11561	3.09156	3.10694	3.11053
	b	-0.02331	-0.03227	-0.02881	-0.02629	-0.02725	-0.0256	-0.02309	-0.01067	-0.01032
	r^2	0.91368	0.88729	0.91073	0.87085	0.95228	0.93298	0.89111	0.91877	0.88618
	n	31	61	64	64	62	58	67	31	40