Hormone Induced Spermatogenesis and Spermiation in Barramundi, *Lates calcarifer* (Bloch).

by Glenn Schipp, B.Sc.(Hons)

A thesis submitted in fulfilment of the requirements for the degree of Master of Applied Science

Department of Aquaculture

University of Tasmania

July 1993

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF TABLES

LIST OF TABLES

LIST OF TABLES

ACKNOWLEDGMENTS

Sincere thanks are extended to my research supervisors, Dr Colin Shelley, Dr K. Leong Wee and Prof. Nigel Forteath for their advice and encouragement with the planning of this study and the preparation of the manuscript.

Thanks also to John Wood, Haydn Russell, Adrian Donati, Graham Williams and Graham Baulch for their expert technical assistance during the course of the project and for their patience when things went wrong.

To Chris Kuo, my co researcher and author of the companion thesis on reproduction control in female barramundi, thank you for your advice and assistance during the course of this project.

To Karen - thank you for your assistance with editing the thesis and your tolerance of my frequent absences for 'spawning duty'. For a while at least, you can have a break from having your life ruled by the sex life of a fish!

This research was supported by the Department of Primary Industry and Fisheries, NT, Australia under the barramundi aquaculture programme.

DECLARATION

I certify that this dissertation contains no material which has been accepted for the award of any other degree or diploma in any institute, college or university and that to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due reference is made in the text of the dissertation.

Glenn Schipp
July 1993

ABSTRACT

Between June 1990 and July 1993 a variety of reproductive hormones were tested for their effect on spermiation, spermatogenesis and spawning performance in male barramundi, *Lates calcarifer*. The study originated from problems experienced with male barramundi spawners during the 1988/89 spawning season.

The hormones and dose rates tested in the spermiation experiments were: irradiated carp pituitary extract (iCPE), in 2 intramuscular injections, 24 hours apart, at 8 mg kg^{-1}; luteinising hormone releasing hormone analogue (LHRHa, des-Gly^{10}[d-Ala^{6}]-LH-RH-ethylamide), injected at either 25 or 50 μg kg^{-1}; LHRHa at 25, 50 or 100 μg kg^{-1} plus pimozide (a dopamine antagonist) at 1.0 mg kg^{-1}; Ovaprim SC (A commercial preparation of salmon gonadotrophin releasing hormone analogue and dopamine antagonist) at 0.1 ml kg^{-1}; 17α, 20β-dihydroxy-4-pregnen-3-one (17α-20βP), a progestin steroid at 1.0 mg kg^{-1}; and Sustanon '250', a combination of testosterone esters, at 0.5 or 1.0 ml $fish^{-1}$.

The results of the spermiation experiments suggest that, LHRHa at 25 μg kg^{-1}, Ovaprim and 17α-20βP have the best potential for the development of a hormone therapy for increasing spermiation.

Results from the spermiation experiments were tested during actual spawning trials to see if hormone therapy influenced spawning success. It was found that the fish were just as likely to spawn if they had not received hormone treatment. In addition, it was determined that male barramundi do not have to be in running ripe condition prior to addition to the spawning tank. Interaction with the ovulating female appears to be one of the most important factors influencing spawning success.

The spermatogenesis experiments examined the effect of chronic hormone therapy over periods longer than one month. In five of the spermatogenesis experiments the fish were implanted with slow releasing cholesterol pellets containing one of the following hormone combinations: 17α-methyltestosterone (17-MT), 100, 200 or 400 μg kg^{-1}; LHRHa, 25, 50 or 100 μg kg^{-1}; LHRHa + 17-MT, 25+100, 50+100 or 100+100 μg kg^{-1}. The other experiments examined the implantation of 10 mg of either 17-MT or 19-Norethisterone acetate in silicone pellets and daily, oral administration of 12.5 mg kg^{-1} 17-MT. The experiments were conducted at different times of the year to investigate seasonal response.

Of all the hormone treatments tested, only 25 μg kg^{-1} LHRHa pellets showed any real potential and only during the peak breeding season.

Based on these trials it is not possible to develop a protocol, using hormone therapy that reliably enhances the breeding condition of male barramundi. Recent success overseas and in Quennsland with environmental control of barramundi breeding provides the best means for control of reproduction.

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Over the past twenty years, the farming of bony fish (Teleosts) has rapidly gained momentum around the world. To keep pace with the increasing interest in fish farming, more scientific study is being devoted to improving the knowledge and techniques of manipulating fish reproduction (Harvey and Hoar 1979; Lam 1982; Bromage 1988).

1.1.1 History of Induced Spawning

Fish culture has been practiced for many centuries. The early Chinese dynasties, Ancient Romans and medieval European monastries all engaged in the production of the common carp, *Cyprinus carpio*, in earthern, freshwater ponds (Harvey and Hoar 1979; Pillay 1990). Marine fish farming is believed to have started in Indonesia during the 15th century when young milkfish, *Chanos chanos*, and other species were trapped in tidal earthern ponds (Bromage 1988). These early methods of fish farming involved little interference or manipulation of the breeding cycle (Lam 82; Pillay 1990).

Destruction of wild spawning habitats, unseasonal climatic conditions, excessive wild harvests and failure of domesticated fish to spawn, has often lead to shortages in the supply of eggs, larvae or fingerlings, collectively referred to as 'seed' (Roberts *et al.* 1978; Matty 1985).

The reliance on 'natural spawnings' for seed supply remained until the 1930's, when the Argentinian endocrinologist, B. A. Houssay, used injections of fresh, fish pituitary glands to induce premature birth of the young of small viviparous fish (Lam 1982). Von Ihering (1937) further investigated the injection of pituitary glands and successfully developed a reliable technique for spawning induction in carp. The injection of pituitary glands or pituitary gland extracts to induce spawning is called hypophysation (Davy and Chouinard 1981). Hypophysation of a variety of carp species became widely practiced in China and India by the early 1950's and is still commonly practiced today (Chaudhuri 1960, 1976; Saad and Billard 1987).

Development of fish spawning techniques has escalated rapidly since the 1950's. This escalation has lead to the emergence of fish endocrinology as an applied science (Matty 1985; Billard 1989) with considerable emphasis on the development of synthetic reproductive hormones (Lam 1982), improvement of hormonal therapies (Crim *et al.* 1988; Sherwood *et al.* 1988; Garcia 1990; Kuo 1991) and on achieving a better understanding of the role of environmental factors in the reproductive process (Roberts *et al.* 1978; Shireman *et al.* 1978; Lam 1983; Kungvankij 1987).

1.1.2 Reproduction Control

The artificial induction of spawning enables seed to be produced from fish that do not normally spawn in captivity and helps avoid the ecological and logistical problems associated with the harvest of fry from wild populations (Bromage 1988). Even when fish do breed in captivity, induced breeding can be utilised to achieve greater

control over fry production (Davy and Chouinard 1981; Lam 1982). The total control of spawning means that not only can seed supply be guaranteed but that spawning times can be altered to suit the individual requirements of farmers and facilitate the use of hybridisation and genetic selection techniques to ultimately improve the quality of fish produced (Thorgaard 1983, 1986).

Von Ihering's technique of hypophysation utilised the fact that the reproductive system is not usually fully interupted during a fish's normal period of breeding (Harvey and Hoar 1979; Lam 1982). In many fish, the maturation process continues in captivity and usually remains unimpaired up to the final stages of gamete development. Reproduction is only inhibited at the point of gamete release due to the absence of normal environmental cues when the fish are kept in captivity. Hormone therapy has made it possible to intervene successfully at the stage where environmental cues are lacking, thereby forcing the final maturation and release of gametes (Yamazaki 1976). Without hormone treatment, gametes in captive fish usually undergo atresia whereby oocytes and sperm degenerate and are resorbed (Zohar 1989). Atresia can be amplified and accelerated in fish that are stressed (Billard *et al.*, 1981; Lacanilao *et al.* 1985) and care must be taken to minimise stress levels in broodstock.

Gonadal development can be divided into two major phases, gametogenesis, the formation of gametes in the gonad (oogenesis and spermatogenesis) and gamete release (ovulation and spermiation). Both phases are governed by the endocrine system, but each has its own set of causal factors (Davy and Chouinard 1981). The presence of these two major phases means that reproductive control can be directed at either the short term process of gamete

release or the longer term and more involved process of gamete maturation (Pillay 1990).

Early studies on fish reproduction focussed almost exclusively on achieving release of mature gametes (Von Ihering 1937; Clemens and Grant 1965; Yamazaki and Donaldson 1969) and it is only in the last twenty years that more attention has been paid to achieving control over gametogenesis through the manipulation of the fish's environment and/ or hormone therapy (Roberts *et al.* 1978; Shireman *et al.* 1978; Borg 1981; Lee *et al.* 1986a; Lee *et al.* 1986b).

These experiments have lead to a better understanding of the effects of the environment on the endocrine control of fish reproduction (Yamazaki 1976; Billard *et al.* 1982a; Lam 1982; Nagahama 1987).

1.2 THE ROLE OF ENVIRONMENT IN REPRODUCTION

The reproductive cycles of almost all fish are regulated by environmental stimuli such as temperature, photo-period, salinity, food supply and rainfall (Donaldson 1973; Roberts *et al.* 1978; Shireman *et al.* 1978;).

1.2.1 Temperature and Photoperiod

Temperate and coldwater teleosts are seasonal breeders and production of the young usually occurs at an optimal time of the year, maximising survival (Lam 1983). In temperate zones the most important cyclic environmental factors are daylength and temperature (Bhattacharya and Banerjee 1990). Daylength is usually the primary determinant of spawning periodicity in temperate fish (Zohar 1989),

although some species such as the orange-red medaka, *Oryzias latipes*, are controlled by temperature (Awaji and Hanyu 1988).

In tropical regions the photoperiod hardly varies throughout the year and is unlikely to play a major role in reproductive development (Lam 1983). Even though temperature only varies slightly in accordance with the wet and dry seasons, it is believed to be the main regulator of spawning periodicity (Lam 1983; Zohar 1989). Most tropical species have extended spawning seasons. Within these spawning seasons many species also have periods of peak spawning activity (Lam 1983). Spawning peaks are usually associated with rainfall or flooding during the monsoonal season (Lam 1983; Davis 1987). Rainfall and flooding are unlikely to regulate gametogenesis, but may be important in the synchronisation of final maturation and spawning (Zohar 1989). The lunar cycle may also be important for the synchronisation of the spawning, as is the case with barramundi, *Lates calcarifer*, (Moore 1979; Davis 1987).

Recently, Thouard *et al.*, IFREMER Centre Oceanologique du Pacifique, Tahiti (pers. comm.) achieved year round control of spawning of barramundi in Tahiti through the regulation of photoperiod and temperature. They did not attempt to separate the effects of photoperiod from temperature but based on the comments of Lam (1983) it is likely that in the future, year round spawning of barramundi may be achieved by temperature regulation alone.

1.2.2 Nutrition

Changes in seasonal abundance and quality of food may significantly influence gamete development (Ali 1987). It is well known that plankton undergoes seasonal changes in species composition and number which can have profound effects up the food chain, particularly in the tropics where the waters have a low productivity (Lam 1983).

Even though dietary nutrients influence gonadal growth, fecundity and egg quality in fish (Watanabe *et al.* 1984a; Mourente and Odriozola 1990) the specific nutritional requirements of broodstock fish are not yet known (Hardy 1988).

It is known that captive fish require diets high in protein for full gonadal maturation (Watanabe *et al.* 1984b; Ali 1987). Highly unsaturated fatty acids (HUFA's), vitamin E, and trace metals are also important dietary elements for successful reproduction (Kanazawa 1985). It is generally agreed that quantity and quality of feed as well as feeding regime are important for breeding success (Davy and Chouinard 1981; Maneewong 1987).

The reproductive condition of broodstock fish may be influenced by the unintentional inclusion of naturally occurring sex steroids in their diet. Various sex steroids have been found in significant quantities in fish meal used for the preparation of broodstock diets (Pelissero *et al.* 1989). Sex steroids and other reproductive hormones are also contained in fresh fish and squid used as broodstock feed.

Most studies on hormonal induction of reproduction either attempt to eliminate nutrition as a variable by offering the fish a high quality diet, rich in protein (Garcia

1990; Barry *et al.* 1991; Kuo 1991) and in some cases with extra vitamins and minerals added (S. Fielder, Sea Harvest, pers. comm.) or, more usually, they ignore consideration of dietary effects altogether (Nacario 1987; Almendras *et al.* 1988a). In the literature, failure of hormone therapies to induce spawning is rarely discussed as being the result of inadequate diet, although this may be a possibility (Pillay 1990).

1.2.3 Salinity

Variations in salinity do not appear to be important in the maturation of milkfish (Nash and Kuo 1976; Lee 1985), grey mullet, *Mugil cephalus* (Lee and Weber 1986); European sea bass, *Dicentrachus labrax* (Brusle and Roblin 1984) or barramundi (Kuo 1991). Salinity can be important for spawning, as milkfish will not spawn under a salinity of 8 ‰ (Lee 1985) and the larvae of most of these species will not survive in water with a salinity of less than 15 ‰ (Lee 1985; Kungvankij *et al.* 1986; Maneewong 1987).

1.2.4 Social Factors

Factors associated with the presence of the opposite sex and courtship behaviour may be important in sychronising spawning (Lam 1982; Billard *et al.* 1989). Data from numerous studies, points to the existence of some form of chemical mediation in the reproductive behaviour of a variety of fish species (Liley and Stacey 1983). Most studies confirm that a chemical product from one individual elicits an approach, or in some instances a more specific sexual response in another (Kyle *et al.* 1979 1985; Billard *et al.* 1989). In the majority of cases the chemical acts as a 'gamete releaser' but a few chemicals

with priming or maturational effects have also been detected (Van Weerd and Richter 1991). It is likely that chemical signals do play an important role in reproduction by facilitating orientation and arousal, thereby ensuring synchronisation of potential partners (Kyle *et al.* 1985). Chemical communication may play a major role in fish that spawn at night or in turbid waters. There is an increasing body of evidence linking the release of the progestin steroid, 17α, 20β-dihydroxy-4-pregnen-3-one (17α-20βP) and prostaglandins from ovulating females with the stimulation of spermiation and sperm release in males (Zhao and Wright 1988; Loir and Billard 1990; Liley and Rouger 1990).

Physical interaction may also be important for gamete release. Oviposition in goldfish, *Carassius auratus*, is triggered by the male 'pushing' against the oviduct of the female (Partridge *et al.* 1976). This behaviour has also been witnessed in spawning barramundi (Garrett and Connell 1991).

1.2.5 Tank Size

In captive fish, the size of the holding tank may influence the reproductive performance (Lacanilao *et al.* 1985). Provision of sufficient space for the fish to mature and spawn is very important for large broodstock such as milkfish (Lee 1985) and barramundi (Kuo 1991). The fish must have sufficient space, so that they are not stressed by crowding and can manoeuvre for spawning and fertilisation of the eggs (Kungvankij *et al.* 1986).

For economic reasons it is important to determine the smallest suitable size for maturation and spawning tanks (Lee 1985). Obviously the smaller the tank the cheaper it will be to construct and operate. The minimum size for

milkfish maturation tanks has been determined at 25,000 litres volume, 1 metre depth and stocked with 10 broodstock. The shape of the tank is not important (Lee 1985).

1.2.6 Environment and Spermiation

Spermiation is the key stage of male reproduction, resulting in the release of good quality spermatozoa at the time that the most favourable environmental conditions occur (Billard 1986). Despite the recognised importance of the spermiating stage there is a paucity of information relating to the effects of environment on spermiation (Lam 1982). It is known that environmental effects vary according to species and the stage of development of the testis (Billard 1986), although available evidence suggests that generally, spermiation is less dependent on environmental modulation than oocyte maturation and ovulation (Lam 1983). This is confirmed by observations made at the Darwin Aquaculture Centre during 1988/89 when male fish were observed to have mature testes well outside the normal period of breeding. Female barramundi sampled at the same time were not in breeding condition. In *Plectroplites ambiguus* spermiation can occur at temperatures below 23.6°C but ovulation cannot (Lake 1967). In the three-spined stickleback, *Gasterosteus aculeatus*, spermatogenesis is independent of temperature but androgen secretion, spermiation and reproductive behaviour are all temperature and photo-period dependent (Baggerman 1980; Borg 1981).

Billard (1986) concludes that spermiation and spermatogenesis depend more on temperature than photoperiod. Spermiation in some species such as the salmonids is favoured by low temperatures, while other

species such as cyprinids depend on fluctuating temperatures for the onset of spermiation (Billard *et al.* 1982a). It is possible that barramundi also depend on fluctuating temperatures for spermiation. Researchers in Thailand have developed a natural spawning technique for barramundi where the water level and temperature are varied daily to simulate the naturally occurring tides (Kungvankij 1987).

1.2.7 The Influence of Environment on Controlled Fish Breeding

Apart from directly influencing reproductive development, the fish's environment (both external and internal) may influence the effectiveness of hormone induced breeding (Crim 1982; Billard *et al.* 1987). For example, Kulikova and Fedulina (1987) found, not surprisingly, that the temperature and salinity of artificial holding tanks for Black Sea golden mullet, *Liza aurata*, needed to closely match those of the natural spawning grounds for successful spawning inducement using hormones.

In concert with annual spawning cycles the endocrine organs have been demonstrated to have annual fluctuations in activity (Zohar and Billard 1984; Zohar 1989). Daily variations in organ receptivity and production has also been proven (Zohar 1986). Samples collected daily from wild, breeding, blue cod, *Parapercis colias*, showed that the fish were not spermiating until after 15:00 hours each day (Pankhurst and Kime 1991).

Billard *et al.* (1987) noted different responses in plasma gonadotrophin (GtH) in common carp, depending on the time of day hormone treatment was administered. Morning treatment caused an elevated level of plasma GtH, evening

treatment did not. Conversely, Zohar (1986) reported a 100% improvement in spawning response of female gilt-head sea bream, *Sparus aurata*, treated in the evening as opposed to morning injections.

Response to hormone treatment may also depend on temperature (Kaya 1973; Saad and Billard 1987) as well as several other variables including age, sex and season (Billard *et al.* 1987). Therefore, whenever hormone therapy is used for fish breeding, it is important to consider the effect the environment may have on the results.

The implications of a better understanding of the influences of environment on reproduction are a better control of broodstock, more reliable hormone therapies, a relatively chemical free spawning process and less stress on the fish. To date, hormone therapies have been unable to provide total control of the maturation process in fish.

1.2.8 Environmental Manipulation

Manipulation of the environment is another way of inducing fish to mature and breed. This method has been shown to be promising in the maturation of salmonids (Shehadeh 1972); red drum, *Sciaenops ocellata* (Roberts *et al.* 1978, 1988); European sea bass (Carrillo *et al.* 1989); barramundi (Thouard *et al.*, pers. comm.) and in spawning barramundi after they have reached a critical stage of maturation (Kungvankij and Suthemechaikul 1986). The effectiveness of environmental manipulation depends on the age and sex of the animal and the season in which the manipulation is attempted (Roberts *et al.* 1978).

The negative side to environment manipulation for fish breeding is that controlled environment facilities are often expensive to set up and operate and therefore, total hormonal control, if it can be achieved, may offer a relatively low cost alternative.

1.3 MALE FISH REPRODUCTION

Many hormones are involved in the control of maturation and spawning but those produced by the hypothalamus, pituitary and the gonads are of primary importance.

1.3.1 The Hypothalamo-Pituitary Gonadal Axis

Appropriate sensory receptors convey environmental stimuli to the brain in the form of neural inputs which are then processed by the pineal gland and/ or the hypothalamus. The pineal gland probably controls gonadotrophin production through the hormone melatonin (Zohar 1989) and the hypothalamus influences GtH release by way of releasing hormones (Harvey and Hoar 1979; Matty 1985). The releasing hormones stimulate the production of gonadotrophin and its subsequent release into the vascular system of the adenohypophysis of the pituitary (Harvey and Hoar 1979). The releasing hormones are the starting point of the pathway for the hormonal control of reproduction (Peter 1982).

Once released, gonadotrophin is carried by way of the systemic circulation to the gonads where it regulates the production of the sex steroids. The sex steroid hormones, androgens, oestrogens and progestins are the direct mediators of gonadal development and are also linked with the development of secondary sexual characteristics such

as colouration and behaviour (Lam 1982; Liley and Stacey 1983; Nagahama 1987; Bromage 1988).

The pathway leading from environmental stimuli to gonadal control is known as the Hypothalamo-pituitary gonadal axis, HPG, (Shehadeh 1972; Liley and Stacey 1983; Weil and Crim 1985). A summary of the HPG pathway as it applies to fish is presented in Figure 1.

1.3.2 The Hypothalamus and Pituitary

The first evidence for the stimulatory regulation of gonadotrophin secretion in fish came from studies demonstrating that hypothalamic extracts induced GtH release (Crim *et al.* 1976). The role of the pituitary and hypothalamus in the control of gonadotrophin secretion has now been well documented (Yamazaki and Donaldson 1969; Fontaine 1976; Peter and Crim 1979).

The pituitary gland is a neuro-epithelial, complex structure that is present and functional in all vertebrate groups. The pituitary has two major sections, the neurohypophysis, which is derived from nervous tissue and the adenohypophysis that has an epithelial origin. The neurohypophysis is in very close contact with all parts of the adenohypophysis and the transition from neural information to hormonal control takes place at the interface between the two (Matty 1985). A generalised diagram of the teleost pituitary is presented in Figure 2.

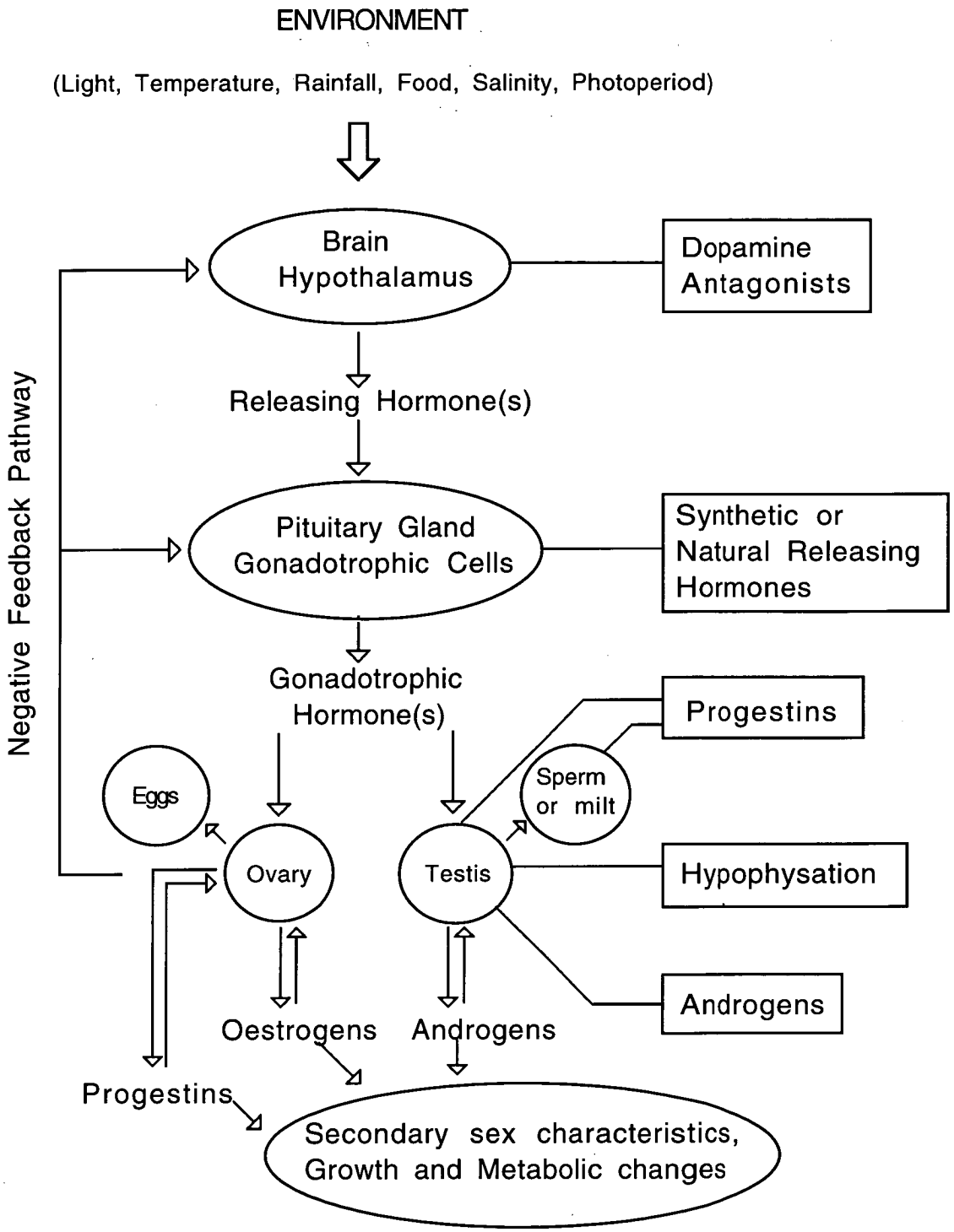

Figure 1. The major steps in the control of reproduction in bony fish. The boxes indicate the sites of action for the artificial control of reproduction in male fish (Modified from Bromage 1988).

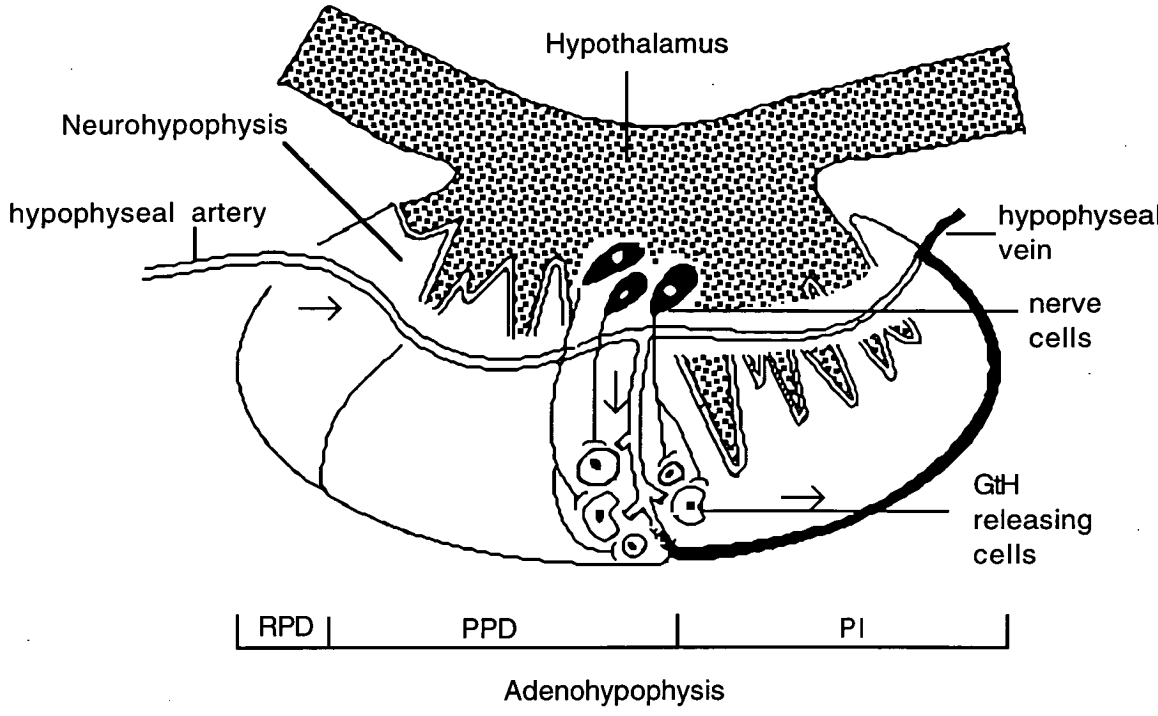

Figure 2. Schematic representation of a sagittal section of the teleost pituitary gland. The arrows show the direction of blood flow.

Adenohypohysis: RPD = rostral pars distalis; PPD = proximal pars distalis; PI = pars intermedia (Modified from Harvey and Hoar 1979; Van Oordt and Peute 1983).

The neurohypophysis of teleosts is composed of hypothalamic neurosecretory fibres, glial cells and blood vessels. The nerve fibres are mainly non-myelinated and terminate in the central area of the neurohypophysis close to the blood vessels. Some fibres extend into the adenohypophysis and this interdigitation is characteristic of the teleost pituitary (Romer and Parsons 1977; Harvey and Hoar 1979, Ball 1981). The axons of the hypothalamic neurones respond to nervous signals from the brain by releasing chemical messengers at the axon terminal, thus bridging the gap between nervous and hormonal information.

The adenohypohysis of teleosts is composed of the rostral and proximal pars distalis and pars intermedia. These contain many different types of cells, some of which have been identified with the production of specific hormones. The rostral pars distalis has been associated with production of Thyroid stimulating Hormone (TSH), Prolactin and Adrenocorticotrophin (ACTH) production; the proximal pars distalis with growth hormone, gonadotrophic

hormone(s) and TSH and the pars intermedia with Melanin-Dispersing Hormone (MSH) (Matty 1985). The gonadotrophin producing cells of teleosts have probably received more attention than any other cell of the fish pituitary. Many studies have investigated the effects of pituitary removal (hypophysectomy), and hormone replacement therapy on circulating levels of GtH and gonad development (Sundararaj and Nayyar 1967; Yamazaki and Donaldson 1969; Billard *et al.* 1982a; Ueda *et al.* 1985).

1.3.3 Mode of Action of Gonadotrophins

The primary role of pituitary gonadotrophin in the physiological regulation of gonadal function is well established (Nagahama 1987). GtH levels in the plasma slowly increase over the period of maturation and ovulation/ spermiation is preceded by a sharp increase (Harvey and Hoar 1979; Zohar and Billard 1984). In most cases gonadotrophin action on gonad development is not direct. GtH stimulates the synthesis of gonadal steroid hormones which in turn mediate various stages of gametogenesis including spermatogenesis and spermiation (Nagahama 1987; Loir and Billard 1990). The effect of GtH on testicular hydration may also be mediated by prostaglandins (Stacey and Goetz 1982).

1.3.4 The Testes

The testis is usually an elongated paired organ lying dorsally in the body cavity and prolonged posteriorly by a vas deferens. The vas deferens ends at the level of the genital papilla between the rectum and the urinary ducts (Billard *et al.* 1982a).

Two types of testicular structures are found in fish, lobular and tubular. Roosen-Runge (1977) stated that lobular and tubular were synonymous but Billard *et al.* (1982a) disagree.

1. The Tubular type.

The tubules are regularly orientated between the external tunica propria (blind end) and a central cavity into which the spermatozoa are released. Spermatogonia are located in cysts at the blind end of the tubule. The tubular structure is not very common and is only found in the atheriniforme fish (Grier *et al.* 1980) (Figure 3a).

2. The Lobular type.

The lobular type of testis is more common in teleosts and is the type found in barramundi (Davis 1982). The lobular structure differs from the tubular in that the cysts are positioned along the tubes, they remain at the same place and the spermatozoa are released into a central lumen. The term lobule is suitable because the diameter of the tube is variable and looks histologically like a lobe (Billard *et al.* 1982a, Figure 3b).

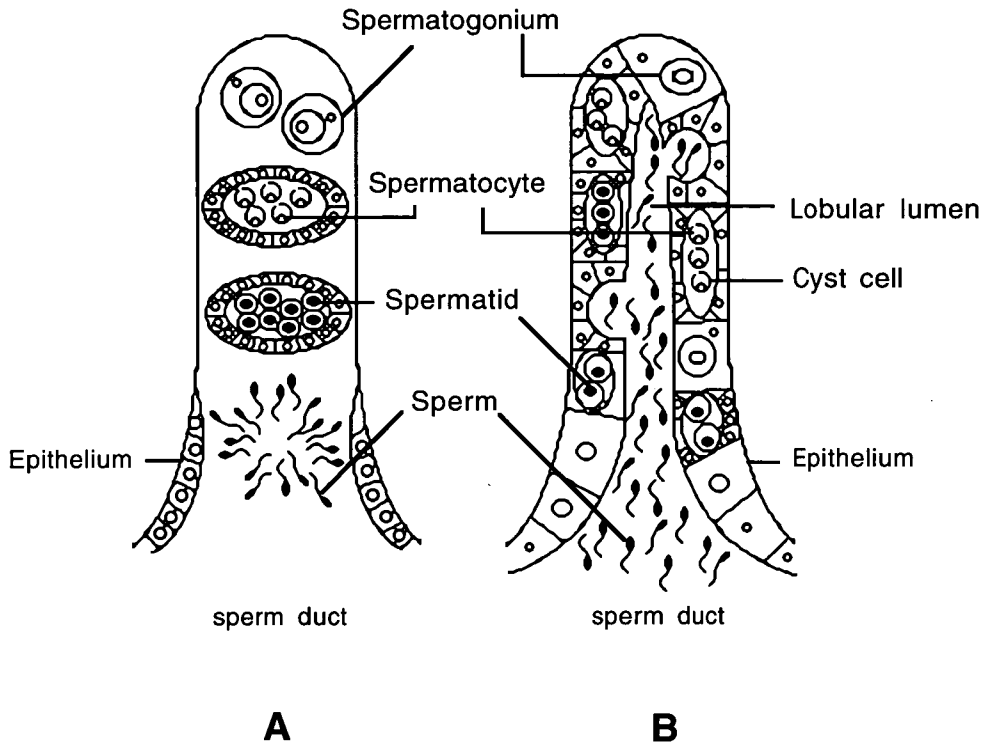

Figure 3. Stylised diagram of the two types of testicular structure found in fish. **A.** The tubular type. **B.** The lobular type (Adapted from Nagahama 1983)

In the lobules, spermatogenesis takes place within individual cysts which contain daughter cells of a primary spermatogonia. The cysts are associated with other 'support cells' that aid its development. The support cells have often been referred to as 'Sertoli cells', however, Roosen-Runge (1977) believes that 'Sertoli cell' should be reserved for mammalian testes and that cyst cell is the more appropriate term for fish.

The mature testes of most teleosts contain numerous large lobules filled with ripe spermatozoa. The interstitium between the lobules consists of interstitial cells, fibroblasts and blood vessels.

1.3.5 Steroid Production

Endocrine secretory cells are characteristically situated in the interstitial tissues of the testis and are sometimes referred to as Leydig cell homologues (Nagahama *et al.* 1982; Zohar 1989), although Billard *et al.* (1982a) believe they are not necessarily homologous. These interstitial cells are thought to be site of GtH controlled androgen production (Nagahama *et al.* 1982; Nagahama 1987; Shetty and Satyanarayana Rao 1990).

Other proposed sites for steroid production are the cyst cells, the interrenal, the sperm duct and the seminal vesicle (Zohar 1989), although Loir (1986) disagrees with regard to the cyst cells. Together with the interstitial cells they are responsible for the production of androgens, progestins and steroid conjugates.

1.3.6 The Androgens

The androgens have been shown to be directly responsible for maturation of the gametes. The most important androgens produced by fish testes are testosterone (T) and 11-ketotestosterone (11-KT) (Billard *et al.* 1982a; Zohar 1989). Radioimmunoassays have shown that T levels slowly increase in the plasma during spermatogenesis, reaching the maximum just prior to spermiation. 11-KT levels are also high during spermatogenesis and decrease during spermiation when the levels of progestin increase sharply (Nagahama 1987). The relative function of the different androgens in the regulation of spermatogenesis remains unclear. Matty (1985) believes that it is difficult to say that endogenous androgens play a true physiological role in maintaining spermatogenesis but it is true to say that they have a beneficial effect on spermatogenesis.

1.3.7 The Progestins

Progesterone (PRG) has been isolated from the gonads but not from the plasma and so probably does not play a role in male reproduction except as a precursor. PRG has a key position in the pathway of steroid synthesis (Matty 1985, Figure 4).

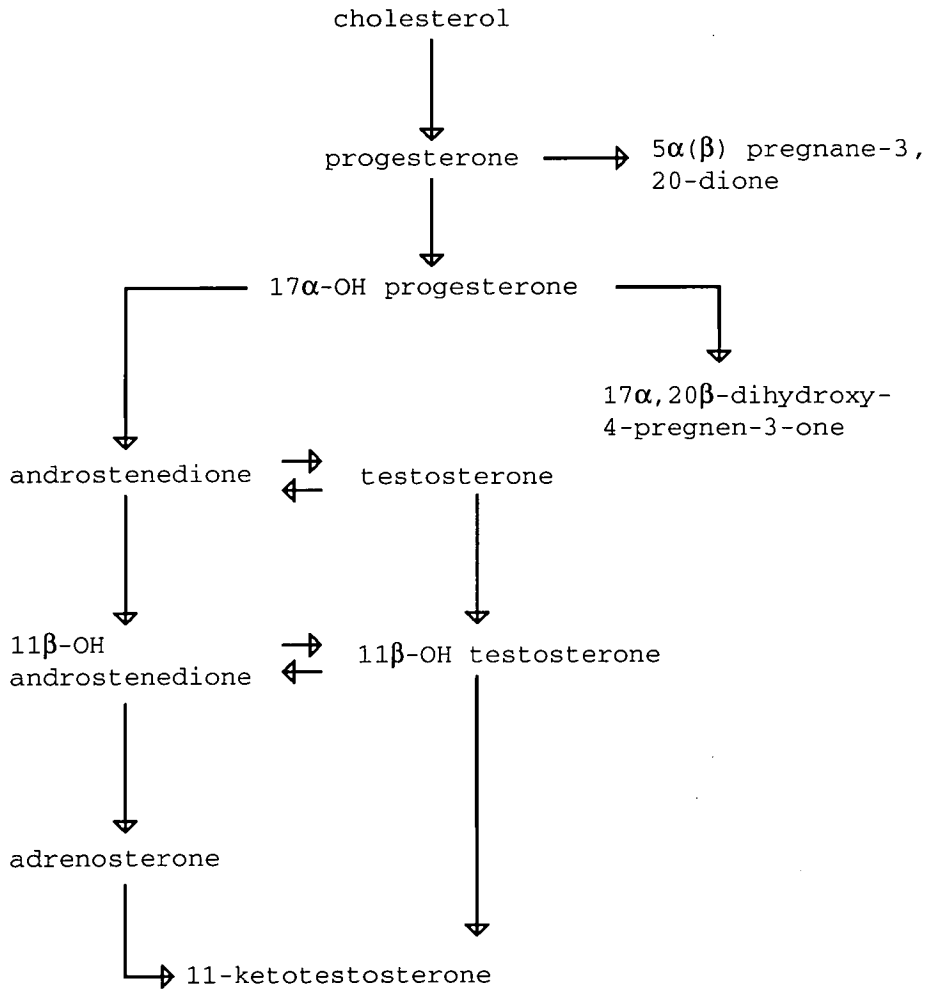

Figure 4. Pathway of steroid synthesis (from Matty 1985).

The progestins, principally 17α, 20β-dihydroxy-4-pregnen-3-one (17α-20βP) have been shown to play a role in the synchronisation of behavioural and maturational events at the time of spawning (Zhao and Wright 1988; Liley and Rouger 1990). Plasma levels of 17α-20βP were found to be low during spermatogenesis and dramatically elevated at the onset of spermiation in several salmonids and the goldfish (Ueda *et al.* 1991, 1983; Nagahama 1987; Zhao and Wright 1988). Further, treatment with 17α-20βP induced precocious spermiation in eels (Yamauchi 1990), amago salmon, *Oncorhynchus rhodurus*, and goldfish (Ueda *et al.* 1985). Miura *et al.* (1991) found that 17α-20βP was responsible for increasing sperm activity by raising the pH of the seminal plasma.

17α-20βP also plays an important role in ovulation in female fish (Scott *et al.* 1982; Liley and Rouger 1990) and has been used in induced breeding of common carp (Jalabert *et al.* 1977). Richter *et al.* (1985, 1987) used injections of 17α-Hydroxyprogesterone (17αP) to induce breeding in African catfish, *Clarias gariepinus*. 17αP is a precursor of 17α-20βP and is cheaper and more readily available.

As with other aspects of teleost steroidogenesis the actual production sites of 17α-20βP remain unclear, although it has been determined that 17α-20βP production depends on the interaction between the sperm and the soma of the testis (Nagahama 1987).

1.3.8 Spermatogenesis

There are two types of spermatogenesis in vertebrates, one in fish and amphibians and another in reptiles, birds and mammals. In fish and amphibians the development of

spermatogenic clones proceeds within cysts which are absent in other vertebrates (Roosen-Runge 1977).

It appears that the nervous and endocrine controls superimposed on spermatogenesis in vertebrates are typically more complex than in other animals. The complexity of spermatogenesis has been more completely studied in mammals, particularly the rat (Roosen-Runge 1977) while the morphology and physiology of the process in fish remains relatively poorly understood (Ueda *et al.* 1983, 1985).

Until recently, spermatogenesis has been interpreted as a linear progression of cell development ultimately leading to the production of a spermatozoon (Roosen-Runge 1962). In the old view of spermatogenesis the sperm cells develop independently of each other and their environment (Hickman *et al.* 1979). According to Roosen-Runge (1977) the new view of spermatogenesis recognises that male germ cells "never develop alone". Their development is intimately linked to each other and their environment (the body of the animal). In most cases the cells are linked to some kind of support cell so that the immediate environment not only contains and conditions the process of spermatogenesis but is inseparable from it.

Roosen-Runge (1977) defines four stages of development for male teleost gametes, Table 1.

The first stage of development is the production of primordial germ cells. These cells usually become recognisable in the embryo and are extra gonadal. The cells of both sexes appear alike in morphology and behaviour and are capable of mitotic division and often amoeboid movement that may assist them in reaching their gonadal location (Zohar 1989).

Table 1. Stages in the development of male gametes (After Roosen Runge 1977).

Term	Name of Cells	Characteristics
1. Primordial development	Primordial germ cells	Not sex determined, extra gonadal, mitotic.
2. Pre-spermatogenesis	Gonocytes, 'stem cells'	Sex determined, gonadal, mitotic, some cellular differentiation.
3. Spermatogenesis		
(a) Spermatocyto-genesis, mitotic, spermatogonial stage.	Spermatogonia	Mitotic clone formation, cellular differentiation prominent.
(b) Spermatocytic or meiotic stage	Spermatocytes I and II	Post mitotic. Two meiotic maturation divisions and often cell growth.
(c) Spermatid stage or Spermiogenesis	Spermatids	Post meiotic, haploid, no multiplication, highly specific differentiation into spermatozoa.
4. Spermiation	Spermatozoa	Release from the germinal tissue. Maturation of sperm cells may still continue after release.

The second stage of development occurs within the gonad and is often called pre-spermatogenesis. During this phase the germ cells or gonocytes, undergo periodic mitotic activity accompanied by subtle morphological changes and are interspersed with periods of degeneration (Hilscher *et al.* 1974). This is the stage in which direct interaction

between the cells and the soma begins and it is also this stage which predetermines the number of spermatozoa to be produced.

During the first two stages of development the germ cells advance to a level of differentiation that primes them for entrance into spermatogonial mitosis, meiosis and subsequent stages of differentiation.

The third stage of development, spermatogenesis, can be further subdivided into three phases, defined according to structural characteristics of the germ cells and their nuclei (Zohar 1989).

The first phase of spermatogenesis is spermatocytogenesis, where the germ cells, now called spermatogonia, divide and multiply. The multiplication of spermatogonia is independent of GtH levels in the blood plasma (Chang 1987). The number of mitotic divisions varies slightly between species and usually numbers between three to five. During successive mitotic divisions the cells differentiate and the nucleus becomes preconditioned for meiosis. The final mitotic division produces primary spermatocytes.

The maturation stage of spermatogenesis involves two meiotic divisions. The primary spermatocytes undergo the first meiotic division producing secondary spermatocytes. The second division follows rapidly from the first, producing spermatid cells with the haploid number of chromosomes.

After meiosis, the last stage of spermatogenesis, spermiogenesis, begins. Immediately after the second meiotic division the spermatid appears as a relatively small undifferentiated cell. Developing spermatids undergo

major morphological changes; the organisation of the head, neck piece, mid piece and flagellum. Between species, spermatids may vary in the shape of the nucleus, length and number of the flagella (Hickman *et al.* 1979; Stoss 1983).

Teleosts possess 'primitive' spermatozoa, which are small, flagellated cells with a spheroid or conical nucleus; few, usually four, mitochondria surrounding the subnuclear region of the flagellum and no acrosome (Stoss 1983). The lack of an acrosome, the 'spring-loaded' mechanism that aids penetration of the egg, may be related to the presence of a micropyle in teleost eggs (Roosen-Runge 1977). As the primitive spermatozoon develops, the cellular cytoplasm may be 'sloughed' to reduce cell volume.

The primitive type of spermatozoa appears to have originated early in evolution and is found in all animals that fertilise externally in water.

The point that marks the end of spermatogenesis is very ill-defined. For mammals the term spermatozoon is used to describe the sperm cell once it has been released from the seminiferous epithelium, although newly released sperm may continue to mature morphologically and physiologically. In other species the sperm may be released soon after spermatogonial divisions and continue their maturation outside the epithelium (Roosen-Runge 1977). For the purposes of the present discussion the end of spermatogenesis will be identified as the time of release of spermatids from the epithelium.

The fourth stage of gamete development, spermiation, is defined as the release of spermatozoa into the sperm ducts accompanied by thinning of the semen (hydration) and leads

to a state of functional maturity and expression of fluid sperm through the genital pore (Yamazaki and Donaldson 1969; Ueda *et al.* 1985). The dilution of the sperm is hormone dependent and hydration probably increases intra lobular pressure, forcing the sperm towards the vas deferens where they can be stored (Billard *et al.* 1982a).

Nervous or neurohormonal mechanisms are involved in the natural process of sperm release which is more specifically the expulsion of sperm through the genital papilla (Demski and Hornby 1982). These mechanisms are logically involved in gamete release to facilitate synchronisation of spawning between males and females.

In most fish species the spermatozoa are immotile in the testis and in many cases, also in the seminal plasma (Stoss 1983). Activity is not induced until the factors that suppress motility are neutralised by environmental conditions during spawning. Teleost gametes generally have a very short survival time in fresh water. Atlantic salmon have a sperm motility time of less than 2 minutes and for trout it may be as brief as 30 seconds (Terner 1986). Salt-water fish generally have sperm with a longer motility time, being 2 minutes in European sea bass and gilt head sea bream (Billard 1986) and up to several days in the case of the Pacific herring *Clupea harengus pallasi* (Stoss 1983). Barramundi sperm have a survival time of approximately 8 minutes (Leung 1987). The generally short survival time of fish gametes necessitates that their release is simultaneous (Billard *et al.* 1982a).

1.3.9 Aspects of Spermatogenesis Peculiar to Fish

Spermatogenesis is a very diversified process in teleost fish. This diversity is evident in the dynamics of spermatogenesis, (which may be continuous, quasi continuous or very seasonal); spermatogenic production and spermiation yield (Billard 1986).

In fish, spermatogenesis has only been investigated in the teleosts and even there the coverage has not been broad. There is little information available on the endocrine control of spermatogenesis in fish with continual breeding capabilities (Billard *et al.* 1982a). Most of the information has been obtained from seasonal breeders such as common carp and salmonids. Common carp differ from salmonids in that they produce sperm all year round (Billard *et al.* 1982a). The endocrine pattern of spermatogenesis in common carp is similar to salmonids but seems different with regard to spermiation (Billard *et al.* 1982a). The carp require fluctuating temperatures for spermiation while salmonids require the temperature to be both cool (less than 12° C) and constant.

The initiation of a new spermatogenic cycle only seems possible when the sperm have been eliminated from the testis either by expulsion or resorption (Billard *et al.* 1982a). The spermiation process has two stages, initiation and amplification, which involve different levels of GtH and steroid balance. 11-KT is the major plasma steroid, but its superior role is yet to be proven (Billard *et al.* 1982a), especially because it is not found in all fish (Matty 1985).

1.3.10 Quantification of Sperm Production

Few quantitative data are available regarding the dynamics of the spermatogenic process in teleosts. The number of spermatogonial generations has been estimated to generally be between five and seven (Billard 1969) and may be as high as 14 (Billard 1986). With each successive generation the cysts cells increase in diameter as the number of clones they contain increases.

Death is frequent among germ cells, particularly during early spermatogonial stages. The result is an output of spermatozoa of only 65% of the theoretical yield. The productivity of the guppy, *Poecilia reticulata,* is in the order of 150 x 10^6 spermatozoa per gram of testis per day (Billard 1969) and common carp are estimated to produce 1.9 x 10^{12} spermatozoa per kilogram body weight per year (Saad and Billard 1987).

There is very little information regarding the duration of spermatogenesis and its stages. In the guppy, Billard (1969) found that the development from early leptotene stage of meiosis to spermatozoon is 14.5 days at 25°C. The spermatogonial stage lasted 21.75 days and the whole of spermatogenesis, 36 days at 25°C.

Teleosts testes may show extreme seasonal variations in size and histological appearance. For instance, in the perch, the average weight of the testes in the mature male varies from 0.12 to 5.88% of body weight (Billard *et al.* 1982a). Barramundi testes also show marked seasonal variation, changing from thin thread-like organs in the non-breeding season to thick, cream coloured organs during the breeding season (Davis 1982).

1.3.11 Spermiation

Spermiation in goldfish seems to be androgen dependent but in salmonids it is possible that GtH initiates spermiation along with other factors although, what the other factors are is not clear (Billard *et al.* 1982a).

The number of spermatozoa that can be harvested from the common carp is low compared to the estimates of the number produced during spermatogenesis and remaining in the testis (Billard *et al.* 1983). The poor yield of spermiation is intriguing and has practical implications for fish farmers (Billard *et al.* 1982a). In induced reproduction and artificial insemination it is desirable to increase the number of available spermatozoa by stimulating spermiation. Carp pituitary extracts were originally used for this purpose (Clemens and Grant 1964, 1965) but more recently, releasing hormones, sex steroids and mammalian gonadotrophins have been applied (Billard *et al.* 1987; Shehadeh *et al.* 1973; Pocsidio 1978).

The range of hormones that can be injected or implanted to induce spermatogenesis and/ or spermiation is limited compared to the female situation (Donaldson and Hunter 1983). Even so, the culturist still has the opportunity of intervening with hypothalamic, pituitary or gonadal hormones. As the endocrine control of the testis is relatively simple compared to the ovary, it is generally perceived to be easier to induce an immature male teleost to undergo spermatogenesis and spermiation than it is to induce an immature female to undergo vitellogenesis, maturation and ovulation (Donaldson and Hunter 1983).
In a number of cultured species the male spermiates spontaneously, eg rainbow trout, *Oncorhynchus mykiss* (Billard *et al.* 1984) and golden snapper *Lutjanus johni* (observed at the Darwin Aquaculture Centre). In others the

captive male fails to spermiate or produces only a small volume of viscous milt. In most cases involving mature males the volume and quality of the milt can be markedly improved by a single injection of an appropriate hormone. If the male is immature and fails to produce milt, a series of injections or one or more hormone pellet implantations may stimulate spermatogenesis and subsequent spermiation (Clemens and Reed 1967; Garcia 1990).

1.4 HORMONE ENHANCED SPERM PRODUCTION

1.4.1 The Releasing Hormones

Since the early 1970's, releasing hormones and their superactive analogues have become increasingly popular in the induction of fish reproduction (Nacario 1987).

Luteinizing hormone releasing hormone (LHRH) was first isolated from mammalian hypothalami in 1971 (Donaldson and Hunter 1983). LHRH is a decapeptide with the following amino acid sequence.

pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

1 2 3 4 5 6 7 8 9 10

The hormone was found to lack species specificity and Breton and Weil (1973) were able to induce GtH release by LHRH injection in the common carp. Synthetic LHRH was also used successfully to induce spawning in goldfish (Lam *et al.* 1975).

Soon after the discovery and artificial synthesis of LHRH, several analogues (LHRHa) of the hormone were synthesised (Fujino *et al.* 1974). These analogues proved to be up to 50 times more effective in inducing gonadotrophin release

(Peter 1980). The super activity of the analogues is possibly due to a slower rate of digestion by enzymes (Donaldson and Hunter 1983; Zohar 1986) and an increased binding affinity with GtH producing cells (Zohar 1986).

Direct comparison of the early studies on LHRH and LHRHa are often not possible because the treatments varied considerably in the amount of hormone injected, the purity of hormone, the analogue used, the site of the injection (Donaldson and Hunter 1983; Weil and Crim 1983), the method of administration and the solvent or carrier (Takashima *et al.* 1984). Nevertheless it was quite clear that LHRHa treatment had a positive effect on the induction of spawning and gamete release in female fish (Lee *et al.* 1987). These early studies largely ignored the effect of LHRHa on male fish and the little information that is available is from its effect on male amphibians (Licht 1974; Billard *et al.* 1975).

LHRH analogues have now become one of the main tools used in the artificial propagation of fish (Lam *et al.* 1975; Lee *et al.* 1985; Lee *et al.* 1986b; Nacario 1987; Garcia 1990). Two analogues used by Weil and Crim (1983), Des-Gly10-[D-Ala^6]-LHRH ethylamide and 6-D2-Napthylalanine-LHRH were both effective at stimulating sperm in salmon, *Salmo salar*, but the results were not quantified. Single injections of LHRHa have significantly increased the volume of sperm produced by male rabbitfish, *Siganus guttatus* (Garcia 1991); sturgeon, *Acipenser* spp. (Goncharov *et al.* 1991) and pacu, *Piaractus mesopotamicus*, (Rosa *et al.* 1988). LHRHa used in combination with the androgen 17α-methyltestosterone (17-MT) has a significant effect on maturation in milkfish (Lee *et al.* 1986a; Lee *et al.* 1986e).

Much less attention has been given to induced spawning using natural fish releasing hormone (GnRH) and its analogues (GnRHa), although they have been shown to be suitable (Donaldson *et al.* 1983). Zohar (1986) suggests that more attention should be paid to fish releasing hormones because of their potentially higher activity levels when compared to mammalian releasing hormones. A species specific response has been demonstrated for at least one teleost releasing hormone. Carolsfeld *et al.* (1988) induced spawning in the Pacific herring with a mammalian analogue but not with salmon GnRHa. Wherever possible it is advisable to use a range of LHRH analogues when conducting preliminary investigations on new culture species.

1.4.2 Application Methods of LHRHa

Commonly, the hormone is administered through an injection that is either intra muscular or intra peritoneal. The former route is favoured because intra peritoneal injections pose the risk of damage to the internal organs or loss of hormone into the gut (Harvey and Hoar 1979). The type of vehicle used to carry LHRHa also varies: sesame oil (Takashima *et al.* 1984), propylene glycol (Weil and Crim 1983) and sterile saline (Billard *et al.* 1983; Chang and Peter 1983; Chang *et al.* 1991) have all been used successfully in experimental studies.

LHRHa treatment often requires repeat injections and this may cause stress to the fish through frequent handling. Severe stress has been demonstrated to be capable of overriding the effect of hormone treatment, preventing successful spawning (Billard *et al.* 1981; Lacanilao *et al.* 1985).

Several researchers have developed methods of chronic hormone treatment through slow release pellets (Dziuk and Cook 1966; Lee *et al.* 1985; Lee *et al.* 1986c; Sherwood *et al.* 1988). The pellets are usually manufactured using either a matrix of cholesterol (Lee *et al.* 1985) or silicone (Weil and Crim 1983; Billard *et al.* 1987) and are implanted into either the back muscle or peritoneal cavity of the fish. The pellets are capable of prolonged hormone release (Dziuk and Cook 1966; Tamaru *et al.* 1990), do not cause foreign body reactions (Dziuk and Cook 1966) and have proven to be very effective in minimising stress to the fish and inducing spawning (Crim *et al.* 1988; Sherwood *et al.* 1988).

There is one report of oral administration of LHRHa. Spotted seatrout, *Cynoscion nebulosus*, were induced to spawn by adding LHRHa to their diet (Thomas and Boyd 1989). The hormone had to be administered in high doses which is an expensive practice.

1.4.3 Dose Rates

A summary of the dose rates used for the induction of spermatogenesis and spermiation, and the responses recorded, is tabulated in Appendix 1.

1.4.4 Disadvantages of Releasing Hormones

Peter (1980) believes that LHRHa administered at high dose rates can cause suppression although he does not mention what constitutes a high dose. This was refuted by Carolsfeld *et al.* (1988) who found no inhibition of response to high doses of LHRHa (800-1000 $\mu g\ kg^{-1}$) in the

Pacific herring. Billard and Marcel (1980) argued that LHRH and its analogues were not very promising as a replacement for pituitary preparations. They saw problems such as: time of treatment, species specificity, inhibitory effect of LHRHa and mode of administration. Billard's statement is somewhat surprising given that he has used LHRHa successfully on numerous occasions.

1.4.5 The Gonadotrophins

Gonadotrophins are hormonal glycoproteins and those used for fish reproduction can be divided into two categories depending on whether they are piscine or mammalian in origin (Donaldson and Hunter 1983).

1.4.6 Fish Gonadotrophins

There is now clear evidence for multiple gonadotrophins in teleosts (Matty 1985; Nagahama 1987), although there is still much confusion and controversy surrounding their actual function (Loir and Billard 1990). The first gonadotrophin is carbohydrate poor and its role in chum salmon, *Oncorhynchus keta*, is to promote the uptake of vitellogen into the oocyte (Nagahama 1987). The other teleost GtH is carbohydrate rich, has a molecular weight of between 25,000 and 40,000 (Nagahama 1987) and consists of two sub-units similar to other vertebrate GtH (Donaldson 1973). The term gonadotrophin is usually reserved for the carbohydrate rich molecule.

There is now evidence for two carbohydrate rich gonadotrophins. In chum salmon, GtH 1 is considered to be responsible for controlling early reproductive events, including spermatogenesis and GtH 2 controls spermiation

and ovulation (Kawauchi *et al.* 1989; Hew and Xiong 1991). Loir and Billard (1990) dispute the role of GtH 1 in male reproduction, believing that there is no clear evidence at this stage.

Fish gonadotrophins are usually more potent than mammalian GtH in maintenance or regeneration of spermatogenesis in hypophysectomised fish although response to GtH is species specific in some teleost families (Billard *et al.* 1982a).

Fish gonadotrophins used for breeding induction can be subdivided according to their level of purity (Hunter and Donaldson 1983). They range from aqueous extracts of fresh, or acetone dried, whole pituitaries through to partially purified extracts prepared by either gel filtration or affinity chromatography. Purification techniques result in hormone that is more active per unit weight but with less total activity than the original whole pituitary. Acetone drying eliminates the need for donor fish to be available on site but are expensive to buy (Lee *et al.* 1988). Fish pituitary homogenates are also expensive to buy and not always readily available (Billard *et al.* 1987).

1.4.7 Effect of GtH on Spermatogenesis and Spermiation

Generally GtH preparations are only used to encourage gamete release, there are very few reports of long term administration of pituitary extracts being effective in promoting gonadal development. Clemens and Reed (1967) induced both spermatogenesis and spermiation in goldfish after treatment every third day with partially purified carp pituitary.

There are also very few reports on the actual response of male fish to hypophysation. Many operators simply give the male fish a precautionary injection at the time the female receives hers (Matty 1985). This apparently ensures satisfactory spermiation at the time of ovulation. Late in the spawning season some males may need two injections to elicit a spermiation response (Bhowmick *et al.* 1977).

In the common carp, injections of an aqueous extract of acetone dried carp pituitaries gave a dose dependent responses at doses up to 22 mg kg^{-1} and induced spermiation within 24 hours (Clemens and Grant 1965; Saad and Billard 1987). Yamazaki and Donaldson (1969) induced a dose related spermiation response in hypophysectomised goldfish with a single injection in the range 0.1-3.0 mg kg^{-1}. Doses up to 50 mg kg^{-1} have been use for hypophysectomised goldfish (Billard 1977). The northern pike, *Esox lucius* had its milt production increased by 3-6 times in volume, when injected with 0.5 - 3.0 mg crude pituitary extract kg^{-1}; up to 11 times with partially purified salmon gonadotrophin and 7 times with fresh pike pituitaries (Billard and Marcel 1980). The peak response was at two days and lasted for 10-14 days. Carp pituitary extract has been demonstrated to stimulate spermiation in barramundi (Schipp *et al.* 1989).

Temperature can significantly affect the response of the testis to pituitary extracts, the higher the temperature the higher the response (Saad and Billard 1987). In the rainbow trout Clemens and Grant (1965) induced a seminal thinning response at 15°C. Kaya (1973) failed to induce a spermiation response in the green sunfish at 10°C but was able to induce spermiation after two weeks at 21°C.

There is one report of the stimulation of milt production by oral dosing of GtH to goldfish (Suzuki *et al.* 1990).

Oral administration of hormones is very rarely cost effective because of the large quantities of hormones needed.

A summary of the dose rates used and the effect on sperm production is presented in Appendix 1.

1.4.8 Method of Administration

The normal methodology involves two intramuscular injections of pituitary extract (Zohar 1989). The first injection, the priming dose, is usually a fraction (< 50%) of the second, resolving dose. Procedural variations exist between operators. Variations include: choice of donor species, homogenisation technique, ambient temperature, addition of other compounds (eg. addition of glycerine to slow dispersion rate), location of injection, number of pituitary equivalents injected, time of injection, interval between injections, determination of suitability of recipient and use of anaesthetic. This great range of variables has meant that not all the variables have been fully tested for one single species (Shehadeh 1972).

1.4.9 Standardisation of Hypophysation Doses

Most studies on fish gonadotrophins have used crude extracts or whole pituitary preparations (Pien and Liao 1975; Rothbard and Rothbard 1982). These preparations are very rarely assayed for gonadotrophic activity which has made it very difficult to quantify and compare the results of different studies (Shehadeh 1972). The activity of gonadotrophic preparations may vary according to the species (Yamamoto 1973); sex (Breton *et al.* 1978); size and reproductive condition of the donor fish. When

injected into goldfish, carp pituitary extract is 36 times more active in stimulating adenyl cyclase activity than GtH from *Oncorhynchus* sp.(Fontaine *et al.* 1972). Activity also varies according to the storage conditions of the hormone (Donaldson *et al.* 1978). Lam (1982) suggested that government or private enterprise should set up banks of assayed pituitaries. To date this suggestion has not been taken up although Argent Chemicals (US) and Syndel Laboratories (Canada) now offer non-assayed, partially purified carp and salmon pituitary products.

1.4.10 Advantages of Hypophysation

It is still difficult to standardise doses for fish gonadotrophin preparations and the whole process remains imprecise. Despite this limitation the methods of hypophysation as developed by Houssay (as cited in Lam 1982) and Von Ihering (1937) are still widely practiced today (Hunter and Donaldson 1983). The technique still offers several advantages. It is relatively simple and if donor fish are available it requires little in the way of capital equipment and funds. It does not require refrigerated storage and the dose rate can be easily calculated from the weight of the donor and recipient fish.

Although optimal responses have been obtained with homoplastic pituitaries (pituitaries from the same species), any quantitative differences that exist due to phylogenetic specificity in fish are, by and large, of little practical significance in fish culture procedures. The general practice when using donors and recipients that are phylogenetically distant is to increase the dose (Harvey and Hoar 1979). The problems of sex-related differences can also be overcome by mixing the pituitaries

of both sexes and increasing doses to above the threshold limits (Shehadeh 1972).

Combining LHRHa and carp pituitary homogenates to induce spawning in grey mullet is the most cost effective option. It reduces the amount of CPH used and improves the reliability of LHRHa (Lee *et al.* 1988).

1.4.11 Disadvantages of Hypophysation

If donors are not available then the hormone can be expensive to purchase, plus the size, and reproductive condition of the donors is often unknown. Argent Chemicals advise there are 220 pituitaries per gram of their product but do not give details of the sex, size or reproductive condition of the donor fish (Argent Chemical Co. pers. comm.). Another disadvantage is that the recipient fish may develop an immune response to the hormone if it is used repeatedly (Hunter and Donaldson 1983) or a lesion may occur at the site of injection (Lee *et al.* 1988). Comparison of treatments is difficult because often the fish are in different reproductive conditions to begin with and these are rarely quantified (Lam 1982).

Injection of fresh pituitary material also offers the possibility of the transfer of disease (Donaldson and Hunter 1983). According to the Australian Quarantine and Inspection Service, pituitary gland extracts offer a potential route for the entry of viral fish diseases into the country. Their answer to this threat was to ban the import of pituitary extracts into Australia, unless the extracts had been irradiated. Radiation treatment makes it even more difficult and expensive to procure the hormone.

1.4.12 Mammalian Gonadotrophins

Mammalian gonadotrophins are either of pituitary or placental origin. Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) are derived from the pituitary and Human Chorionic Gonadotrophin (HCG) from the placenta. LH and FSH have not been found to be effective in stimulating reproduction in male fish (Billard *et al.* 1982a). HCG has stimulated spermiation in several teleosts, *viz:* goldfish (Billard 1976); rainbow trout (Clemens and Grant 1965); milkfish (Juario *et al.* 1981) and the European eel, *Anguilla anguilla*, (Bieniarz and Epler 1977; Amin 1986; Khan *et al.* 1987). HCG is only potent in a limited number of species and is not effective in stimulating sperm production in the common carp (Courtois *et al.* 1986; Billard *et al.* 1987) or barramundi (Schipp *et al.* 1989).

Another problem with HCG is that it may cause an immune response (Lam 1982), although Van der Kraak *et al.* (1989) found a lack of antigenicity in silver carp and goldfish and suggested that the problem may not be as severe as implied in the literature.

1.4.13 The Feedback Pathway

Gonadotrophin secretion is regulated by a negative feedback pathway (see Figure 1). Circulating gonadal steroids interact with steroid binding sites in the hypothalamus and pituitary, regulating the secretion of gonadotrophin. For example, a rise in steroid levels causes a decrease in gonadotrophin secretion (Billard *et al.* 1987; Harvey and Hoar 1979).

There is also evidence for a gonadotrophin release inhibitory factor, GRIF (Peter 1983). It is likely that dopamine, originating from the anteroventral preoptic region of the brain acts as the GRIF in fish (Peter 1983). Some researchers have examined ways of blocking the production of GRIF as a means of increasing the secretion of gonadotrophin from the pituitary gland (Chang and Peter 1983; Chang *et al.* 1983; Billard *et al.* 1987).

The gonadotrophin release inhibitory factor (GRIF) (Peter 1982) can itself be inhibited through the application of dopamine antagonists such as pimozide (Billard *et al.* 1984, 1987), eglonil and metoclopramide (Glubokov *et al.* 1991). Dopamine antagonists have been shown to elevate GtH in goldfish (Chang and Peter 1983; Billard *et al.* 1987) and to potentiate the effect of LHRHa in goldfish (Chang *et al.* 1983); common carp, (Billard *et al.* 1987); bream, *Abramis brama* (Glubokov *et al.* 1991); rainbow trout and brown trout, *Salmo trutta*, (Billard *et al.* 1984). Dopamine antagonists such as pimozide must be used with caution because the effect of continued high doses is not known and it is advisable to use higher doses of LHRHa and less pimozide (Billard *et al.* 1987).

Recently an analogue of salmon GnRH has been combined with the dopamine antagonist domperidone and sold commercially as Ovaprim® by Syndel Laboratories in Canada. The results achieved in several carp species have been far superior to hypophysation techniques (Harker 1992). The dose rate is species dependent and in females ranges from 0.25-0.7 ml kg^{-1} and in males from 0.1-0.2 ml kg^{-1}. Only a single injection is required.

Examples of studies using dopamine antagonists combined with LHRHa are shown in Appendix 1.

1.4.14 Androgens

Application of sex steroids may have a positive effect on GtH production in immature fish (Crim and Evans 1979). This suggests that they may be part of the mechanism for the onset of sexual maturity (Peter 1982).

Steroids have the benefit of commercial synthesis, usually have an indefinite storage life and are uniform in quality (Shehadeh *et al.* 1973). Steroids were first demonstrated to induce spermiation by Yamazaki and Donaldson (1969). A single intra peritoneal injection of 17-MT induced spermiation in hypophysectomised goldfish. The response was dose dependent, with a minimum effective dose of 50 mg kg^{-1}. Billard (1976) found 17-MT to be more effective than testosterone propionate. 17-MT has also induced spermiation in the grey mullet (Shehadeh *et al.* 1973). Another steroid, 11-ketotestosterone (11-KT) has induced spermiation in the sockeye salmon, *Oncorhynchus nerka* (Idler *et al.* 1961). In the rainbow trout, spermiation was induced with testosterone but not 11-KT. A commercial preparation of steroid (testosterone propionate, testosterone phenylpropionate, testosterone isocaproate and testosterone decanoate) induced spermiation for up to seven days in mature milkfish (Juario *et al.* 1981) but failed to induce spermatogenesis in fish in regressed breeding condition (Lacanilao *et al.* 1985).

It has been widely reported that administration of 17-MT on a regular basis can have a positive effect on spermatogenesis. This has been demonstrated for grey mullet (Shehadeh *et al.* 1973; Weber and Lee 1985); milkfish (Lee *et al.* 1986d) and channel catfish, *Ictalurus punctatus* (Simone 1990). Conversely, it has been reported that administration of 17-MT can have an inhibiting effect

on gametogenesis in rainbow trout (Billard and Richard 1982) and the three-spined stickleback (Borg 1981).

1.4.15 Methods of Administration

Similar to LHRHa, a variety of methods have been used to administer the steroids: injection, either single or multiple (Shehadeh *et al.* 1973; Saad and Billard 1987) and pelleted, either cholesterol (Kuo 1991) or silicone (Lee *et al.* 1986c, 1986e).

Another method and one that is commonly used with steroids, is oral administration, where the hormone is incorporated in the feed, (Lee *et al.* 1986d; Weber and Lee 1985). Oral administration of 17-MT to grey mullet has induced spermatogenesis and maintained the fish in breeding condition for up to 55 weeks (Weber and Lee 1985; Lee and Weber 1986; Fang and Lee 1989). Androgenic steroids have also been administered by first dissolving them in ethanol and then adding them to the water in the fish tank (Borg 1981).

1.4.16 Other Steroids

In goldfish and amago salmon, 17α-20βP is the most effective steroid for the induction of spermiation (Ueda *et al.* 1983). Japanese eels, *Anguilla japonica* have also been stimulated to produce sperm by 17α-20βP injections (Yamauchi 1990).

Injections of progesterone proved to be 10 times more effective than 17-MT in inducing sperm release in the goldfish (Billard 1976). Progesterone has not been found in the plasma of male teleosts, therefore conversion of

progesterone to 17α-20βP is the likely explanation of its effectiveness in the goldfish (Loir and Billard 1990). Progesterone is not as effective as pituitary extracts for stimulating spermiation in the male pike (Billard and Marcel 1980).

1.4.17 LHRHa and Steroid (Combined)

Long term administration of 17-MT either by silicone implant or dietary addition had little effect in the male spotted scat, *Scatophagus argus* (Barry *et al.* 1991). However when this treatment was followed by an implant of LHRHa, spermiation was significantly increased.

Garcia (1990) combined LHRHa and 17-MT in cholesterol pellets to advance maturation and spawning in barramundi.

1.5 BARRAMUNDI

1.5.1 Introduction

Barramundi is an important coastal, estuarine and freshwater fish in the Indo-Pacific region (Davis 1987).

It supports extensive commercial and recreational fisheries in Australia and Papua New Guinea and provides the basis of an expanding aquaculture industry in Asia, where it is also subject to wildstock exploitation (Pearson 1987; Grey 1987).

The species is distributed from the Arabian Gulf to China and Taiwan, and to Papua New Guinea and northern Australia. In Australia, barramundi are found as far south

as the Noosa River (26°30' S) on the east coast and the Ashburton River (22°30' S) on the west coast (Grant 1982; Morrissy 1985 (both as cited in Grey 1987)).

As to be expected with a species that occupies a wide geographic range, *Lates calcarifer* comes under a diverse group of common names. Although 'barramundi' is the accepted common name in Australia (derived from the aboriginal word 'burramundi' meaning 'large scales'), it has been variously called giant perch, cock-up and barramundi. It is also known as 'anama' in Papua New Guinea, 'sea bass' in Thailand and the Philippines and 'Bhekti' in India (Grey 1987).

1.5.2 Taxonomy

The taxanomic classification of barramundi is shown in Table 2.

Table 2. Taxonomic classification of barramundi (Source Grey 1987).

Phylum	Chordata
Subphylum	Vertebrata
Class	Pisces
Subclass	Teleostomi
Order	Perciformes
Family	Centropomidae
Genus	*Lates*
Species	*calcarifer* (Bloch)

1.5.3 Biology

Studies on Barramundi in northern Australia and Papua New Guinea have identified that it has a complex life history (Moore 1979; Davis 1982).

1.5.4 Breeding Season

The starting and completion times for the barramundi spawning season varies from river to river and year to year (Garrett *et al.* 1987). The start of the breeding season for barramundi in the Darwin area coincides with the return to large spring tides and increasing water temperatures in mid to late August (Grey 1987) and the breeding season is usually completed by March. Fish kept under artificial conditions have spawned in May (Schipp and Kuo, unpublished data) and fish suitable for breeding induction have been found as late as June (Schipp pers. obs.). In northern Queensland, barramundi usually commence breeding in October and spawning is completed by March (Garrett *et al.* 1987).

Populations of Barramundi in southern Queensland and the central coast of Western Australia have breeding seasons of shorter duration than those of the Northern Territory and North Queensland (Garrett 1987).

1.5.5 Sexual Maturity

Barramundi mature sexually at 2-3 years of age (James and Marichamy 1987). In the wild most of the fish mature initially as males and participate in one or more spawning seasons before undergoing a sexual inversion (protandry), becoming functional females by the next breeding season

(Moore 1979; Davis 1982). Captive barramundi have exhibited a higher percentage of primary females (up to 100%) than that found in the wild (Stuart Fielder, Sea Harvest, pers. comm).

Generally fish < 80 cm length are males and those > 100 cm are females (R. Griffin, Northern Territory Department of Primary Industry and Fisheries, pers. comm). This is not always the case, as sexually precocious (fish that mature and change sex at a smaller than usual size) populations of barramundi are known to occur in the NT and Queensland (Davis 1982).

1.5.6 Spawning Grounds

Although barramundi are euryhaline, barramundi eggs and larvae will only survive in brackish or salt water (salinities between 15 and 40 ppt) and for this reason all breeding takes place in river mouths and bays near areas of suitable nursery habitat (Garrett *et al.* 1987). Areas such as mangrove swamps and low-lying land that becomes flooded during spring tides and monsoonal rains provide ideal habitats for juvenile barramundi.

Australian barramundi, unlike barramundi in Papua New Guinea do not undergo a long range spawning and post spawning migration. Instead it is believed that mature males and females congregate locally within a river system (R. Griffin, pers. comm.). Other fish may arrive at the spawning grounds later in the breeding season aided by monsoonal flooding of upstream billabongs (Grey 1987).

1.5.7 Spawning Behaviour

Spawning takes place at night around the time of the slack tide and appears to be related to the lunar cycle (Garrett *et al.* 1987). The nights following full and new moons are the periods of greatest spawning activity.

Each female may release many millions of eggs (the highest reported number is 40 million, although 3-6 million is more common (Davis 1987) as she swims in tandem with one or more males that release a cloud of sperm to fertilise the eggs in the water. The actual release of the eggs lasts only a few seconds and the males may immediately 'pirouette' around the females tail or swim closely alongside, releasing their sperm (observed at the Darwin Aquaculture Centre.). Most wild females are believed to spawn only once in a season (Garrett 1987), however hatchery stock have spawned up to five times in a single season (Kuo 1991).

1.5.8 Reproduction and Juvenile Development

The eggs are positively buoyant and are capable of being fertilised for only the first few minutes before they 'water harden'. Once fertilised the eggs will hatch between 12 and 18 hours later, depending on the water temperature (Grey 1987; Russell *et al.* 1987; Garrett and Rasmussen 1987).

Barramundi larvae live on their yolk sac for the first 40-44 hours and then feed on microscopic plankton. They continue to feed on plankton for a number of weeks, moving on to larger prey as they grow rapidly. Barramundi are also cannibalistic and many of the juveniles are eaten by

other barramundi not much larger than themselves (Trendall 1990).

The surviving juveniles may remain in their nursery habitat for most of the wet season. As water levels in the nursery swamps begin to fall, most of the young fish will move upstream into fresh water and stay there for two to three years until they mature (Grey 1987).

1.5.9 Environmental Requirements

Barramundi eggs and larvae are known to have a narrower range of tolerance of temperature and salinity than juveniles and adults (Grey 1987). The optimum temperature and salinity ranges for larvae are 26-30° C and 28-32 ppt (Russell *et al.* 1987).

Barramundi fingerlings (fish larger than 20 mm) are much more tolerant of environmental changes and have been known to survive in water with a salinity over 50 ppt and at temperatures up to 42°C (F. Baronie, Dampier Salt Co., pers. comm.).

The optimum temperature for growth of juvenile and adult barramundi is considered to be between 28°C and 32°C and the optimum salinity range is 0-36 ppt (Russell *et al.* 1987).

1.6 BARRAMUNDI AQUACULTURE

1.6.1 History

One of the main features of barramundi is its popularity, both as an eating and sporting fish. In northern Australia, barramundi support commercial and recreational fishing industries worth many millions of dollars (Grey 1987).

Its popularity, demand and biological attributes have made barramundi an obvious choice for the development of aquaculture technology. Apart from the characteristics that endear it to the consumer, the fish is fast-growing and euryhaline. The latter fact is a valuable attribute for a species being raised in areas subject to monsoonal conditions.

Techniques for the culture of barramundi were first developed at the Songkhla Marine Laboratories in Thailand in the early 1970's (Wongsomnuk and Manevonk 1973) and considerable progress in aquaculture techniques for the species has been achieved since that time.

Culture of *L. calcarifer* has now spread throughout southeast Asia, Australia and French Polynesia. Techniques for spawning induction have become more reliable and the culture fishery is expanding rapidly (Grey 1987).

Australia's involvement with barramundi culture began in 1984 with the establishment of a research programme at the Northern Fisheries Research Centre (NFRC) in Cairns (Pearson 1987). The thrust of the initial research was to produce fingerlings for the enhancement of rivers and estuaries for recreational fishing.

Initial breeding programmes in Queensland used eggs and sperm hand stripped from wild spawners at Weipa in North Queensland. Later spawnings have used captive as well as wild spawners.

1.6.2 Barramundi Aquaculture in the Northern Territory

In 1988, the Northern Territory government established a pilot barramundi hatchery at Stokes Hill in Darwin, now called the Darwin Aquaculture Centre (DAC). The aim of the hatchery was to investigate and refine the techniques of barramundi breeding under Northern Territory environmental conditions. Any fingerlings produced by the hatchery were to be used to foster the development of barramundi aquaculture in the Northern Territory.

Since that time several private barramundi farms have been established with the first farmed product appearing on local menus in November 1991. The NT government continues to actively support the industry through the supply of barramundi larvae and technical assistance.

1.6.3 Induced Spawning of Barramundi

In Thailand, fertilised eggs are produced by either hand stripping wild or captive broodfish, or by induced spawning under controlled conditions in tanks (Maneewong 1987). The latter method is preferred because of the better quality and number of fertilised eggs produced (Fortes 1987; Maneewong 1987).

1.6.4 Hand Stripping

Spawning fish are sourced from either captive broodstock or are collected directly from the spawning grounds. If the wild fish are collected in a running ripe condition they are hand stripped immediately, otherwise hormone induction is used (Kungvankij 1987). HCG is one of the most commonly used hormones for spawning induction and is often used in conjunction with pituitary extract (Maneewong 1987). The male fish are not normally injected and are often hand stripped at the spawning grounds (Maneewong 1987). The sperm is then stored chilled, for up to 7 days and used for fertilisation when an ovulated female becomes available (Kungvankij 1987).

Eggs from an ovulated female are stripped by firstly anaesthetising the fish and laying her on a table. A container is placed under the fish's genital pore and the eggs are extruded by gripping the abdomen between thumb and forefinger and stroking from near the pectoral fins towards the genital pore. The eggs should be released in a steady flow (Maneewong 1987). To achieve fertilisation, sperm from two to three males is carefully mixed in with the eggs and left for 2-3 minutes before rinsing well with clean sea water at a salinity of 28-32 ‰ (Maneewong 1987; Kungvankij 1987; Garrett *et al.* 1987).

1.6.5 Tank Spawning

Tank spawnings are induced by either hormonal or environmental manipulation (Kungvankij 1987).

1.6.6 Hormone Induction

Fish that are induced hormonally have their reproductive condition checked by cannulation prior to treatment. For successful spawning, males must have sperm present and females must have oocytes greater than 420 µm in diameter (Garrett and Connell 1991; Kuo 1991).

While HCG was originally the hormone of choice for spawning induction of barramundi, LHRHa has now become more commonly used (Garcia 1990; Garrett and Connell 1991). In the Philippines, LHRHa is used via injection in saline solution or by implantation in cholesterol pellets (Fortes 1987; Garcia 1990; Almendras *et al.* 1988a). The cholesterol pellet provides a longer and more complete spawning (Garcia 1990), while the injection is easier and quicker to prepare (Garrett and Connell 1991).

Once induced, the fish are placed in spawning tanks at the ratio of 2:1 (males:females). Prior to spawning, the fish display pre-spawning behaviour, where the male fish pair with the female and continually rub themselves against the females genital papilla. If this behaviour is not exhibited the fish may still spawn but fertilisation is unlikely (Garrett and Connell 1991). A fatty scum appears on the surface of the water just before spawning and indicates that spawning is imminent (Kungvankij 1987; Schipp, pers. obs.). The fish usually spawn 30-36 hours after hormone induction and the fertilised eggs float in the spawning tank from where they are collected.

1.6.7 Environmental Induction

This technique has been developed through observations of natural phenomena that occur when barramundi spawn in the wild.

Kungvankij (1987) reports that there are several steps necessary for successful spawning:

1. Selection of ripe spawners.

2. The salinity of the water is changed to simulate migration to the spawning grounds.

3. The water temperature is decreased to simulate the temperature drop experienced after rain.

4. The water level in the tank is dropped and rapidly refilled to simulate tidal movement,

5. The manipulations follow the correct moon cycle for spawning in the wild.

The fish normally spawn the night after the manipulation is performed.

1.7 HORMONE INDUCTION OF SPERMATOGENESIS AND SPERMIATION IN MALE BARRAMUNDI

The effect of spawning induction on female barramundi has been well documented (Almendras *et al.* 1988a; Garcia 1990; Kungvankij 1987; Garrett and Rasmussen 1987; Garrett and Connell 1991) but there are very few reports on the effects of induction on male fish.

Nacario (1987) injected males and females with LHRHa to induce spawning, but the males already had flowing milt. No attempt was made to quantify the effect of injection on the males. Similarly, Almendras *et al.* (1988) chose males with running milt which were injected with LHRHa and the results were not quantified.

LHRHa administered at a dose rate of 10-25 μg LHRHa kg^{-1} improved milt quality in mature males (Garrett and Connell 1991). LHRHa administered in cholesterol pellets may have advanced the onset of breeding condition in male barramundi (Garcia 1990).

1.7.1 Induction of Spermatogenesis in Barramundi

Recent advances in hatchery technology have resulted in an increased reliability of supply of barramundi fry (Kungvankij 1987; Garcia 1990; Garrett and Rasmussen 1987; Kuo 1991), but so far these have only been available during the normal breeding season. Artificially lengthening the breeding season for barramundi broodstock may benefit the culture of the species by providing fingerlings throughout the year or at least for a greater portion of the year (Fortes 1987; Garrett and Connell 1991). Lengthening the breeding season for barramundi logically involves induced maturation of females (Kuo 1991) and males.

As noted above, reports from the Philippines suggest that it may be possible to lengthen the period of breeding for barramundi by several months. Garcia (1990) used the hormones LHRHa and 17-MT to induce barramundi to spawn two months prior to the peak breeding season in the Philippines. From Garcia's results it is hard to distinguish any significant difference between 17-MT and

LHRH as both treatments apparently advanced spermatogenesis. With Garcia's experiment it is also important to note that 66% of the control fish also matured early. Repetition of this experiment may give more meaningful results.

It is not known whether barramundi are seasonally responsive to hormone treatment. Garcia (1990) used this as a possible reason for failure to spawn after treatment.

1.7.2 Induction of Spermiation in Barramundi

Initial attempts at breeding barramundi at the Darwin Aquaculture Centre relied on hormone induction and hand stripping of wild caught and captive spawners. Female fish were forced to mature through two injections of HCG given 24 hours apart. On a number of occasions it was possible to strip several litres of eggs from the females but fertilisation was not possible due to a lack of sperm in the males. HCG administered to male fish had no effect on sperm production, neither did LH and FSH (Schipp *et al.* 1989).

Wild, male fish caught in running ripe condition were 'dry' when brought to the hatchery. During 1989, preliminary investigations were carried out to find the most suitable hormone therapy for the induction of sperm release in barramundi. Carp pituitary extract (CPE) and LHRHa were both tried, with CPE having the most significant effect (Schipp *et al.* 1989).

1.8 EXPERIMENTAL AIMS

1.8.1 Spermatogenesis

The study of spermatogenesis in barramundi had four experimental aims. Firstly, to repeat the experiments conducted by Garcia (1990) to verify that hormone treatment could have a positive effect on spermatogenesis (maturation) in male barramundi. Secondly, to test the effect of different dose rates of LHRHa and 17-MT. Thirdly to test the effect other hormones known to promote spermatogenesis in other fish species and fourthly to examine whether or not the effect of hormone treatment was seasonally variable.

1.8.2 Spermiation

The spermiation experiments were based on the results of preliminary trials at the Darwin Aquaculture Centre during 1989. The aim of the spermiation experiments was to develop a protocol for the effective stimulation of sperm release in mature male barramundi. Various reproductive hormones, known to increase spermiation in other fish species were tested for their effect on barramundi.

Experiments were also carried out to examine the effect of hormone therapy on the spawning success of the fish.

Even though hand stripping of fish is no longer carried out at the DAC, this study potentially benefits those workers conducting experiments on the cryopreservation of barramundi sperm (Leung 1987) and may also result in higher fertilisation percentages of tank spawned fish.

CHAPTER 2

EXPERIMENTAL PROTOCOL

The experiments undertaken for this study can be divided into two categories, those designed to stimulate spermatogenesis and those aimed at stimulating spermiation and increasing the fertilisation rates of tank spawned fish.

Essentially the experimental protocols were the same for both sets of experiments. The protocols are listed below. Any features peculiar to an experiment or set of experiments are identified in the reports and discussion of the experiments in the following chapters.

2.1 THE ENVIRONMENT

All experiments were undertaken at the Darwin Aquaculture Centre which is located adjacent to the port of Darwin and as such is situated within the normal geographic range of barramundi in Australia.

2.1.1 The Tanks

The fish were maintained in circular fibreglass tanks, connected to a flow through sea water supply. The sea water was sand filtered to approximately 40 µm to remove large suspended particles and marine animals. Two tank volumes were used: 45,000 litres (6 metres diameter x 1.8 metres deep - one tank) and 7,500 litres (3.6 metres diameter x 800 mm deep - ten tanks).

The salinity of the sea water was adjusted to 28-30 parts per thousand which has been identified as the optimum salinity range for the survival of barramundi larvae (Russell *et al.* 1987). It is routine practice for all broodstock at the Darwin Aquaculture Centre to be maintained and spawned in this salinity. The exchange rate of the water was kept at 200-300% daily. The tanks were cleaned every day. Aeration was supplied to the tanks and distributed via either ceramic aeration domes (Universal Grinding Wheel Company, Stafford, England) or rubber spa aeration discs. The dissolved oxygen in the water was always maintained close to saturation.

2.1.2 Water Temperature

Earlier studies (Kuo 1991), indicated that the minimum water temperature for successful maturation of female barramundi is 28°C. As water temperature was not one of the variables to be tested in the current experiments, where necessary, the temperature was artificially raised above 28°C.

Raising of the water temperature was achieved by a solar heating system attached to the 45,000 litre (45T) and two of the 7,000 litre (7T) tanks. Water was withdrawn from the tanks and pumped though a solar heating array on the hatchery roof and returned to the tanks. A 6°C temperature rise could easily be obtained using this system. At night, when the solar system was inoperable, swimming pool insulating blankets and submersible, 1 and 2 kW bar heaters were used to maintain the temperature. One 2 kW heater was used per 7T tank and two 2 kW heaters and one 1 kW heater in the 45T tank. The heating system was only used during the months March to August, when the ambient water temperature dropped to approximately 24°C.

Periodically, between experiments, the salinity of the water was lowered to fresh water for the treatment and/ or prevention of the marine protozoan, *Cryptocaryon irritans* (Schipp and Pearce 1991).

2.2 THE FISH

2.2.1 Broodstock Supply

Fish used in the experiments were obtained from two sources, either by gill netting wild adult fish in the waters of Shoal Bay near Darwin (Schipp 1991b) or from a captive population of barramundi bred at the Darwin Aquaculture Centre. The fish were examined prior to the commencement of each experiment to ensure that they were free from all injuries and not visibly suffering from disease.

All fish were tagged with individually numbered dart tags for identification.

2.2.2 Broodstock Diet

The fish were maintained on a high quality diet of freshly frozen mullet, garfish, whiting and squid. The proportion of squid to fish was kept at approximately 1:2. The fish were fed to satiation once daily.

While it is acknowledged that nutrition can influence reproductive performance (Section 1.2.2), it was beyond the scope of this study to examine the effects of nutritional manipulation on reproduction. Instead, the fish were fed what was believed to be a consistently high

quality diet in an attempt to minimise the influence of nutrition as a variable. Prior breeding trials at the Darwin Aquaculture Centre had indicated that this diet did not hinder reproductive development or performance.

2.2.3 Fish Handling and Anaesthesia

All fish used in these experiments were anaesthetised using 2-phenoxyethanol (2PE) prior to examination or administration of hormones (Schipp 1991a). To enable handling of the fish, the water in the holding tanks was lowered to approximately 0.4 metres deep. The fish were surrounded by a small, knotless drag net and then individually lifted with a piece of cotton bed sheet into a fibreglass tub containing 100 litres of sea water and 28 ml of 2PE (280 ppm). The fish were deemed to be sufficiently anaesthetised when they floated 'belly up' in the anaesthetic bath. The time to reach anaesthesia varied with fish size but was usually achieved within five minutes.

Once anaesthetised the fish were placed onto a stainless steel table and had their eyes covered with a damp cloth to further reduce levels of stress. In this position the fish could be examined and/ or operated on as needed.

After treatment the fish were returned to their tank and floated near a bubbling air stone. Recovery from the anaesthetic was always rapid and complete.

2.2.4 Gonad Sampling

Males for gonad sampling were anaesthetised and prepared as outlined in 2.2.3.

The reproductive condition of the fish was determined in two ways. Firstly by attempting to hand strip a sample of sperm from the fish. If this was unsuccessful the second method involved inserting a small length of cannula tubing into the gonad for the removal of a sperm sample.

2.2.5 Hand Stripping

Hand stripping of a sperm sample from the fish was performed by placing the fish on its side and holding it firmly with its genital pore close to the edge of the table.

Urine was expelled from the fish by gently squeezing the area around the urinogenital pore and blotting it dry with paper towel. Any faeces expelled at this time were wiped away.

The fish's abdomen was held in a firm grip between the thumb and fingers and then the abdomen was squeezed and the hand slid from near the pectoral fins towards the genital pore.

If a sperm sample was extruded it was scored according to the method of Shehadeh *et al.* (1973).

(1) = traces of viscous milt.
(2) = small amounts of running (hydrated) milt.
(3) = copious running milt.

If a (3) was recorded, the volume of the sample was also noted.

If sperm was not extruded after three or four attempts, cannulation of a sperm sample was attempted.

2.2.6 Cannula Sampling

To sample the gonad, a 15 cm length of polyethylene cannula (Clay Adams PE100) was attached to a 20 gauge needle and 12 ml syringe. The cannula was carefully inserted through the genital opening and into the gonad. Suction was applied to the cannula by gently pulling back the plunger on the syringe and simultaneously withdrawing the tubing. If a sperm sample was not obtained the process was repeated.

Once a sample was withdrawn the syringe was marked for later identification and examination under a microscope.

The scoring system for cannula sampling was:

(F) = fluid in cannula (also applied to situations when no sample obtained).

(S) = sperm in sample.

2.2.7 Microscopic Examination of Sperm Samples

Sperm samples from the fish were examined microscopically prior to the commencement of hormone therapy. A small drop of sperm was placed onto a microscope slide and a similar sized drop of brackish sea water (20 ppt) was added to activate the sperm (Billard 1978). A cover slip was quickly put in place and the sample examined under 40X and 100X power. The time delay from the addition of the saline until microscopic examination was kept to a minimum so that the period of greatest sperm activity was not missed (Terner 1986).

The sperm was observed for activity and scored using an arbitrary scale between 0 and 5 (Billard 1978).

0 - no activity.
1 - 20% of spermatozoa active, very little directional movement. High percentage of sperm simply vibrating in the same spot.
2 - 40% active.
3 - 60% active.
4 - 80% active.
5 - 100% active, strong directional movement, majority of the sperm swimming strongly in a straight line across the field of view.

2.2.8 Selection of Experimental Fish

Generally, confirmation that the sample contained sperm was necessary for the fish to be chosen for experiment. Exceptions to this were several fish from which a sperm sample was not obtained but their very recent spawning history identified them as being male. A number of the spermiation experiments used fish that only possessed gametes in the spermatid stage. These spermatids had no activity and the fish were used to assess the effectiveness of the hormones to induce final maturation and release of spermatozoa. Fish to be used in the experiments had not been the subject of previous experiments for at least six weeks, to ensure that the residual effects from previous hormone treatments were minimal. Obviously it would have been preferable to use fish that had not previously been exposed to exogenous hormone treatment, however this was not possible and the six weeks latency period was considered the best compromise.

Once selected for experimentation, the fish were randomly assigned to the treatment groups and tanks. Wherever possible the minimum number of fish in a treatment group was kept at three.

It is recognised that the small number of fish available for experimentation placed restrictions on the interpretation of results. It was not possible, for example, to apply tests of statistical significance to any of the trials. Despite this, the trials were still important because they provided indications of the best methods and were a starting point for the development of practical solutions to the problems of induced breeding in male barramundi.

2.3 THE TREATMENTS

Four different methods were used for the administration of hormones to the fish during the experiments. Fish undergoing spermiation trials received hormone therapy via injection, while fish used in the majority of the spermatogenesis experiments were treated using either silicone or cholesterol pellet implants. Fish in two of the spermatogenesis experiments were treated orally by having the hormone incorporated in their food.

2.3.1 Injection

Injections for the fish were prepared in the following way:

1. Needle size: 23 gauge when the solvent was isotonic saline and 19 gauge when the solvent was

oil or the hormone was particulate or difficult to dissolve.

2. Syringe: 3 ml luer lock.

3. Solvents/ carriers: Depended on the hormone being used. Details are given under the relevant experiments.

4. Hormones were administered per kilogram body weight and were injected either once only or daily for two or three days.

5. The fish were always injected intramuscularly in a position approximately 3 cm ventrally from the mid point between the first and second dorsal fin rays.

2.3.2 Pellets

Small pellets consisting of hormone mixed with a matrix of either cholesterol or silicone were used for experiments into the enhancement of spermatogenesis. These pellets act to release small amounts of hormone consistently for periods of up to six months (Jensen *et al.* 1978; Lee *et al.* 1985; Tamaru *et al.* 1990) and therefore are ideal vehicles for administering hormones during attempts at manipulating the long term process of spermatogenesis.

2.3.3 Cholesterol Pellet

The method for manufacture of the cholesterol pellet was based on Lee *et al.* 1985, with modifications for ease of

manufacture and implantation. The full details of pellet manufacture are given in Appendix 2.

Two sizes of pellets were produced, 3 mm x 1 mm and 5 mm x 1 mm. The two sizes gave more flexibility in matching the appropriate dose to the weight of the fish.

2.3.4 Silicone Pellets

The technique for the manufacture of the silicone pellet implants was modified from Jensen *et al.* (1978) and Lee *et al.* (1986c). Silicone tubing[1] was cut into the appropriate number of 5 cm lengths. One of the open ends of each the 5 cm lengths was sealed by injecting a small quantity of elastomer[2] into the tubing using a 20 gauge needle attached to a 3.0 ml syringe (Figure 5A). The elastomer was left to cure at room temperature for one hour.

To manufacture pellets with a final hormone concentration of 10 mg, the hormone mix was prepared by first dissolving 100 mg of 17-MT in 0.2 ml of 100% ethanol. To this was added 0.1 ml of castor oil. The hormone mix was stirred well and loaded into a 1.0 ml tuberculin syringe with a 20 gauge needle attached. The needle was inserted all the way into the previously prepared 5 cm tubes and 0.03 ml of the hormone mix was carefully injected (Figure 5B and C). The needle was slowly withdrawn as the mixture was injected.

The tubes were sealed in a similar manner to the placement of the original silicone plug (Figure 5D). The final operation involved trimming the ends of the tubing until the total length measured 2 cm (Figure 5E).

---

[1] Dow Corning, Medical-Grade Tubing, 0.058 inch ID, 0.077 inch OD. Cat. No. 602-235.

[2] Dow Corning, Medical Adhesive Silicone Type A. Cat No. 891.

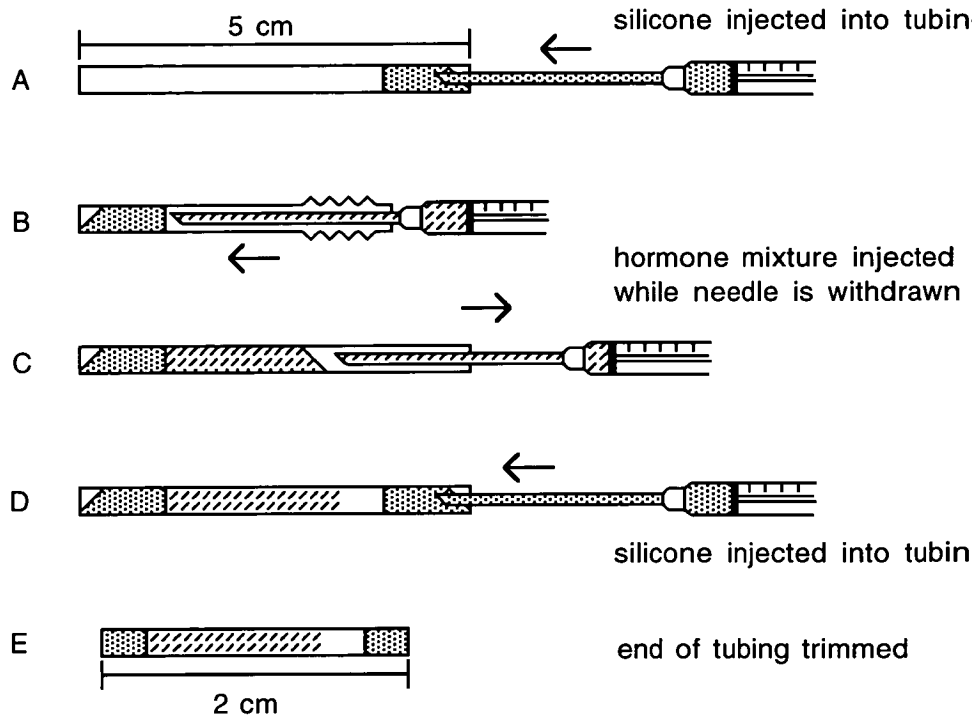

Figure 5. Detail of the manufacture of a silicone pellet. A: injection of elastomer plug, B and C: injection of hormone mixture, D: injection of second elastomer plug, E: finished pellet.

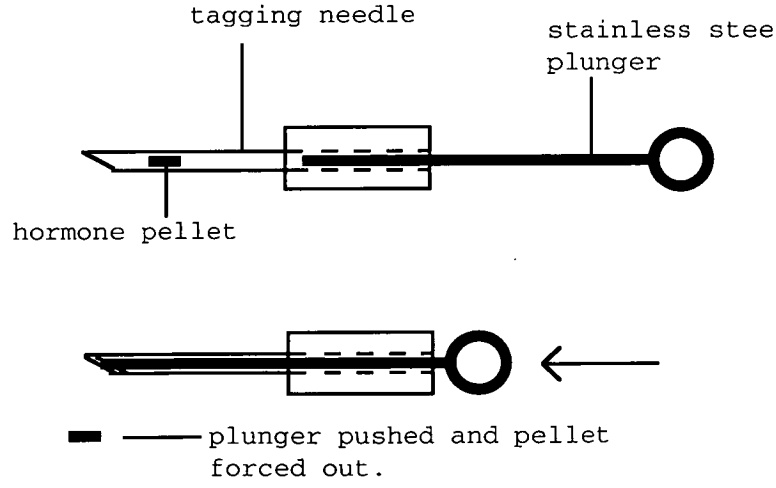

Figure 6. Fish tagging needle used to implant hormone pellets into male barramundi.

2.3.5 Pellet Implantation

Both types of pellets were implanted using the same method. The equipment used for implantation included:

1. Dart tagging needle with an internal diameter of 1.1 mm and a stainless rod to match the internal diameter of the needle. The rod was pushed through the needle and acted as a plunger (Figure 6).
2. A scalpel.
3. 100% ethanol.

Prior to the operation, the tagging needle was sharpened to a fine point to ensure easy penetration through the fish's muscle. The tip of the needle was sterilised in the ethanol, air dried and a pellet loaded into the hollow shaft. The position for the implant was the same as for injection; approximately 3 cm ventrally from the mid point between the first and second dorsal fin rays. At the implant point a single scale was removed using the handle of the scalpel. A small incision was made in the exposed skin and the needle was inserted through the skin and 2-3 cm into the muscle. The stainless rod was pushed through the needle, pressing the pellet into the muscle. The needle was removed and the wound site swabbed with Betadine® antiseptic.

2.3.6 Oral Administration

Two of the spermatogenesis experiments involved the incorporation of 17-MT into the diet of the fish. The experiment was based on Weber and Lee (1985). 17-MT was mixed with cod liver oil and injected into the stomach cavity of small mullet fed to the barramundi.

2.4 Assessment of Experiments

A flow chart of the protocol used for the assessment of the experiments is presented in Figure 7.

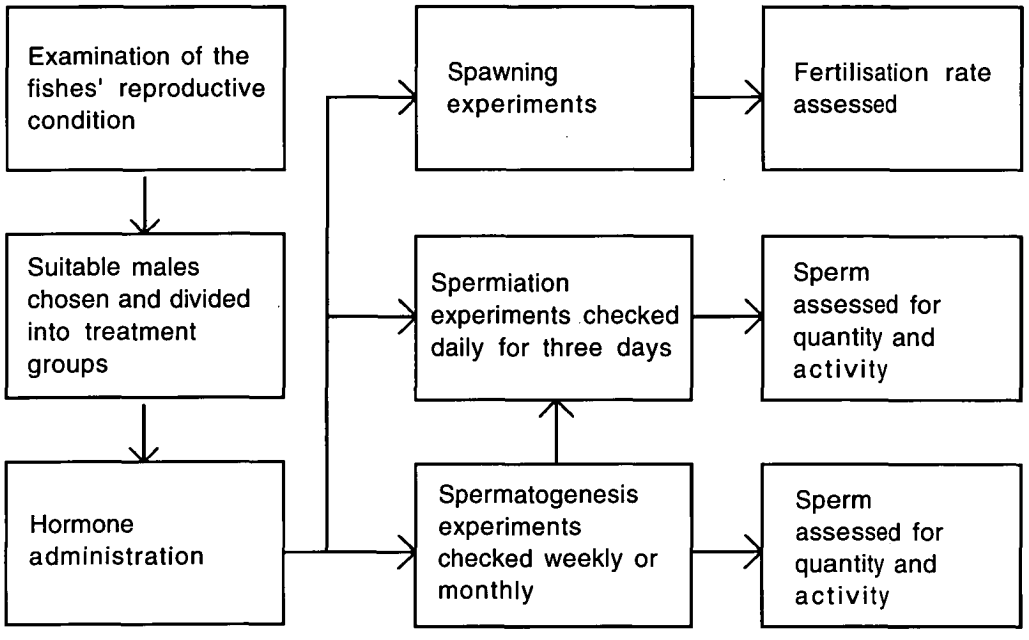

Figure 7. Flow chart of the protocol followed for the experiments.

2.4.1 Spermatogenesis

Spermatogenesis experiments using hormone pellets were checked weekly for five weeks, the approximate effective life of the pellets (Sherwood *et al.* 1988). The same assessment procedure used for the spermiation experiments was used for the spermatogenesis experiments.

The fish were fed throughout the experiments.

2.4.2 Spermiation

Spermiation experiments were assessed daily for three days following the first injection. The fish were examined at the same time each day, usually early morning.

Fish to be examined were prepared as previously described under section 2.2.3 and the quantity and quality of milt was scored as per sections 2.2.4 to 2.2.7.

The fish were not fed during the experiments.

2.4.3 Spawning

The effect of the hormone treatments on spawning was assessed by observing spawning frequency and fertilisation rates between treatment groups.

CHAPTER 3.

HORMONE INDUCED SPERMATOGENESIS

The spermatogenesis experiments were undertaken between June 1990 and July 1993. There were a total of eight experiments in this series and they examined the effects of chronic hormone treatment on spermatogenesis.

3.1 EXPERIMENT 1, JUNE/ JULY 1990, 17-MT CHOLESTEROL PELLET IMPLANTS

3.1.1 Introduction

Recent trials undertaken in the Philippines suggest that it may be possible to lengthen or advance the peak period of breeding for barramundi by two to three months. Garcia (1990) incorporated the hormones LHRHa and 17-MT into slow releasing cholesterol pellets to induce barramundi to spawn two months prior to their peak breeding season. The hormones were equally effective, whether they were administered singly or together.

It has also been widely reported that administration of 17-MT on a regular basis can have a positive effect on spermatogenesis in other teleost fish. This has been demonstrated for grey mullet (Shehadeh *et al.* 1973; Weber and Lee 1985); milkfish (Lee *et al.* 1986d) and channel catfish (Simone 1990).

The limitation on the number of male fish available for experimentation at the Darwin Aquaculture Centre restricted the number of hormone variables tested in each

experiment. For this reason, the first spermatogenesis experiment only investigated the effect of 17-MT.

Single cholesterol pellets, containing one of a range of concentrations of 17-MT were implanted into the fish. The experiment was based on the work of Garcia (1990) and compared the dose rate of 100 μg kg^{-1}, with two higher doses, 200 and 400 μg kg^{-1} respectively. Garcia conducted his experiments within the breeding season for barramundi in the Philippines. Experiment 1 was conducted in June and July 1990, well before the start of the breeding season for barramundi in northern Australia. It was thought that higher doses of hormone may be required to elicit a response during this time.

The effect of the hormone treatment was examined weekly over a four week period. It was planned to repeat the hormone implant at the end of the first month, however, at this time, the fish contracted a *Cryptocaryon* infection causing the termination of the experiment.

3.1.2 Methods

DAY 0

1. Sixteen male barramundi weighing between 3.0 and 5.4 kilograms were selected for the experiment. Due to the regressed breeding condition of the fish at the start of the experiment, the majority of the fish were chosen on the basis of their body weight. Fish less than 6.0 kg should still be males (R. Griffin, pers. comm).

2. The fish were randomly assigned to one of four experimental groups.

(i) Control group: no treatment (N=4).
(ii) 100 μg 17-MT kg^{-1} in a cholesterol pellet (N=4).
(iii) 200 μg 17-MT kg^{-1} in a cholesterol pellet (N=4).
(iv) 400 μg 17-MT kg^{-1} in a cholesterol pellet (N=4).

3. The fish were weighed and distributed between two tanks. Two controls were allocated to each tank.

4. The hormone/ cholesterol pellets for the fish were manufactured on day 0, ready for implantation on day 1. The method for manufacture of the cholesterol pellet was based on Lee *et al.* (1985) and is detailed in Appendix 2.

DAY 1

6. The fish were caught and anaesthetised and had their maturation level assessed as per the method and scoring system outlined in sections 2.2.4 to 2.2.7.

7. The fish were implanted with cholesterol pellets of the appropriate dosage implanted and returned to the water.

DAY 8 ONWARDS

8. The reproductive condition of the fish was reassessed on days 8, 15, 22 and 29.

The fish were due for re implantation of the pellets on the 29th day but the presence of a *Cryptocaryon* infection forced the termination of the experiment.

3.1.3 Results

The results of experiment 1 are shown in Table 3.

At the start of the experiment only two fish were able to have a sperm sample withdrawn by cannula. All the other fish were in regressed breeding condition. At the end of the first week none of the fish had a sperm sample withdrawn. After two weeks, 4 fish had sperm samples extracted, including both controls. Both controls also had a sperm sample taken in the third week, as did one of the treated fish. At the conclusion of the experiment, 1 control and 2 treated fish had sperm samples withdrawn.

At the start of the experiment, gonad dissections of two, recently deceased male fish confirmed that they were not in breeding condition.

3.1.4 Discussion of Experiment 1

From this experiment it would appear that 17-MT administered on its own, in cholesterol pellets, at dose rates of 100, 200 or 400 $\mu g\ kg^{-1}$, does not advance spermatogenesis in barramundi outside their normal breeding season. At any stage during the course of the experiment, a maximum of four fish (including both controls) may have been suitable for induction of spermiation and/ or spawning. By the conclusion of experiment, three fish were considered suitable for induction, one of these was a control. Examination of sperm activity for the few fish that could be cannulated did not reveal any differences between control and treated fish. These results indicate that there was no difference in response between control and treated groups.

Table 3. Results of a single intramuscular implant of 17α-methyltestosterone on the reproductive condition of male barramundi.

Fish No.	Weight	Treatment	Reproductive Condition				
		17-MT	Day 1	Day 8	Day 15	Day 23	Day 29
072	3.8	Control	F	F	S	S	S
058	5.4	Control	F	F	S	S	F
2147	3.1	Control	F	F	F	F	F
080	4.0	Control	F	F	F	F	F
069	3.2	100 μg kg^{-1}	F	F	F	F	F
2149	3.0	100 μg kg^{-1}	F	F	F	F	F
063	3.8	100 μg kg^{-1}	S	F	F	F	F
2146	2.8	100 μg kg^{-1}	F	F	S	F	F
071	3.4	200 μg kg^{-1}	F	F	F	F	F
2150	3.8	200 μg kg^{-1}	F	F	F	F	F
078	4.4	200 μg kg^{-1}	F	F	S	S	S
067	3.7	200 μg kg^{-1}	F	F	F	F	F
856	3.4	400 μg kg^{-1}	S	F	F	F	F
073	4.8	400 μg kg^{-1}	F	F	F	F	F
2144	3.0	400 μg kg^{-1}	F	F	F	F	F
2143	3.2	400 μg kg^{-1}	F	F	F	F	S

F = fluid in cannula sample, S = sperm in cannula sample.

The results do not support the findings of Garcia (1990). However, there are two important differences between the two trials. Firstly, Garcia's experiment was conducted over three months. This was not possible in experiment 1 because of the occurrence of the *Cryptocaryon* infection. Secondly, as pointed out before, Garcia's experiment was conducted within the normal breeding season for barramundi in the Philippines. Experiment 1 was conducted outside the breeding season for NT barramundi. The fish may respond differently to hormone treatment depending on the time of year or the condition they are in when they are implanted. Garcia did not state if his male fish were able to have a sperm sample cannulated and therefore the condition of the fish at the start of the experiment was not defined.

Another consideration with Garcia's experiment was that a large proportion of his control fish also matured at the same time as the treated fish. Although apparently statistically different, the response of the controls was based on a smaller sample number than the treated groups. If Garcia had repeated his experiment, with larger sample numbers the results may be more meaningful.

The fact that Garcia's control fish did have a high maturation rate, is interesting in itself. As mentioned in the section 1.7.2, male barramundi in the Northern Territory have exhibited poor maturation rates in captivity. Garcia kept his broodstock in floating sea cages. It has been reported for the Red Sea bream, *Pagrus major*, that confinement in tanks inhibits reproductive development but confinement in sea cages, apparently, does not (Dr Soon Kil Yi, Korean Ocean Research and Development Institute, pers. comm.). This may also be true for barramundi. The present investigation did not aim to study the effect of holding conditions on maturation levels but

it is obviously something that should be examined in the future.

3.2 EXPERIMENT 2, MARCH/ APRIL 1991, 17-MT AND LHRHa CHOLESTEROL PELLET IMPLANTS.

3.2.1 Introduction

The second experiment was again based on the work of Garcia (1990), who suggested that LHRHa was also effective in advancing the maturation of male fish, when it was used on its own or in combination with 17-MT.

Experiment 2 investigated the effect of a single implant of a cholesterol pellet containing varying doses of LHRHa, either singly or in combination with 17-MT. The dose rate chosen for 17-MT, 100 $\mu g\ kg^{-1}$, was the same as that used by Garcia (1990) and the LHRHa dosage was based on successful spermiation trials at the Darwin Aquaculture Centre during 1989/90. The experiment was conducted during March, which is considered to be late in the spawning season for Northern Territory barramundi (Davis 1987).

3.2.2 Methods

1. Sixteen male fish ranging in weight between 5.0 and 9.5 kg were used in this experiment.

2. The fish were maintained in three 7,000 litre fibreglass tanks, with flow through sea-water @ 30 ppt salinity and ambient temperature (30-32°C).

3. The fish were divided into five experimental groups:

   (i) Control - no treatment (N=4).

(ii) Pelleted LHRHa at the rate of 25 μg kg^{-1} (25L) (N=3).

(iii) Pelleted LHRHa at the rate of 50 μg kg^{-1} (50L)(N=3).

(iv) 25L + 17α–methyltestosterone at the rate of 100 μg kg^{-1} (25L+17-MT)(N=3).

(v) 50L + 17α–methyltestosterone at the rate of 100 μg kg^{-1} (50L+17-MT)(N=3).

4. The reproductive condition of the fish was examined prior to the implantations and again 28 days later. The maturation level of the fish was assessed as per the method and scoring system outlined in sections 2.2.4 to 2.2.7.

3.2.3 Results

The results of experiment 2 are shown in Table 4.

At the start of the experiment, 10 of the 16 fish had a sperm sample withdrawn by cannulation. This included 2 of the 4 control fish.

After 28 days, 2 of the 3 fish that had received 25L pellets and 1 fish that had received a 25L+17-MT pellet, produced small quantities of viscous milt by hand stripping. All three fish that had received 50L+17-MT pellets had a sperm sample withdrawn by cannula at the end of the experiment. This represented and increase in condition for one of them and no change in condition for the other two. All other treated fish were in regressed breeding condition at the conclusion of the experiment as were all four controls.

Table 4. The effect of a single implantation of a cholesterol pellet containing varying doses of LHRHa and 17α-methyltestosterone during March/ April 1991.

Fish No.	Weight	Treatment	Reproductive Condition	
			Day 0	Day 28
2149	6.2	Control	S	F
058	8.6	Control	F	F
886	9.4	Control	S	F
2135	7.6	Control	F	F
2110	5.6	25L	F	F
2113	7.0	25L	S	2
095	8.6	25L	S	2
2147	5.2	50L	S	F
2143	7.0	50L	S	F
063	6.6	50L	F	F
2150	6.4	25L+17-MT	F	F
078	6.8	25L+17-MT	S	2
069	7.0	25L+17-MT	S	F
2146	5.0	50L+17-MT	F	S
2137	8.4	50L+17-MT	S	S
895	8.0	50L+17-MT	S	S

25L = 25 μg kg^{-1} LHRHa, 50L = 50 μg kg^{-1} LHRHa, 25L+17-MT = 25 μg kg^{-1} LHRHa + 100 μg kg^{-1} 17-MT, 50L + 17-MT = 50 μg kg^{-1} LHRHa + 100 μg kg^{-1} 17-MT.

F = fluid in cannula sample, S = sperm in cannula sample, 2 = small amount of sperm expressed by hand stripping.

3.2.4 Discussion of Experiment 2

The results from this experiment indicate that pelleted LHRHa, at the dose rate of 25 μg kg^{-1}, may increase the volume of sperm produced by the fish within one month of implantation during the breeding season. This result appears to irrespective of the inclusion of 17-MT in the

pellet. Three of the six fish that received pellets containing 25 μg kg^{-1} LHRHa were in moderate running ripe condition after 1 month. Two of the other fish receiving this dose did not increase in condition whilst the third decreased.

The other hormone treatments tested did not induce an improvement in reproductive condition and the control fish finished the experiment in a regressed state.

The use of LHRHa, incorporated into cholesterol pellets at the dose rate of 25 μg kg^{-1}, shows promise for increasing the maturation rate of male barramundi within the normal period of breeding.

Experiments 3, 4 and 5 further examined the effects of LHRHa administered at other times of the year.

3.3 EXPERIMENT 3, JUNE 1991, LHRHa CHOLESTEROL PELLET IMPLANTS

3.3.1 Introduction

Experiment 2 demonstrated that LHRHa may be effective in advancing the maturation of male barramundi. In that experiment, a single cholesterol pellet implant of LHRHa, at a concentration of 25 μg kg^{-1} body weight, either singly, or in combination with 100 μg kg^{-1} 17-MT produced expressible sperm in 50% of the fish, one month after they were treated.

The third spermatogenesis experiment aimed to further test the effects of LHRHa implants. Two dose rates were used for this experiment, 25 μg kg^{-1}, the successful rate from the March experiment and 50 μg kg^{-1}, chosen because of the

possible need for a higher hormone dose during the non-spawning season.

This experiment took place in June, which is outside the normal breeding season, (Davis 1987).

3.3.2 Methods

1. Fifteen mature male fish, ranging between 5.2 and 12.0 kg in size were chosen for this experiment. The fish were maintained in either one of two 7,000 litre fibreglass tanks or a 45,000 litre fibreglass tank.

   All tanks were heated to maintain the temperature above a minimum of 28°C as per section 2.1.2.

2. The fish were divided into three experimental groups:

   (i) Control - no treatment (N=5).
   (ii) Pelleted LHRHa at the rate of 25 μg kg^{-1} (25L) (N=5).
   (iii) Pelleted LHRHa at the rate of 50 μg kg^{-1} (50L) (N=5).

3. The reproductive condition of the fish was checked prior to the implantations and again 28 days later. The maturation level of the fish was assessed as per the method and scoring system outlined in sections 2.2.4 to 2.2.7.

3.3.3 Results

The results of experiment 3 are shown in Table 5.

Treatment with either of the two doses of LHRHa did not stimulate sperm production in comparison to control fish Seven of the nine fish in the 45,000 litre tank, including both controls were able to have a sperm sample cannulated after 28 days. This represented an increase in condition for two of the fish, one control and one 50L treated and no change in condition for the other five. Two fish in the 45,000 litre tank were identified as female at the end of the experiment.

In the seven thousand litre tanks, two controls, one 25L treated and one 50L treated stayed in a regressed condition. For the other two fish, one, 50L treated, improved in condition and the other, 50L treated, maintained condition.

None of the fish in this experiment were able to be hand stripped at any stage.

3.3.4 Discussion of Experiment 3

Unlike the results achieved in experiment 2, treatment with pelleted LHRHa during the non-spawning season did not clearly influence the volume of sperm produced by the fish. None of the fish were capable of having a sperm sample hand stripped at the conclusion of this experiment.

Table 5. The effect of a single implantation of a cholesterol pellet containing varying doses of LHRHa, during June 1991.

Fish No.	Weight	Treatment	Reproductive Condition	
			Day 0	Day 28
2113	7.2	Control^{45T}	S	S
2149	7.4	Control^{45T}	F	S
095	8.4	Control^{45T}	S	S
2120	12.0	25 L^{45T}	S	S
063	6.8	25 L^{45T}	F	S
2121	5.8	25 L^{45T}	F	female
2110	6.8	50 L^{45T}	F	female
078	6.6	50 L^{45T}	F	S
2122	5.2	50 L^{45T}	S	S
2150	6.8	Control^{7T1}	F	F
2143	8.0	25 L^{7T1}	F	F
2147	5.8	50 L^{7T1}	F	F
069	7.4	Control^{7T2}	F	F
2137	9.2	25 L^{7T2}	S	S
2146	5.4	50 L^{7T2}	F	S

F = fluid in cannula sample, S = sperm in cannula sample.
female = sample of oocytes withdrawn in the cannula, sex of fish had previously been impossible to determine.

45T = kept in a 45,000 litre tank during the experiment.
7T1 = kept in 7,000 litre tank, number 1, during the experiment.
7T2 = kept in 7,000 litre tank, number 2, during the experiment.

There is the possibility that the volume of the experimental tanks may have had an influence on the results of this experiment. At the end of the fourth week, all of the male fish, including the controls, in the 45,000 litre tank were capable of having a sperm sample cannulated. This was in contrast to the 7,000 litre tanks where only a third of the fish could be cannulated. In the 7,000 litre tanks there was an average of one fish per 2,500 litres of water and in the 45,000 litre tank there was one fish per 5,000 litres of

water. It is possible that the difference in the response of the fish was due to the lower density in the larger tank. The fish in the larger tank were also more isolated from interference by human visitors and consequently may have experienced less stress during the course of the experiment. Whatever the reason, it once more highlights the significant role that the environment can play in influencing fish reproduction.

3.4 EXPERIMENT 4, AUGUST 1991, LHRHa AND 17-MT CHOLESTEROL PELLET IMPLANTS

3.4.1 INTRODUCTION

The fourth experiment commenced prior to the 1991/92 spawning season and compared the effects on spermatogenesis of the doses of LHRHa and 17-MT (100 μg kg^{-1} each) used by Garcia (1990) with the dose of LHRHa used with some success in experiment 2.

The fish were implanted once and their reproductive condition was assessed prior to implantation and again after 14 and 28 days. The experiment terminated at 28 days as some of the fish were required for spawning.

3.4.2 METHODS

1. Sixteen fish, ranging between 3.0 and 8.8 kg in size were chosen for this experiment. The fish were maintained in one 45,000 litre fibreglass tank.

2. The fish were divided into four experimental groups:

   (i) Control - no treatment (N=4).
   (ii) Pelleted LHRHa at the rate of 25 μg kg^{-1} (25L)(N=6).

(iii) Pelleted LHRHa at the rate of 100 μg kg^{-1} plus 100 μg kg^{-1} 17-MT (100L+17-MT)(N=3).

(iv) 100 μg kg^{-1} 17-MT (17-MT)(N=3).

3. The reproductive condition of the fish was checked prior to implantation and again 14 and 28 days later. The maturation level of the fish was assessed as per the method and scoring system outlined in sections 2.2.4 to 2.2.7.

3.4.3 Results

The results of experiment 4 are shown in Table 6.

Two fish produced free flowing sperm during the course of the experiment. One of these fish had received a pellet containing 100L+17-MT and the other was a control.

Five of the fish, two controls, two 17-MT treated and one 100L+17-MT treated were identified as females at the conclusion of the experiment. Of the remaining fish, all stayed at the same condition level except for one fish, 25L, which improved condition after 14 days.

3.4.4 Discussion of Experiment 4

The fourth experiment again failed to produce a clear distinction between control and treated groups. One of the control fish was found to be in running ripe condition during the second week of the experiment and one of the 100L + 17-MT treated fish was in running condition at the end of the fourth week.

Table 6. The effect on spermatogenesis in barramundi of a single cholesterol pellet containing either LHRHa, 17-MT or a combination of the two, implanted prior to the 1991/92 spawning season.

Fish No.	Weight	Treatment	Reproductive Condition		
			Day 0	Day 14	Day 28
2144	8.0	Control	F	F	female
2112	9.4	Control	S	3(0.5)	S
061	8.8	Control	F	female	-
2147	5.0	Control	F	F	F
2124	3.0	25L	F	F	F
095	8.6	25L	S	S	S
889	8.0	25L	S	F	S
2115	7.0	25L	F	S	S
2121	8.4	25L	S	S	S
734	4.8	25L	S	S	S
895	8.0	100L+17-MT	S	S	S
063	6.6	100L+17-MT	F	2	3
2128	6.2	100L+17-MT	F	female	-
2136	7.6	17-MT	F	female	-
2113	7.0	17-MT	S	S	S
2150	6.4	17-MT	F	F	female

F = fluid in cannula sample, S = sperm in cannula sample, 2 = thick fluid sperm expressed by hand pressure, 3 = copious free flowing sperm, figure in brackets represents volume obtained in millilitres, female = sample of oocytes withdrawn in the cannula, sex of fish had previously been impossible to determine.

Unfortunately the results of the experiment were compromised by the fact that five of the sixteen fish turned out to be females. As discussed in section, 4.2.4, this is one of the frustrating aspects of working with barramundi and the accidental inclusion of newly changed females is unavoidable given that cannula sampling often fails to are distinguish them from males in regressed

breeding condition. The main discussion for this chapter and section 5.4.3 discuss this problem in more detail.

3.5 EXPERIMENT 5, MAY/ JUNE 1992, LHRHa CHOLESTEROL PELLET IMPLANTS

3.5.1 Introduction

Experiment 5 examined the effect of cholesterol pellet implants containing LHRHa administered over a two month period. Two doses of LHRHa were used in this experiment, 25 μg kg^{-1}, the dose used with moderate success in experiment 2 and 100 μg kg^{-1}, the dose tested by Garcia (1990). The inclusion of 17-MT in previous experiments did not appear to have a beneficial effect, therefore 17-MT was not included in this experiment.

The experiment was conducted in May and June which is outside the normal period of breeding.

3.5.2 Methods

1. Fourteen fish, ranging between 2.8 and 5.4 kg in size were chosen for this experiment. The fish were maintained in one 45,000 litre fibreglass tank.

2. The fish were divided into three experimental groups:

   (i) Control - no treatment (N=4).
   (ii) Pelleted LHRHa at the rate of 25 μg kg^{-1} (25L)(N=5).
   (iii) Pelleted LHRHa at the rate of 100 μg kg^{-1} (100L) (N=5).

3. The pellets were implanted twice, first on day 1 and again on day 28.

4. The reproductive condition of the fish was checked prior to implantation and again 28 days later. The maturation level of the fish was assessed as per the method and scoring system outlined in sections 2.2.4. to 2.2.7.

3.5.3 Results

The results for experiment 5 are shown in Table 7.

The four control fish finished the experiment in a regressed breeding state. This represented a decrease in condition for two of the controls and no change for the others. Of the fish that received 25L pellets, two were more easily cannulated after 56 days than they were at the start of the experiment (this is a subjective analysis and difficult to quantify), and the other 3 had decreased in condition. One of the 100L fish slightly improved its condition during the course of the trial, the other four decreased in condition.

None of the fish were able to be hand stripped during this experiment.

3.5.4 Discussion of Experiment 5

Once again the results from this experiment do not clearly show that LHRHa hormone treatment is effective at enhancing the maturation of male barramundi. Only three of the ten fish treated showed any sign of improvement in reproductive condition and even then it was only based on

a subjective assessment that the fish had a sperm sample more easily cannulated at the completion of the experiment than at the start. The hormone treatments did not induce any of the fish to become running ripe and the results of this experiment do not support those achieved by Garcia (1990).

Table 7. The effect on spermatogenesis in barramundi of two implantations of cholesterol pellets containing two different doses of LHRHa administered one month apart, during May and June, 1992.

Fish No.	Weight	Treatment	Reproductive Condition		
			Day 0	Day 28	Day 56
712	3.4	Control	F	F	F
716	4.5	Control	F	F	F
789	3.8	Control	S	F	F
705	4.6	Control	S	F	F
701	4.6	25L	S	F	S^1
706	4.0	25L	S	F	F
707	5.4	25L	S	S	S^1
709	2.8	25L	S	S	F
787	4.1	25L	S	F	F
710	5.0	100L	S	F	F
711	3.8	100L	S	S	F
778	4.2	100L	S	F	F
783	5.2	100L	S	S	S^1
790	3.6	100L	S	F	F

F = fluid in cannula sample, S = sperm in cannula sample, S^1 = sperm was easily sampled by cannula and was very active when examined under the microscope.

It would seem from this experiment that LHRHa, administered at either of the doses tested and during the non-spawning season, does not offer a practical method for

improving the reproductive condition of male barramundi broodstock.

3.6 EXPERIMENT 6 JUNE/ SEPTEMBER 1992, 17-MT SILICONE PELLET IMPLANTS

3.6.1 Introduction

The three fish used in this trial were implanted with silicone pellets (Lee *et al.* 1986c) containing 10 mg of 17-MT. It has been demonstrated that sealed silicone pellets containing steroid are capable of slowly releasing hormone over several months (Jensen *et al.* 1978) and can influence the serum steroid profile for at least thirty five days (Tamaru *et al.* 1990). Tamaru *et al.* (1990) determined that the release rate of steroid from the pellet is determined by the initial concentration of steroid. The higher the concentration, the faster the release rate. After an initial surge of hormone during the first few days following implantation the release rate settles down to a relatively constant value. A silicone capsule, 2 cm long and 1.92 mm O.D., containing 10 mg of testosterone will release approximately 750 ng of hormone per day (Tamaru *et al,* 1990). The pellet potentially has a useful life of over 36 years! Obviously, even if the pellet is active over only a portion of this time, it offers the possibility of controlling the reproduction of the fish over an extended period.

Although previous spermatogenesis experiments, sections, 3.1, 3.2 and 3.4, indicated that 17-MT was not effective in inducing spermatogenesis in barramundi this may be due to the type of pellet used to dispense the hormone. Testing the effect of 17-MT distributed from silicone pellets was considered worthwhile.

This experiment investigated the effect of a single implant into male barramundi of a silicone pellet containing 10 mg of 17-MT. The breeding condition of the fish was assessed prior to implantation and at monthly intervals over a four month period. The experiment was also used as a precursor for spermiation trial number 7, section 4.2, testing the effect of LHRHa injections in fish that had been 'primed' with 17-MT for a period of 28 days.

3.6.2 Methods

1. Three fish, ranging between 4.6 and 6.8 kg in size were chosen for this experiment. The fish were maintained in one 45,000 litre fibreglass tank.

   The tank was heated when necessary to maintain the temperature above a minimum of 28°C as per section 2.1.2.

2. The fish were divided into two experimental groups:

   (i) Controls - there were no dedicated controls for this experiment. The experiment ran for three months and with the priorities of the hatchery lying with the production of larvae, the constant demand on the male broodstock for spawning made it impossible to reserve two or three fish for control treatment.

   The control fish reproductive score was based on the average condition of the remainder of the male broodstock

population that were untreated at the time.

(ii) Silicone pellets containing 10 mg 17-MT (N=3).

3. The silicone pellets were prepared according to the method detailed in section 2.3.4.

4. The reproductive condition of the fish was checked prior to implantation and again every 28 days for a total of 84 days. The maturation level of the fish was assessed as per the method and scoring system outlined in sections 2.2.4 to 2.2.7.

5. One of the fish was used in spermiation trial number 7, section 4.2, to assess the effect of LHRHa injections into fish that had been primed by exposure to 17-MT for 28 days.

3.6.3 Results

The results of experiment 6 are shown in Table 8.

The silicone pellets did not have a positive effect on the reproductive condition of the fish treated. The results indicate that if there was an effect then it may have been negative. During the second and third month of the experiment many of the untreated fish in the hatchery were able to have a sperm sample cannulated. The treated fish remained in regressed breeding condition during these months.

Table 8. The effect on spermatogenesis in barramundi of a single implantation of a slow release silastic pellet containing 17-MT, implanted in June 1992.

Fish No.	Weight	Treatment	Reproductive Condition			
			Day 0 June	Day 28 July	Day 56 August	Day 84 Sept.
untreated fish	various	Controls	F	F	S	S
780	4.6	17-MT	F	F	F	F
226	3.6	17-MT	S	F	F	F
2195	6.8	17-MT	S	F^1	F	F

F = fluid in cannula sample, S = sperm in cannula sample, F^1 = this fish used for spermiation trial number 7, section 4.2.

3.6.4 Discussion of Experiment 6

Based on this experiment, long term administration of 17-MT in silicone pellets does not represent a viable means of advancing maturation in male barramundi. All fish in this experiment were in a regressed breeding state after three months. This experiment further supports the results of spermatogenesis experiments, sections 3.1, 3.2 and 3.4, which suggested that 17-MT was not suitable for inducing or maintaining sperm production in barramundi.

As a footnote to this experiment, it was interesting to note that one of the fish treated in this trial proved to be among the best of the spawning males in the 1992/93 spawning season. This particular fish, 2195, participated in six separate spawnings between November 1992 and February 1993 and was still suitable for spawning when cannulated in late May 1993.

Prior to November, it was not possible to extract a cannula sample of sperm from this fish. It is feasible to suggest, that either, 17-MT, administered in silicone

pellets, takes a long time to enhance spermatogenesis and then has a major positive effect, or that the 17-MT actually interfered with reproductive development until November and the fish matured despite the implant, not because of it. Until further trials are undertaken, this will remain unclear.

3.7 EXPERIMENT 7, MAY/ JUNE 1992, ORAL ADMINISTRATION OF 17-MT

3.7.1 Introduction

Several studies on spermatogenesis in male teleosts have examined the effect of long term, oral administration of 17-MT (Weber and Lee 1985, Lee and Weber 1986, Lee *et al.* 1986d, Barry *et al.* 1991). The experiments of Weber and Lee (1985) were particularly successful. Male grey mullet were forced to mature within three weeks by daily administration of 12.5 mg of 17-MT. These fish were then maintained in a spermiating condition for 42 weeks by feeding them 17-MT three times per week. Lee *et al.* (1986d) induced premature spermatogenesis, but not spermiation, in immature milkfish by feeding them 12.5 mg of 17-MT daily for twelve weeks.

Barry *et al.* (1991), were not able to induce spermiation in male spotted scats, *Scatophagus argus*, by oral administration of 17-MT, however, fish that were implanted with LHRHa pellets (35 $\mu g\ kg^{-1}$), following 30 days of feeding with 10 mg kg^{-1} of 17-MT, spermiated copiously. LHRHa implants produced little or no response in fish not previously exposed to 17-MT. This result indicates that spermatogenesis in the scats was significantly enhanced by oral treatment with 17-MT.

In May 1992 a trial was undertaken to test the effect of oral administration of 17-MT to barramundi at the Darwin Aquaculture Centre.

3.7.2 Methods

The trial was conducted during May and June which is outside the normal period of breeding for barramundi.

1. Seven fish, ranging in size from 6.0 to 10.8 kilograms were chosen for this experiment.

2. The fish were fed 17-MT at the rate of 12.5 mg kg^{-1} body weight, every day for a period of 28 days.

   To make the diet, 17-MT was mixed with cod liver oil and injected into the fresh mullet fed to the broodstock. The hormone diet was prepared by first, totalling the weight of fish in the experiment and multiplying this figure by 12.5 mg to give the total weight of 17-MT to be fed per day. The daily ration of 17-MT was weighed and mixed with cod liver oil. The volume of cod liver oil used was calculated so that each mullet was injected with 0.2 ml. The number of mullet injected corresponded to the number of fish in the experiment, that is, one mullet per fish per day. If the fish required extra feed they were fed untreated mullet and squid.

3. The fish were fed between 12 noon and 1 pm every day.

4. At the end of the 28 day period the reproductive condition of the fish was examined and scored according to the system described in sections 2.2.4 to 2.2.7.

5. The fish were then used in a spermiation trial, experiment 7, section 4.2.

3.7.3 Results

The results for experiment 7 are shown in Table 9.

Four days before the conclusion of this experiment, 4 of the fish involved were stolen from the tank. The remaining 3 fish were used in spermiation trial, number 7, section 4.2, where two of them were identified as females.

Table 9. The effect on spermatogenesis in male barramundi of daily, oral, administration of 17-MT, at the rate of 12.5 mg kg^{-1}.

Fish No.	Weight	Treatment	Reproductive	Condition
		17-MT kg^{-1} day^{-1}	Day 0	Day 30
2150	8.0	12.5	F	F^1
2137	10.8	12.5	F	stolen
2110	10.0	12.5	F	stolen
2196	8.0	12.5	F	stolen
2200	6.8	12.5	F	stolen
2194	6.0	12.5	F	F^1
2180	8.4	12.5	F	F

F = only fluid sample obtained.
Stolen = four fish were stolen from the tank two days before the completion of the experiment. F^1 = fish subsequently found to be female.

3.7.4 Discussion of Experiment 7

Unfortunately the theft of most of the fish involved in this experiment, and the subsequent finding that two of

the remaining fish had changed sex to female made it impossible to conclude anything from this trial. The one remaining male fish was in regressed breeding condition at the conclusion of the experiment but it is possible that this was due to the trauma experienced when the other fish were forcibly taken from the tank. The only option available was to repeat the experiment 12 months later.

3.8 EXPERIMENT 8, MAY/ JUNE 1993, ORAL ADMINISTRATION OF 17-MT AND IMPLANTATION OF SILICONE PELLETS

3.8.1 Introduction

Experiment 8 repeated the method used in experiment 7. The fish were fed 17-MT in their diet, similar to the experiments of Weber and Lee (1985) and Lee *et al.* (1986d) This experiment also included fish treated in the same manner as experiment 6, where the fish were implanted with silicone pellets containing 17-MT.

One difference in this experiment was that two of the fish were implanted with silicone pellets containing 19-norethisterone acetate[3] (19-NE). 19-NE is a synthetic anabolic steroid which has androgenic activity (Varadaraj 1990). It has been found to be 15 times more potent than 17-MT in sex reversal studies in the Japanese medaka, *Oryzias latipes*, (Varadaraj 1990) and was included in this trial to compare its effect in inducing spermatogenesis against the effect of 17-MT.

[3] Purchased from Sigma Chemical company, catalogue number: N 6127.

3.8.2 Methods

The fish used in this experiment were divided into two groups. Experiment 8a fish received a silicone pellet implant containing steroid and experiment 8b fish were fed 17-MT injected in mullet.

The method for experiment 8a was:

1. Six fish, ranging in size from 7.0 to 11.0 kg were chosen for this experiment.

2. The fish were further divided into three groups:

   (i) Control - no treatment (N=2).
   (ii) Silicone pellet containing 10 mg 17-MT (N=2).
   (iii) Silicone pellet containing 10 mg 19-NE (N=2).

3. The silicone pellets were manufactured according to the method described in section 2.3.4.

4. The reproductive condition of the fish was checked prior to implantation and again on day 28. The reproductive condition of the fish was examined and scored according to the system described in sections 2.2.4 to 2.2.7.

5. The fish were then used in experiment 9, section 4.3, of the spermiation trials.

The method for experiment 8b was :

6. Five fish, ranging in size from 5.0 to 8.0 kg were chosen.

7. The fish were fed 17-MT at the rate of 12.5 mg kg^{-1} body weight, every day for a period of 28 days.

8. The diet was prepared and fed as described in section 3.7.2.

9. At the end of the 28 day period the reproductive condition of the fish was examined and scored according to the system described in sections 2.2.4 to 2.2.7.

10. The fish were then used in experiment 9, section 4.3, of the spermiation trials.

3.8.3 Results

The results for experiments 8a and 8b are shown in Table 10.

The results for both experimental groups were the same. None of the fish in this experiment were considered to be in breeding condition after 28 days of treatment. Sperm samples could not be cannulated from any of the fish at the conclusion of the experiment.

3.8.4 Discussion of Experiment 8

The results for experiments 8a and 8b were negative after one month. Implantation of a silicone pellet containing either 17-MT or 19-NE failed to maintain or induce spermatogenesis in the treated fish. These results confirm earlier experiments, sections 3.1, 3.2, 3.4, 3.6 and 3.7, and indicate that treatment with 17-MT is not a suitable or practical method for inducing maturation in male

barramundi either in or out of the normal breeding season. Although only trialled with two fish it would seem that 19-NE, a potent androgen, is also not suitable for breeding control in male barramundi.

Table 10. The effect on spermatogenesis in male barramundi of: silicone pellet implants containing either 17-MT or 19-NE or daily, oral, administration of 17-MT, at the rate of 12.5 mg kg^{-1}.

Fish No.	Weight	Treatment	Reproductive Condition	
			Day 0	Day 30
8a 782	9.0	Control	F	F
392	7.0	Control	S	F
701	8.0	17-MT	F	F
2195	8.8	17-MT	S	F
2152	11.0	19-NE	S	F
395	7.0	19-NE	F	F
8b 226	5.8	17-MT fed	F	F
227	8.0	17-MT fed	F	F
398	7.1	17-MT fed	S	F
780	5.0	17-MT fed	F	F
871	6.8	17-MT fed	F	F

F = fluid in cannula sample, S = sperm in cannula sample.

The silicone pellet implants are capable of releasing hormone for a very long time and it will be necessary to follow the progress of the treated males during the next spawning season. As mentioned in the discussion in section 3.6.4, one of the males previously treated with 17-MT in a silicone pellet became an excellent breeder 6 months after treatment. It remains to be seen if this pattern is repeated with fish from this experiment.

Oral administration of 17-MT also failed to induce spermatogenesis. It is obvious from this experiment that barramundi, unlike grey mullet and milkfish (Weber and Lee 1985; Lee *et al.* 1986d) are not suitable candidates for oral treatment with 17-MT. The results of this part of the experiment are consistent with those of Billard *et al.* (1982b) who found that oral treatment with 17-MT inhibited gametogenesis in male rainbow trout. 17-MT has also been found to also block spermatogenesis in the three-spined stickleback (Borg *et al.* 1986).

There were several obvious problems with oral treatment of barramundi not least of which was ensuring that each fish received its required dose of hormone each day. Quite often this was impossible to regulate as one fish would out compete the others and consume two or three. As it was not practical to house each fish individually it can only be assumed that the numbers of fish consumed averaged out over the course of the experiment. This was certainly indicated by the body weights of the fish before and after the trial. All fish remained approximately the same weight.

Further evidence for the failure of the treatments trialled in experiments 8a and 8b, was provided in experiment 9, section 4.3 of the spermiation experiments. Injections of Ovaprim did not induce spermiation indicating the fish were not mature.

It should also be noted that the oral administration of high doses of androgens such as 17-MT is potentially dangerous to the health of the fish. A feeding trial was conducted with juvenile barramundi at the Darwin Aquaculture Centre between May and June 1990. Apart from stunting the growth of the fish that received 17-MT at the rate of 12.5 mg kg^{-1}, the hormone may also have been

responsible for enlargement of their livers, spleens and kidneys (Schipp unpub. data). These symptoms are common in humans who abuse steroids and continued use can lead to organ disease or failure (Cedaro 1992). It is possible that continued use in fish may also lead to organ failure.

3.9 GENERAL DISCUSSION OF THE SPERMATOGENESIS EXPERIMENTS

The experimental series examining the effects of hormone therapy on the maturation rate of male barramundi failed to produce any results of practical significance for the barramundi farming industry. Although each of the experiments repeated experimental protocols used successfully overseas with barramundi or other species of fish, the results were not reproducible in Northern Territory barramundi. At this stage it would appear that environmental control (Thouard *et al.*, pers. comm.) offers the best chance for inducing and maintaining maturation in male barramundi.

The fact that none of the control fish matured when kept on their own in small heated tanks has implications for the environmental control of breeding. If environmentally controlled breeding is to be undertaken then these experiments indicate that male fish should be housed in a tank with a large volume and must not be kept separate from females. The interaction with the females is likely to be important because of their pheromonal influence (Suquet *et al.* 1992).

One of the recurring problems with this series of experiments was that several of the fish had undergone, or were undergoing, sex change to female and this was not able to be detected prior to the start of the trials. When

dealing with only a limited number of fish this can severely compromise the results of the experiment. Until reliable sex control or sex detection techniques become available this problem will remain. Larger sample sizes may have helped reduce the effect of including newly changed females in the experiments but this was beyond the budget or holding capacity of the hatchery.

Examination of sperm activity did not produce any differences between the different hormone groups. All fish that had sperm in their cannula samples had active sperm and could not be distinguished using the scoring system in section 2.2.7. This result is in accordance with Ngamvongchon *et al.* (1987) who did not detect any difference in sperm activity following hormone treatment in common carp. The only apparent difference detected by cannulation was in experiment 5 when three of the fish were more easily cannulated at the end of experiment than at the start. This was a subjective test and may be due to differences in cannula technique or the position of the cannula in the gonad.

If these experiments are to be repeated then it is important that the progress of the injections/ implants be followed at the molecular level using radioimmunoassay techniques so that the effect of the hormones can be more precisely understood. At this stage the daily, monthly and yearly variations in hormone levels for barramundi are not known, neither is the change in hormone profile during the sex change process.

As mentioned previously, this series of experiments followed successful experiments from overseas. None of these studies have examined the effects of injections of 11-ketotestosterone (11-KT) despite the reported importance of this steroid during the maturation phase of

many male fish (Koldras *et al.* 1990; Ueda *et al.* 1991). 11-KT is itself a potent androgen and has induced the formation of testicular tissue when implanted into female goldfish (Kobayashi *et al.* 1991). Future experiments may wish to investigate the effect of injections or implants using 11-KT.

CHAPTER 4.

HORMONE INDUCED SPERMIATION

4.1 IRRADIATED CARP PITUITARY EXTRACT

4.1.1 Introduction

Experiments conducted at the Darwin Aquaculture Centre during 1989 demonstrated that carp pituitary extract (CPE) injected at a concentration of 8 mg kg^{-1} body weight, was able to stimulate spermiation in male barramundi (Schipp *et al.* 1989).

Following on from these early studies, a series of experiments were planned in an attempt to refine the techniques of hypophysation in barramundi. The experiments aimed at determining the optimal dosage of CPE and the frequency of administration.

During 1990, the importation of CPE into Australia was prohibited by the Australian Quarantine and Inspection Service (AQIS). CPE was banned in the belief that its use in fish reproduction was a possible method of transmission for virus particles. After persistent lobbying, permission to import CPE was eventually given, providing the CPE was irradiated with gamma rays at a minimum strength of 60 kGys[4]. This suggested rate of irradiation was not believed to have any effect on the activity of the hormone (Australian Animal Health Laboratory, pers. comm.).

---

[4] 60 kGys (60 kiloGrays) is the new terminology for the expression of gamma irradiation strength. 1 megaRad (old terminology) = 10kGys.

Rather than attempt to refine the hypophysation technique, as proposed earlier, using the irradiated CPE (iCPE) the decision was made to retest the effectiveness of CPE once it had been irradiated. Unfortunately, the activity of the iCPE could not be compared with non-irradiated CPE as stocks of the latter were exhausted.

4.1.2 Methods

Trials testing the spermiation response of barramundi injected with iCPE at a dose rate of 8 mg kg^{-1} were performed in December 1990, August 1991 and February 1992. This corresponded to peak, early and late spawning season respectively.

The method followed was the same for each experiment. The experiments compared the effectiveness of two injections of iCPE, given 24 hours apart.

For each experiment:

1. Five mature male barramundi, ranging in size from 2.6 to 10.2 kg, were chosen for treatment.

2. The fish were divided into two groups:

   (i) Control (N=2) - untreated fish.
   (ii) iCPE treated (N=3) - injected with 8 mg kg^{-1} iCPE[5] in 0.9% sterile saline.

[5] The CPE was supplied by Argent Chemical Laboratories in Canada. and was irradiated at a strength of 60 kGys at the Australian Animal Health Laboratories, Geelong, Victoria, prior to delivery to Darwin.

3. The reproductive condition of the fish was assessed prior to injection and 24 hours later. The system for scoring reproductive condition is set out in sections 2.2.4 to 2.2.7.

4.1.3 Results

Experiment 1, December 1990 (refer to Table 11)

Injections of iCPE failed to induce a spermiation response in this experiment. Both control fish regressed in condition during the course of the experiment with only fluid being extracted in the cannula sample after 48 hours. The first treated male also regressed in condition. Thick sperm was hand stripped from the second treated fish prior to injection but the volume did not increase, nor the viscosity decrease, following injection. The spermatozoa stripped from this fish had an activity score of 80%, both before and after injection. The other treated male also failed to improve in breeding condition.

Experiment 2, August 1991 (refer to Table 12)

Two of the fish were in running ripe condition 24 hours after the first injection. They produced 0.5 and 0.3 ml of free flowing sperm, respectively. The second injection of iCPE did not induce the production of more sperm in these fish. When the fish were removed from the experimental tank, 4 days after the completion of this experiment, both these fish had running sperm again. The activity level of the sperm produced by these two fish was very high, > 80%.

No sperm was produced by the other treated fish or the two controls.

Experiment 3, February 1992 (refer to Table 13)

The condition of the fish remained constant throughout the experiment. All of the fish had sperm samples withdrawn by cannula before and during the experiment. There was no increase in quantity or quality following treatment The activity level of the sperm in the cannula samples remained between 60-80% for the three days.

Table 11. The effect on spermiation in male barramundi of two intramuscular injections of gamma irradiated CPE, given 24 hours apart during December 1990.

Fish No.	Weight	Treatment	Reproductive Condition		
			0 Hrs	24 Hrs	48 Hrs
072	6.0	Control	2	S	F
074	8.0	Control	S	F	F
088	7.9	iCPE	S	F	F
078	6.2	iCPE	2	2	2
067	6.0	iCPE	F	F	F

F = fluid in cannula sample, S = sperm in cannula sample, 2 = thick fluid sperm expressed by hand pressure.

Table 12. The effect on spermiation in male barramundi of two intramuscular injections of gamma irradiated CPE, given 24 hours apart during August 1991.

Fish No.	Weight	Treatment	Reproductive Condition		
			0 Hrs	24 Hrs	48 Hrs
2135	10.2	Control	S	F	F
2146	5.4	Control	S	F	F
2143	9.0	iCPE	S	3 (0.5)	F
2110	7.8	iCPE	S	3 (0.8)	F
2130	4.4	iCPE	F	N/S	F

F = fluid in sample, S = sperm in cannula sample, 3 = copious free flowing sperm, figure in brackets represents volume obtained in millilitres.

Table 13. The effect of two intramuscular injections of gamma irradiated CPE in male barramundi, given 24 hours apart during February 1992.

Fish No.	Weight	Treatment	Reproductive Condition		
			0 Hrs	24 Hrs	48 Hrs
713	4.0	Control	S	S	S
720	3.5	Control	S	S	S
709	2.6	iCPE	S	S	S
707	4.5	iCPE	S	S	S
701	4.0	iCPE	S	S	S

S = sperm in cannula sample.

4.1.4 Discussion of the iCPE Experiments

The most notable feature of the experiments investigating the effects of injections of iCPE was the variability of the results.

Injections of iCPE into mature male fish during December 1990, failed to elicit a spermiation response. Prior experiments at the Darwin Aquaculture Centre had demonstrated that injections of non-irradiated CPE into male fish during the spawning season normally produced a significant elevation in the quantity of milt available within 24 hours. If the activity of the hormone was not compromised by the radiation treatment, an elevation in milt levels would have been expected.

Injections of iCPE during August 1991 did elicit a strong spermiation response, with two of the three fish able to be hand stripped after 24 fours. This was comparable with results achieved previously, using non-irradiated CPE. The spermiation response could not be sustained after the

second injection which was also comparable to results achieved with normal CPE.

The third experiment, at the end of the 1991/92 breeding season again failed to induce spermiation after either injection. It is important to note that injections of LHRHa given to other fish at the same time as this experiment did produce a response.

This poses the question of why the variability?

There are several possible explanations as to why the activity of the iCPE and the response of the fish was so variable:

1. The first explanation is obviously that gamma radiation destroys or reduces the activity of the hormone. Staff at the Australian Animal Health Laboratory (AAHL) suggested that the radiation level of 60 kGys should not effect the activity of the hormone but they could not guarantee it. Very little is known about the effect of gamma irradiation on biological material (P. Le Blanc Smith, AAHL, pers. comm.). If the radiation was to blame, then it could reasonably be expected that the activity of all of the hormone would be affected, this does not explain why one trial worked and two did not. Although slight variations exist in the radiation dose distributed within the treatment vessel, this variation would be insufficient to explain the major differences observed between the experiments (P. Le Blanc Smith, AAHL, pers. comm.). It must be assumed that the radiation treatment was consistent in its effect and all bottles received an approximately similar dose. Without the benefit of an assay of hormone activity, it is impossible to speculate further on the effect of radiation on the hormone.

2. The variability in response may also lie with the fish. Even though the majority of the fish had a sperm sample withdrawn by cannulation prior to the commencement of the experiments, the amount of sperm stored in the testes and available for spermiation was not quantifiable. As standardised techniques for quantifying the reproductive condition and capability of the fish do not exist, this remains a problem with these types of experiments.

3. Two of the fish injected in the December 1990 had been treated with injections of CPE, six and 10 months prior to this trial. The development of specific immunity to repeated treatment with fresh pituitary preparations has been reported in other fish species (Donaldson and Hunter 1983). There is the possibility that fish used in the December trial had developed an immunity to CPE injections and therefore did not respond. This reasoning cannot be applied to the fish from the February trial as they had not previously received CPE.

4. Another factor affecting the results may be the hormone itself. The hormone supplied by Argent has a batch number but does not have details of the reproductive condition, sex or size of the donor fish. This lack of information on the source and likely activity of the hormone has long been recognised as a problem with the hypophysation technique (Shehadeh *et al.* 1973). The hormone was imported in ten, 1 mg bottles and a new bottle was opened for each trial. There is the possibility that there was a difference in the activity of the hormone from one bottle to the next. An assay of the activity of the hormone prior to the experiments would have been an advantage but was beyond the scope or the budget of the present study.

5. The fifth possibility is that the response of the fish to the hormone depends on the time of the month or year. Billard *et al.* (1987) demonstrated a diurnal difference in response to hormone injection and it is therefore feasible to suggest that a monthly cycle exists as well. Considering that barramundi are known to spawn naturally in accordance with the new and full moon (Garrett *et al.* 1987) it is perhaps a factor that deserves more attention when planning experiments using barramundi. Future research may examine what role, if any, the time of month plays in the response to the injection.

Prior to the enforcement of gamma irradiation, injections of CPE could be administered to barramundi with a reasonable assurance that a spermiation response could be elicited. The conclusion that can be drawn from the experiments investigating the effects of injecting iCPE into mature male barramundi is that it is now a technique that cannot be recommended with any confidence. Even though it is impossible to lay the blame solely on the irradiation process, it is certainly implicated. Notwithstanding the possible deleterious effects on hormone activity, the inclusion of the irradiation step into the import procedures has added an extra expense to an already expensive hormone.

The choices available to the researcher or farmer wishing to continue with hypophysation of male barramundi are that they either persevere with importing and irradiating CPE, accepting that the results are variable, or that they seek a local source of teleost pituitary material.

4.2 LHRHa

4.2.1 Introduction

Luteinising Hormone Releasing Hormone analogues (LHRHa) have become one of the major tools in artificial fish breeding since they were first synthesised in the early 1970's. Research using LHRHa has examined a wide range of variables including: analogue type, time of day and year, species of fish, hormone solvent/ carrier, combination with other hormones, dosage and method of administration.

Garrett and Connell (1991) indicate that injections of LHRHa into male barramundi at a concentration of 10-25 $\mu g\ kg^{-1}$, routinely increased milt quality and quantity. This has not been the case with barramundi broodstock at the Darwin Aquaculture Centre (DAC). Earlier studies on barramundi at the DAC (Schipp *et al.* 1989), compared the effect on spermiation of cholesterol pellet implants containing of 25 $\mu g\ kg^{-1}$ of LHRHa with LHRHa injections at the same concentration. Both methods of administration were equally effective and trials conducted during April, the end of the barramundi spawning season, induced small elevations in milt quality and quantity. Trials conducted in May, the non-spawning season, only increased milt quality. The variation in response between months indicates that further investigations are required into the treatment of male barramundi with LHRHa to maximise the effect on improving milt production.

Between September 1990 and February 1992, four separate experiments were conducted at the Darwin Aquaculture Centre to test the effectiveness of LHRHa in inducing spermiation in barramundi.

Experiment 4, September 1990, investigated the effect of the addition of pimozide to the LHRHa injection. As discussed in section 1.4.13 of the introductory chapter, pimozide, a dopamine antagonist, can be used to block the production of the gonadotrophin release inhibitory factor (GRIF) (Peter 1983). Several studies have shown that pimozide acts synergistically when used with LHRHa to induce ovulation in goldfish (Chang and Peter 1983), and trout (Billard *et al.* 1984) and to stimulate sperm production in carp, (Billard *et al.* 1987).

Experiments 5 and 6 compared the effect of administering LHRHa on its own, at two different times of the year, peak spawning season and the end of the spawning season respectively.

Experiment 7, investigated the effect of injecting LHRHa into fish that had been 'primed' for one month by continual dosing, either from a slow release pellet or by oral administration, of 17-MT. This experiment was based on the work of Barry *et al.* (1991) who induced spermiation in the male spotted scat, *Scatophagus argus*, by long term administration of 17-MT followed by an injection of LHRHa.

4.2.2 Methods

Trials testing the spermiation response of barramundi treated with LHRHa at a different dose rates were carried out in September 1990, December 1990, February 1992 and June 1992.

Experiment 4, September 1990 LHRHa + Pimozide.

In the first of the LHRHa experiments, 1 mg kg^{-1} of pimozide was combined with one of three doses of LHRHa. The experiment was based on the successful experiment with common carp reported by Billard *et al.* (1987).

The treated fish were compared with control, untreated fish. The shortage of male broodstock precluded the comparison of the pimozide treated fish with fish treated only with LHRHa.

1. Twelve mature male barramundi, ranging in size from 2.8 to 5.4 kg were selected at random and assigned to one of four experimental groups.

   (i) Control (N=3) - untreated fish.
   (ii) 25 µg LHRHa kg^{-1} + 1 mg pimozide kg^{-1}(N=3).
   (iii) 50 µg LHRHa kg^{-1} + 1 mg pimozide kg^{-1}(N=3).
   (iv) 100 µg LHRHa kg^{-1} + 1 mg pimozide kg^{-1}(N=3).

3. The reproductive condition of the fish was assessed prior to injection and again 24 hours post injection. The system for scoring reproductive condition in all four trials is described in sections 2.2.4 to 2.2.7.

4. LHRHa injections were prepared by adding one ml of 80% ethanol into a vial containing 5 mg of LHRHa[6] powder. The dose of LHRHa for each fish was calculated according to body weight and the appropriate volume of dissolved LHRHa was withdrawn from the vial using a micro pipette and mixed with 0.9% sterile saline. The hormone solution was loaded

[6] Analogue = des Gly^{10}[d-Ala^{6}]-LH-RH-ethylamide, Sigma Chemical Co., Cat. No. L4513.

into a 3 ml syringe ready for injection. The method used follows that of Takashima *et al.* (1984), with the addition of dissolving the LHRHa in alcohol.

5. The pimozide solution was prepared by first dissolving 50 mg of sodium metabisulphite in 50 ml of sterile saline. To this was added 100 mg of pimozide[7]. The saline/ metabisulphite solution was shaken vigorously to dissolve the pimozide and the appropriate volume was drawn into the syringe containing the LHRHa. The method of preparation of the pimozide solution follows Chang and Peter (1983).

6. The reproductive condition was examined prior to injection and again 24 hours post injection.

Experiments 5, 6 and 7 December 1990, February 1992 and June 1992.

Experiment 5 compared the effectiveness of a single injection of LHRHa[8] at the rate of 25 $\mu g\ kg^{-1}$ with untreated control fish. The experiment was performed during December 1990, the peak of the spawning season for barramundi and was undertaken simultaneously with an experiment assessing irradiated carp pituitary injections.

Experiment 6 compared the effectiveness of two successive injections of LHRHa given 24 hours apart at the rate of 25 $\mu g\ kg^{-1}$. The experiment was performed during February 1992, near the end of the spawning season for barramundi and was undertaken simultaneously with an experiment assessing injections of irradiated carp pituitary injections or testosterone.

---

[7] Sigma Chemical Co., Cat No. P1793.

[8] For the second and subsequent experiments, LHRHa was purchased from Peptide Technologies in Sydney, Australia.

Experiment 7 investigated the effectiveness of two successive injections of LHRHa, 24 hours apart at the rate of 25 μg kg^{-1}, into fish that had received 17-MT for a period of 30 days. Four fish were involved in this experiment, three of them had been fed 12.5 mg of 17-MT per day and the other fish had been implanted with a silastic pellet containing 10 mg of 17-MT. The number of fish available for this experiment was limited because 4 fish were stolen two days prior to commencement of the injections. This experiment was repeated twelve months later using Ovaprim.

The method followed was similar for each of the 3 experiments:

1. For experiments 5 and 6, five mature male barramundi, ranging in size from 3.3 to 8.0 kg were selected at random and assigned to one of two experimental groups. Four males were used in experiment 7 and there was only one experimental group.

   Experiments 5 and 6:
   (i) Control (N=2) - untreated fish.
   (ii) 25 μg LHRHa kg^{-1} (N=3).

   Experiment 7:
   (i) 25 μg LHRHa kg^{-1} (N=4).

2. The LHRHa injections were prepared in the same manner as those for experiment 1, without the inclusion of pimozide.

3. The reproductive condition of the fish was assessed prior to injection and again 24 hours post injection.

4.2.3 Results

Experiment 4 (refer to Table 14)

Three of the fish exhibited a slight increase in sperm volume following injection. Two of these fish had received 25 μg LHRHa plus 1 mg pimozide kg^{-1} and the other, 100 μg LHRHa plus 1 mg pimozide kg^{-1}. The other treated fish did not respond to hormone treatment.

Experiment 5 (refer to Table 15)

Two of the three treated fish maintained their reproductive condition following injection. Both expressed small quantities of thick fluid sperm. The other treated fish regressed in condition as did both controls.

Experiment 6 (refer to Table 16)

Two of the treated fish in experiment 6 showed an increase in sperm production. One of the fish produced small quantities of viscous milt after each injection and the other fish produced copious quantities of free flowing milt, 1.2 ml after the first injection and 0.6 ml after the second. The condition of all other fish in the experiment remained the same.

Experiment 7 (refer to Table 17)

Unfortunately two of the fish in this experiment were identified as female after the start of the experiment thus reducing the sample size to two. Neither of the two male fish responded to hormone treatment and they both stayed in regressed breeding condition.

Table 14. The effect on spermiation in male barramundi of a single intramuscular injection of various concentrations of LHRHa and 1 mg kg^{-1} pimozide given during September 1990.

Fish No.	Weight	Treatment		Reproductive Condition	
		µg kg^{-1} LHRHa	mg kg^{-1} pimozide	0 Hrs	24 Hrs
071	3.4	Control	1.0	S	F
058	5.4	Control	1.0	F	F
2147	2.8	Control	1.0	S	F
856	3.4	25	1.0	S	2
080	4.0	25	1.0	F	F
069	3.2	25	1.0	F	2
063	3.8	50	1.0	F	F
073	4.8	50	1.0	F	F
067	3.7	50	1.0	F	F
072	3.8	100	1.0	S	F
078	4.4	100	1.0	S	1
2150	2.8	100	1.0	F	F

F = fluid only in cannula, S = small amount of sperm in cannula, 1 = traces of viscous sperm expressed by hand pressure, 2 = thick fluid sperm expressed by hand pressure.

Table 15. The effect on spermiation in male barramundi of a single intramuscular injection of 25 µg kg^{-1} LHRHa given during December 1990.

Fish No.	Weight	Treatment	Reproductive Condition	
			0 Hrs	24 Hrs
072	6.0	Control	2	S
074	8.0	Control	S	F
856	5.0	25 µg LHRHa	2	2
2137	7.2	25 µg LHRHa	S	F
2126	6.0	25 µg LHRHa	2	2

F = fluid only in cannula, S = small amount of sperm in cannula, 2 = thick fluid sperm expressed by hand pressure.

Table 16. The effect of two intramuscular injections of 25 µg kg^{-1} LHRHa in male barramundi, given 24 hours apart during February 1992.

Fish No.	Weight	Treatment	Reproductive Condition		
			0 Hrs	24 Hrs	48 Hrs
713	4.0	Control	S	S	S
720	3.5	Control	S	S	S
787	3.6	25 µg LHRHa	S	2	2
782	4.5	25 µg LHRHa	S	3(1.2)	3(0.6)
711	3.3	25 µg LHRHa	S	S	S

S = small amount of sperm in cannula, 1 = viscous sperm expressed by hand pressure, 2 = fluid sperm expressed by hand pressure, 3 = copious free flowing sperm expressed by hand pressure. Figures in brackets refer to volume of sperm obtained in ml.

Table 17. The effect of two intramuscular injections of 25 µg kg^{-1} LHRHa into male barramundi, previously primed by treatment with 17-MT for 30 days. The injections of LHRHa were given 24 hours apart during February 1992.

Fish No.	Weight	Treatment	Reproductive Condition		
			0 Hrs	24 Hrs	48 Hrs
2194	6.0	50 µg LHRHa	F	female	-
2195*	2195	50 µg LHRHa	F	F	F
2180	2180	50 µg LHRHa	F	F	F
2150	2150	50 µg LHRHa	F	F	female

* = fish that had been treated with 17-MT using a slow release silastic pellet. All other fish given 17-MT orally.

F = Fluid in cannula sample, female = sample of oocytes withdrawn in the cannula, sex of fish had previously been impossible to determine.

4.2.4 Discussion of the LHRHa Experiments

The experiments investigating the effect of injections of LHRHa achieved variable results similar to those already discussed for carp pituitary extract.

Experiment 4 examined the effect of the inclusion of 1 mg of pimozide with the LHRHa. The results show that the spermiation response of the fish to these injections was moderate. Three out of the nine fish treated exhibited an increase in milt volume after 24 hours. Two of these fish had received 25 μg LHRHa kg^{-1} and the other 100 μg kg^{-1}. The dose rate of 25 μg kg^{-1} produced a slightly better response than 100 μg kg^{-1}, with the sperm being more fluid and more easily expressed. This result confirms earlier trials at the DAC (Schipp and Kuo, unpublished data) that showed that 25 μg kg^{-1} was a suitable dose for LHRHa in male barramundi, higher doses did not produce a better response and could be seen as wasting hormone. Our results compare favourably with those reported by Garrett and Connell (1991) who recommend a dose rate of between 10 to 25 μg kg^{-1}.

The significant spermiation response observed when LHRHa and pimozide were injected into common carp by Billard *et al.* (1987), was not repeated in barramundi. Based on this trial, the inclusion of pimozide in injections of LHRHa could not be recommended. This may be just as well given that pimozide is a potentially hazardous chemical. Billard *et al.* (1987) also caution against the continued use of high doses of pimozide. Future experiments may examine the use of other dopamine antagonists such as eglonil and metoclopramide (Glubokov *et al.* 1991).

Experiment 5, conducted in December 1990 produced a mild response to LHRHa injections. Two of the three fish

treated maintained their level of spermiation over the 24 hour period and the third decreased in condition.

Experiment 6, conducted in February 1992, produced the best results of all of the LHRHa trials. One of the three fish treated was induced to produce free flowing sperm over a 48 hour period and another produced expressible sperm, but not in a measurable quantity.

The result in the experiment 7 was a good example of what can go wrong with barramundi experiments. Unfortunately two of the 'males' in this experiment had recently changed sex and this was not detected by the cannula sampling technique. The recent spawning history of the fish indicated that they should be males so this experiment demonstrates the speed with which sex change can take place. Premature sex change was a recurring problem with the spermatogenesis and spermiation experiments and made the interpretation of some of the results difficult because of the extremely small sample size. This is a problem that can only be overcome or alleviated by increasing the number of fish in each experimental group. The fact that two of the four fish were female, combined with the loss of four of the fish intended for this experiment, made the results of limited value. Certainly the two fish that did survive and were definitely male, did not respond to the injections of LHRHa. Whether this was due to them not being in breeding condition or still suffering stress from when the other fish were netted is unclear.

Explanations for the variability in response to injections of LHRHa are likely to be very similar to those described for iCPE. The inability to accurately quantify the condition of the fish prior to experimentation is a major disadvantage for this study and the issue of variation in

response according to the time of the month is also one that must be addressed for LHRHa.

The present experiments were very limited both in their scope and the number of fish available for experimentation. One of the major limitations was that it was only possible to examine the effects of two analogues. The analogue used in section 4.2, des Gly^{10}[d-Ala^{6}]-LH-RH-ethylamide, was chosen because it has commonly been used in previous studies with reasonable results (Billard *et al.* 1983; Weil and Crim 1983; Lee *et al.* 1986a; Garcia 1990). and it was readily available in Australia. In section 4.3, two trials were conducted testing a commercial hormone preparation containing an analogue of salmon gonadotrophin releasing hormone.

There is a definite requirement for further investigations into the spermiation response of male barramundi to treatment with LHRHa. Two of the main areas that need to be examined are the response to different analogues and to injections at different times of the lunar cycle during the spawning season. It is also suggested that further attention be paid to the use of analogues of fish releasing hormones as these have potentially higher activity levels (Zohar 1986). The optimum dose rate would have to be determined for each new analogue tested.

4.3 OVAPRIM

4.3.1 Introduction

Syndel Laboratories in Canada recently began selling a product that contains an analogue of salmon gonadotrophin releasing hormone (sGnRHa) in combination with domperidone, a dopamine antagonist, known as Ovaprim-SC®.

When used in several carp species it has yielded far superior results to hypophysation (Harker 1992).

Syndel Laboratories do not give information on the analogue or concentrations of sGnRHa and domperidone in Ovaprim. Harker (1992) reports that the dose rate of Ovaprim-SC is species dependent and in females ranges from 0.25 - 0.7 ml kg^{-1} and in males from 0.1-0.2 ml kg^{-1}. Only a single injection is required.

Ovaprim has been used in attempts to stimulate sperm production in barramundi broodstock at the Northern Fisheries Research Centre in Cairns. When administered at the dose rate recommended by Syndel Laboratories, 1.0 ml kg^{-1}, a number of the broodstock died post treatment (R. Garrett, Northern Fisheries Research Centre, Cairns, pers. comm.). When administered at a lower dose, 0.1 - 0.5 ml kg^{-1}, a significant increase in sperm production resulted. In February and June 1993, two experiments were conducted at the Darwin Aquaculture Centre to test the effect on spermiation in barramundi of two injections of Ovaprim-SC given 24 hours apart at the rate of 0.1 ml kg^{-1}.

4.3.2 Methods

Experiments 8 and 9

Experiment 8 was performed during February 1993, near the end of the barramundi spawning season and was conducted simultaneously with an experiment assessing the injections of 17α-20βP.

Experiment 9 was conducted in June 1993, during the non-spawning season. The fish used in this experiment had previously been primed with either 17-MT, fed orally or

implanted, or 19-NE, implanted in a silicone pellet (Chapter 3, section 3.8).

The method followed for the experiments was:

1a. In experiment 8, eight male barramundi ranging in size between, 4.2 and 10.2 kilograms were chosen and divided into two experimental groups,

(i) Control (N=2) - untreated fish.
(ii) Ovaprim-SC treated 0.1 ml kg^{-1} (N=6).

1b. In experiment 9, eleven male barramundi ranging in size between, 5.0 and 11.0 kilograms were chosen and divided into two experimental groups,

(i) Control (N=2) - untreated fish.
(ii) Ovaprim-SC treated 0.1 ml kg^{-1} (N=9).

2. The fish were given two injections 24 hours apart.

4. The reproductive condition of the fish was examined prior to injection and again 24 hours post injection. A further examination was performed 48 hours after the second injection. The scoring system for both experiments is described in sections 2.2.4 to 2.2.7.

4.3.3 Results

Experiment 8 (refer to Table 18)

One of the treated fish produced small quantities of viscous milt after the first injection This fish maintained this condition 24 and 48 hours after the second

injection. None of the other fish in the experiment produced expressible sperm

Experiment 9 (refer to Table 19)

Hormone treatment failed to induce a spermiation response in any of the treated fish in experiment 9. All fish were in a regressed breeding condition prior to injection and maintained this condition after treatment.

Table 18. The effect on spermiation in male barramundi of two intramuscular injections, of Ovaprim-SC at the rate of 0.1 ml kg^{-1}, during February 1993

Fish No.	Weight	Treatment	Reproductive Condition			
			0 Hrs	24 Hrs	48 Hrs	72 Hrs
783	7.0	control	F	F	F	F
227	6.8	control	S	F	F	F
780	4.2	0.1 ml kg^{-1}	S	2	2	2
063	10.2	0.1 ml kg^{-1}	F	F	F	F
871	6.4	0.1 ml kg^{-1}	F	F	F	F
392	6.0	0.1 ml kg^{-1}	F	S	F	S
701	6.5	0.1 ml kg^{-1}	F	F	F	F
782	7.5	0.1 ml kg^{-1}	S	S	F	S

F = fluid in cannula sample, S = small amount of sperm in cannula, 2 = fluid sperm expressed by hand pressure.

Table 19. The effect on spermiation in male barramundi of two intramuscular injections of Ovaprim at the rate of 0.1 ml kg^{-1}, during June 1993

Fish No.	Weight	Treatment	Reproductive Condition		
			0 Hrs	24 Hrs	48 Hrs
782	9.0	Control	F	F	F
392	7.0	Control	F	F	F
701	8.0	Ovaprim	F	F	F
2195	8.8	Ovaprim	F	F	F
2152	11.0	Ovaprim	F	F	F
395	7.0	Ovaprim	F	F	F
226	5.8	Ovaprim	F	F	F
227	8.0	Ovaprim	F	F	F
398	7.1	Ovaprim	F	F	F
780	5.0	Ovaprim	F	F	F
871	6.8	Ovaprim	F	F	F

F = fluid in cannula sample, S = small amount of sperm in cannula, 2 = fluid sperm expressed by hand pressure.

4.3.4 Discussion of the Ovaprim Experiments

The first trial on the effect of Ovaprim indicated that this hormone has potential in inducing spermiation in barramundi. The trial showed that one of the two fish that had a sperm sample withdrawn by cannula prior to the experiment could be induced to produce a mild spermiation response within 24 hours and maintain it for a further forty eight hours. None of the other hormones tested for the present study could maintain spermiation for this length of time.

The second spermiation experiment using Ovaprim produced a negative result but this is unlikely to be the fault of the hormone. The fish for the second experiment were

derived from experiments 8a and 8b of the spermatogenesis experiments. These fish did not mature following long term hormone therapy and therefore were not capable of spermiating. The results for this experiment do not support the finding of Barry *et al.* (1991) who were able to induce a significant spermiation response in scats using chronic steroid treatment followed by injections of LHRHa.

The use of Ovaprim was approached with some caution because it was responsible for the death of broodstock at the Northern Fisheries Research Centre in Cairns. For this reason a very low dose of hormone was trialed. Further investigations should examine the effect of slightly higher dose levels to see if the spermiation response can be improved. At the moment, the use of Ovaprim holds promise but the response generated to the dose rate injected in these trials is of limited value.

4.4 17α, 20β-DIHYDROXY-4-PREGNEN-3-ONE

4.4.1 Introduction

The progesterone derivative, 17α, 20β-dihydroxy-4-pregnen-3-one (17α-20βP) has been implicated in the spermiation process in teleost fish (Ueda *et al.* 1983; Nagahama 1987). Plasma levels of 17α-20βP have been found to be low during spermatogenesis and dramatically elevated at the onset of spermiation in several salmonids and the goldfish (Ueda *et al.* 1983; Nagahama 1987).

Despite its implied role in spermiation it was not until 1985 that tests were carried to investigate the effect on spermiation of *in vivo* injections of 17α-20βP. Ueda *et al.* (1985) used injections of 17α-20βP at the rate of 1.0 mg

kg^{-1} to induce precocious spermiation in amago salmon and goldfish.

Treatment of male barramundi with injections of 17α-20βP has not been attempted before. In February 1993, an experiment was conducted at the Darwin Aquaculture Centre to test the spermiation response of male barramundi to two injections, 24 hours apart, of 17α-20βP at the rate of 1.0 mg kg^{-1}.

4.4.2 Methods

Experiment 10

The experiment was performed during February which is near the end of the barramundi spawning season and was conducted simultaneously with an experiment assessing the injections of Ovaprim.

1. Four mature male barramundi, ranging in size from 5.0 to 7.0 kg, were used for this trial and were divided into two experimental groups:

   (i) Control (N=2) - untreated fish.

   (ii) 17α-20βP[9] treated (N=3) - 1.0 mg kg^{-1}. (The steroid was first dissolved in a small amount of 100% ethanol and then mixed with 0.9% saline).

3. The reproductive condition of the fish was examined prior to injection and again 24 hours later. A further examination was performed 48 hours after the

[9] 17α, 20β-diOH-prog was purchased from the Sigma Chemical Company. Catalogue number P 6285.

second injection. The system for scoring reproductive condition is described in sections 2.2.4 to 2.2.7.

4.4.3 Results

Experiment 10 (refer to Table 20)

One of the treated fish expressed a small amount of sperm after 24 hours. The volume of sperm increased after the second injection with 0.8 ml being expressed after 48 hours. None of the other fish in the experiment produced milt.

Table 20. The effect on spermiation in male barramundi of two intramuscular injections, 24 hours apart, of 17α-20βP at the rate of 1.0 mg kg^{-1}, during February 1993.

Fish No.	Weight	Treatment	Reproductive Condition			
			0 Hrs	24 Hrs	48 Hrs	72 Hrs
783	7.0	control	F	F	F	F
227	6.8	control	S	F	F	F
226	5.0	17α-20βP	S	2	3(0.8)	S
390	6.1	17α-20βP	F	F	F	F

F = fluid in cannula sample, S = small amount of sperm in cannula, 2 = fluid sperm expressed by hand pressure, 3 = copious free flowing sperm expressed by hand pressure. Figures in brackets refer to volume of sperm obtained in ml.

4.4.4 Discussion of the 17α-20βP Experiment

From this experiment it was clear that spermiation can be induced in mature male barramundi by an injection of 17α-20βP at the rate of 1 mg kg^{-1}. This result supports the finding of Ueda *et al.* (1985) that injections of 17α-20βP can be used to induce precocious spermiation. It also adds

support to the suggestion that 17α-20βP is naturally involved in the spermiating process (Nagahama 1987).

Previously the role of 17α-20βP in spermiation has been recognised in salmonids (Ueda *et al.* 1983; Ueda *et al.* 1985); goldfish (Ueda *et al.* 1985; Kobayashi *et al.* 1986, as quoted by Nagahama 1987); eels (Yamauchi 1990) and wrasse (Hourigan *et al.* 1991). Now that 17α-20βP has been used to induce spermiation in barramundi, a perciform fish and phylogenetically distant to the others, it implies that the importance of this steroid is widespread throughout the teleost orders.

Further investigations using 17α-20βP may be used to examine the effects of combining it with other hormones and using higher dose rates. Jalabert *et al.* (1977) used 17α-20βP to induce spawning in common carp but the hormone was only effective in inducing ovulation when it had been preceded by a priming dose of pituitary extract. It would therefore be interesting to compare the spermiation results produced by fish receiving injections of 17α-20βP, with those in which the fish received a priming dose of LHRHa or CPE followed by 17α-20βP.

One of the major problems with using 17α-20βP is that the hormone is very expensive. This problem has also been noted by other researchers (Richter *et al.* 1985). One way of reducing the cost may be to inject 17α-hydroxy-progesterone (17αP) instead of 17α-20βP. 17αP is a precursor of 17α-20βP and is much cheaper and more readily available (Richter *et al.* 1985). Injections of 17αP have been used to induce spermiation in goldfish (Billard 1976). It is believed that 17αP, once injected, is converted to 17α-20βP, which then stimulated spermiation (Nagahama 1987).

Future investigations into spermiation in barramundi may also look at the effect of using injections of 17αP.

4.5 SUSTANON '250'

4.5.1 Introduction

Juario *et al.* (1981) demonstrated that a single intramuscular injection of Durandon Forte '250', a long acting testosterone preparation (N.V. Organon Oss, Holland) induced spermiation in male milkfish. Males injected with Durandon Forte '250' produced copious milt, with high sperm motility for up to four days and were in good running condition for a maximum of seven days.

Durandon Forte '250' is supplied in 1.0 ml ampoules, containing arachis oil as the solvent and the testosterone esters, propionate (30 mg), phenylproprionate (60 mg), isocaproate (60 mg) and decanoate (100 mg) (Lacanilao *et al.* 1985).

At the time of the present experiments the name Durandon Forte '250' had been replaced by its manufacturers, Organon, with Sustanon '250'. The concentrations of testosterone esters and the type of solvent remained the same.

Two experiments were conducted at the Darwin Aquaculture Centre to test the effectiveness of Sustanon '250' in inducing spermiation in barramundi. Experiment 11 was conducted in August 1991, at the start of the barramundi breeding season and experiment 12 was carried out during February 1992, at the end of the breeding season.

4.5.2 Methods

Experiments 11 and 12, August 1991 and February 1992

Experiment 11 was undertaken simultaneously with an experiment assessing irradiated CPE injections. Experiment 12 was undertaken simultaneously with an experiment assessing LHRHa and irradiated CPE injections

The method followed was:

1a. For experiment 11, five male barramundi between 4.0 and 10.2 kilograms were chosen and placed into 2 experimental groups:

(i) Control (N=2), untreated fish.
ii) Sustanon '250' treated (N=3), 1.0 ml per fish.

1b. For experiment 12 five male barramundi between 3.2 and 4.3 kilograms were chosen and placed into 3 experimental groups:

(i) Control (N=2), untreated fish.
(ii) Sustanon '250' treated (N=2), 1.0 ml per fish.
(iii) Sustanon '250' treated (N=2), 0.5 ml per fish.

2. The reproductive condition of the fish was assessed prior to injection and again 24 hours and 48 hours post injection. The system for scoring reproductive condition is described in sections 2.2.4 to 2.2.7.

4.5.3 Results

Experiment 11 (refer to Table 21)

The hormone failed to induce a spermiation response in the treated fish. All fish in the experiment were in regressed breeding condition after 24 hours.

Experiment 12 (refer to Table 22)

There was no difference in response between the treated and control fish. All fish stayed at the same level of reproductive condition during the course of the experiment. Examination of sperm activity did not reveal any differences between control and treated fish.

Table 21. The effect on spermiation in barramundi of a single intramuscular injection of Sustanon '250' at the rate of 1.0 ml $fish^{-1}$, during August 1991.

Fish No.	Weight	Treatment	Reproductive Condition		
			0 Hrs	24 Hrs	48 Hrs
2135	10.2	Control	S	F	F
2146	5.4	Control	S	F	F
2122	5.2	Sustanon	S	F	F
078	7.4	Sustanon	S	F	F
736	4.0	Sustanon	S	F	F

S = sperm in cannula sample. F = fluid in cannula sample.

Table 22. The effect on spermiation in barramundi of a single intramuscular injection of Sustanon '250' at the rate of either 1.0 ml or 0.5 ml $fish^{-1}$, during February 1992.

Fish No.	Weight	Treatment	Reproductive Condition		
			0 Hrs	24 Hrs	48 Hrs
713	4.0	Control	S	S	S
720	3.5	Control	S	S	S
780	3.7	1.0 ml Sustanon	S	S	S
712	3.2	1.0 ml Sustanon	S	S	S
705	4.3	0.5 ml Sustanon	S	S	S
716	3.6	0.5 ml Sustanon	S	S	S

S = sperm in cannula sample.

4.5.4 Discussion of the Sustanon '250' Experiments

Injections of Sustanon '250' failed to elicit a spermiation response in the fish tested. The failure to produce a response may be due to one of the following four reasons:

1. The reproductive condition of the fish may have been insufficient for a response to be generated. It is known that Sustanon '250' is only effective in inducing spermiation in mature fish (Lacanilao *et al.* 1985). The difficulty in accurately staging the condition of the fish has already been discussed, however it is unlikely to be a factor here as all fish tested had a sperm sample easily withdrawn from the testes prior to injection. This should indicate that the fish were capable of being forced to spermiate. Other fish receiving LHRHa injections at the same time were induced to produce sperm.

2. The dose rate injected may have been insufficient to induce spermiation. The two experiments on Sustanon '250' used the dose rate found to be effective in milkfish with a body weight of 5-6 kg. As the barramundi tested were approximately the same size and generally, doses for spawning induction using other hormones, such as LHRHa, are very similar between barramundi and milkfish, it was assumed that rate of 1.0 ml per fish would be sufficient.

3. High doses of hormones are thought to be capable of interfering with or suppressing reproduction (Peter 1980). The dose rate of 0.5 ml kg^{-1} was trialed on two fish during the second experiment. The result was negative indicating that it was unlikely that the higher dose was not working because it was suppressing the reproductive system.

4. The fourth explanation for failure is that testosterone esters are not suitable for inducing sperm production in barramundi. Several other species of fish have exhibited poor or non-existent responses to treatment with testosterone: goldfish injected with testosterone propionate (Billard 1976); spotted scats implanted with 17-MT (Barry *et al.* 1991); green sunfish injected with testosterone propionate (Quillier and Labat 1977, as quoted by Donaldson and Hunter 1983).

At this stage, Sustanon '250' cannot be considered as a useful tool for inducing spermiation in barramundi. Further trials may investigate the effects of higher dose rates and a longer series of injections.

4.6 GENERAL DISCUSSION ON SPERMIATION EXPERIMENTS

Based on the results achieved in these trials the indications are that Ovaprim, LHRHa and 17α-20βP (and

possibly 17αP) offer the best prospects for the development of a method for the enhancement of sperm production in barramundi. It is very difficult to draw definitive conclusions as a result of this experimental series and even though some of the hormone treatments failed to produce a positive result, it is possible that alterations to the experimental protocol, (increasing the dose rates, changing the time of month or day of the treatment, increasing the period of administration) may be successful.

These experiments should only be viewed as a starting point. Further experimentation is needed to confirm or deny the results achieved and to investigate the consequences of changing the methods.

CHAPTER 5

SPAWNING

5.1 EFFECT OF HORMONE TREATMENT ON MALE SPAWNING PERFORMANCE

5.1.1 Introduction

Concurrent with experiments testing the effect on spermiation in barramundi of various doses and types of reproductive hormones, trials were carried out using the hormones under actual spawning conditions. Some of the male fish used in the spawnings were injected with reproductive hormones to see if the reproductive performance of the fish, as determined by both spawning frequency and fertilisation rate, was enhanced.

Between January 1990 and May 1993, sixty barramundi spawnings, with a total of 102 spawning nights, were conducted at the Darwin Aquaculture Centre. During these spawnings various hormone therapies were administered to male and female fish in an attempt to develop a protocol for reliable breeding success. For each spawning, data was recorded on the egg number released, egg quality at time of release, fertilisation rate and number of nights of spawning. Results for each of the hormone treatments were then compared with those of fish that had not received hormone therapy.

Summary tables for each the spawnings are listed in Appendix 3.

5.1.2 Methods

Over the past four years the method for setting up a spawning has varied quite considerably. Early spawning attempts were based on hand stripping of milt and eggs from the fish and generally produced poor results. Tank spawning first succeeded in January 1990 and then became the preferred method.

Once the first successful spawning for each season was achieved, it became possible to alter the method of the spawnings and experiment with various hormone treatments and sex ratios.

The generalised method for inducing the fish to spawn was:

1. The reproductive system of the fish was examined by cannulation, as per the method in sections 2.2.3 and 2.2.4. females chosen for spawning had to have an average oocyte diameter of greater than 410 μm and males had to have a sperm sample extracted.

2. Suitable male and female fish were then weighed and returned to a holding tank pending the preparation of the hormone treatments.

3. Female fish were always treated with hormones, usually with LHRHa either by injection or cholesterol pellet.

4. Male fish received one of a variety of treatments based on results of the spermiation trials described in Chapter 4.

   The males received either: no treatment and were put straight in with the female; injections of carp

pituitary extract, either a single dose or two doses 24 hours apart at the rate of 8 mg kg^{-1}; LHRHa at the rate of 25 or 50 µg kg^{-1}; LHRHa at the rate of 25 µg kg^{-1} plus 1 mg kg^{-1} of pimozide; or Ovaprim SC at the rate of 0.1 ml kg^{-1}.

5. Once the fish had received their respective treatments they were put into one of several 7,000 litre fibreglass tanks situated outside the hatchery or into a 45,000 litre fibreglass tank situated inside the hatchery. All tanks were equipped with flow through salt and fresh water supplies and aeration.

6. During the early experiments, the ratio of males to females varied from 1:1 to 4:1. Later experiments used either 2:1 or 3:1.

7. Unless the males were to receive a second injection the fish were left undisturbed in the spawning tanks. The fish usually spawned within 36 hours of treatment. Close to the time of spawning, the water supply to the tanks was turned off to prevent loss of eggs.

8. After the fish had spawned the eggs were left to develop for one hour or until they had reached the 64 cell stage. Egg number was estimated by counting the eggs in several, repeat, 50 ml aliquots and multiplying the average number per litre by the volume of water in the tank.

9. Egg quality and fertilisation rate were examined microscopically.

10. The eggs were collected and distributed to hatching tanks following routine hatchery procedures.

5.1.3 Results

A summary of the results of four years of spawning trials is contained in Table 23.

Table 23. Summary of four years of spawning data for male barramundi at the Darwin Aquaculture Centre.

Male Treatment	Number of Spawning Nights	Average Fertilisation Rate (±SD)	Number of Failures
Nil Treatment	53	67 ± 33	8 (er) 2 (ns)
CPE	22	72 ± 27	2 (er)
25 LHRHa + pim	4	64 ± 6	1 (ns)
25 LHRHa	5	65 ± 23	1 (er) 1 (ns)
50 LHRHa	8	54 ± 40	2 (er)
Ovaprim	10	31 ± 33	5 (er)

CPE = carp pituitary extract, usually administered at 8 mg kg^{-1}, 25 LHRHa + pim = 25 µg LHRHa kg^{-1} + 1 mg pimozide kg^{-1}, 25 LHRHa = 25 µg LHRHa kg^{-1}, 50 LHRHa = 50 µg LHRHa kg^{-1}, Ovaprim = Ovaprim SC at 0.1 ml kg^{-1}.
er = eggs released and fertilisation rate less than 10%.
ns = failed to spawn.

Figure 8 shows the monthly average of egg fertilisation rates achieved for each of the 6 male hormone treatments tested during the spawning season.

The majority of the spawnings were carried out between September and February of each year, which coincides with the normal breeding season for barramundi in the Darwin area. On one occasion, August 1991, the spawning was achieved earlier than in the wild with the aid of experiments conducted on the control of breeding in female fish (Kuo 1991). The actual duration of the breeding season varied from year to year and appeared to be related to the temperature of the water. In years when the water temperature remained high, above 28°C, it was possible to breed the fish in late April.

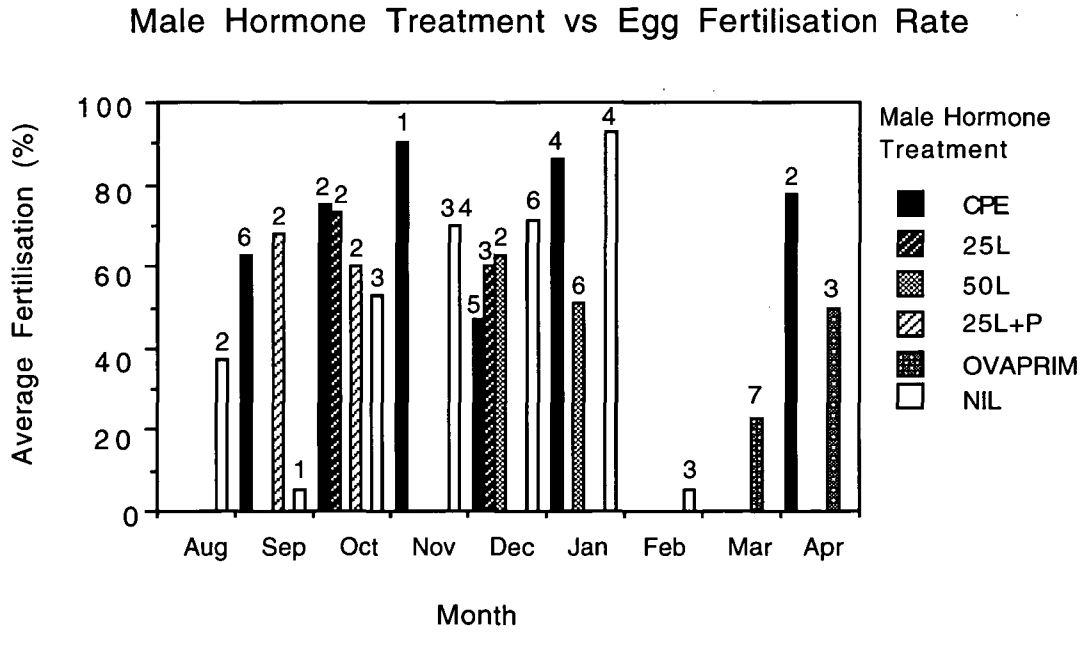

Figure 8. Average fertilisation rate following hormone treatment. Results are shown for each month of the barramundi spawning season.

CPE = carp pituitary extract, usually administered at 8 mg kg^{-1}, 25L = 25 µg LHRHa kg^{-1}, 50L = 50 µg LHRHa kg^{-1}, 25L+P = 25 µg LHRHa kg^{-1} + 1 mg pimozide kg^{-1}, Ovaprim = Ovaprim SC at 0.1 ml kg^{-1}.

The numbers above the columns indicate the number of spawning nights. Not all hormone treatments have been tested for every month of the spawning season.

5.2 DISCUSSION OF THE SPAWNING TRIALS

5.2.1 Effect of Hormone Treatment on Male Performance

From the data presented in Table 23 and Figure 8 there does not seem to be a relationship between hormone treatment and fertilisation rate. While male fish have successfully fertilised eggs after receiving any one of the hormone therapies, fish that have not received hormone treatment have still spawned successfully in five of the nine months of the spawning season. During the other four months, non-treated males have either been tested only once or not at all and so it is possible that they may also spawn successfully during these months.

It is very important to note that only one of the non-treated fish, August 1991, was in a 'running ripe' condition prior to pairing with an induced female. Previously it has been reported that male barramundi need to have running milt to be chosen for spawning (Kungvankij 1987), this appears to be unnecessary. All that is required is for the male fish to have a sample of sperm withdrawn by cannula prior to addition to the spawning tank. The withdrawal of a sperm sample ensures two things, one, that the fish is still a male and two, that it is reproductively mature and capable of spermiating.

An explanation for successful spawning of non-spermiating, non-hormone induced males, probably lies with their close interaction with the ovulating female prior to and during spawning. It has already been reported that male fish exposed to ovulating females can experience a rise in plasma GtH and spermiation levels (Kyle *et al.* 1985). The influence of the female can be both physical, as in courting behaviour providing direct stimulus to the males (Partridge *et al.* 1976; Garrett and Connell 1991) and

chemical, as in the release of pheromone-like compounds (Liley and Stacey 1983; Billard *et al.* 1989) and it is reasonably well established that the latter influences the former (Liley and Stacey 1983).

The progestin, 17α-20βP, has been identified as one of the most important steroids involved in final oocyte maturation and ovulation in female teleosts (Nagahama 1987). A close association has also been observed between 17α-20βP and spermiation in several fish species, including salmonids, (Ueda *et al.* 1983); goldfish, (Nagahama 1987) and barramundi (section 4.4, this study). Billard *et al.* (1989) observed a possible pheromonal stimulation of milt production in common carp and suggested that 17α-20βP may be responsible. A similar suggestion was made by Liley and Rouger (1990) with regard to synchronisation of spawning behaviour in the goldfish.

It is feasible that pheromonal stimulation exists in barramundi and the non-spermiating males are stimulated to release sperm by being in the presence of an ovulating female. While 17α-20βP is implicated in this process, its actual role would have to be elicited from further studies following spawning at the molecular level.

Apart from the evidence on the pheromonal enhancement of spermiation it is also possible that chemical and social interaction may be important during spermatogenesis - a priming effect (Liley and Stacey 1983). Females undergoing oogenesis may release chemical signals and stimulate males to mature (Suquet *et al.* 1992). If this is the case it helps explain why one male fish, in a tank full of females forced to mature through hormone treatment, was running ripe in August 1991 but it does not explain the lack of running ripe males during the rest of the spawning season. Running ripe or not, it is still likely that close

proximity between the sexes is needed for full, 'natural' maturation of the male fish and it is therefore recommended that male and female barramundi broodstock should not be kept separate. This is a concept supported by Van Weerd and Richter (1991).

5.2.2 Spawning Failures

Explanations for spawning failures are many and varied. The study on the influence of hormones on male spawnings was complicated by the fact that many of the spawning female fish were themselves part of hormone trials. A few of the spawnings failed either because the females did not receive enough hormone to induce spawning or received no hormone at all. In these situations, no eggs were released and for this reason 'non spawnings' were not included in the average egg fertilisation percentages in Table 23 and Figure 8. In situations where eggs were released and poorly fertilised the blame can quite often be laid on poor quality eggs. Several of the spawnings undertaken at the Darwin Aquaculture Centre have resulted in poor quality eggs being released on the first night. These eggs are smaller than usual and possess small or multiple oil droplets. Fertilisation rates for these eggs are usually low. It is common for the second night's eggs to be of improved quality with high fertilisation rates. This variance in egg quality may be a result of the way the females are induced. At this stage the evidence suggests that fish receiving hormone injections have a higher chance of releasing poor eggs on the first night, than those that receive cholesterol pellet implants. Another possible explanation for poor quality eggs may be that the diet fed to the fish is inadequate in some way. As reported in the introductory chapter dietary intake of vitamins, minerals and fats can significantly influence

the quality of eggs and larvae produced (Watanabe *et al.* 1984a; Mourente and Odriozola 1990).

On the one or two occasions where the fault was conceivably due to the males, it may have been because the fish had not taken part in a spawning before or were not fully mature prior to the event. If fish with no prior spawning experience have to be used it may be a good idea to combine them with proven spawners, although this also present problems with one fish excluding another from the spawning. This has been observed on at least one occasion at the DAC.

5.2.3 Repetitive Breeding and Sex Change

Spawning records kept at the Darwin Aquaculture Centre show that there is the variation in breeding performance between individual male fish. Information contained in Appendix 3 reveals that some male fish are capable of being used for spawning up to 5 times in a season and over a period of three years. By contrast, other fish, of approximately the same weight, caught in the same river, at the same time, may only be used for one spawning before they change sex to female, often in the same year that they were caught.

The controlling mechanism for sex change in barramundi remains the subject of much confusion. Examination of all of the DAC's spawning records failed to reveal any consistent pattern. The fish were all maintained under similar environmental conditions and fed exactly the same food. Frequent administration of reproductive hormones appears to neither enhance or impede sex change although trials are being undertaken to examine the effect of potent androgens in delaying sex change. There is

speculation that wild males need only participate in one spawning season before they undergo sex change (R. Griffin, pers. comm.) but this is unsubstantiated by hatchery data. Thouard *et al.* (pers. comm.) suggest that sex change can be prevented by denying the fish a 'rest period'. They do not quantify the length of the rest period required, however barramundi at the DAC have changed sex within two months of spawning as males, still within the same breeding season. Until a study is undertaken to monitor blood plasma levels of hormones on a seasonal basis it is likely that factors controlling sex change in barramundi will remain obscure.

The variation in breeding performance and the time of sex change cannot be predicted and it is obvious that a good male breeder is a valuable asset and should be looked after with care.

5.3 GENERAL DISCUSSION OF CONTROLLED SPAWNING OF BARRAMUNDI

Trials with barramundi spawning in the early 1970's relied on the stripping of wild caught fish that were already in running ripe condition (Maneewong 1987). Since that time, improvements in broodstock husbandry and with the development of various reproductive hormones, both synthetic and natural, the process of barramundi spawning has become much more reliable and controlled and no longer requires the capture of wild, spawning, fish.

In the past, most authors in the literature reported that male and female fish were routinely injected or implanted with hormones and the males fish chosen for spawning were usually in running ripe condition (Maneewong 1987 for example).

The spawning data presented in this chapter clearly show that the male fish do not usually require a hormone injection to induce them to spawn and neither do they have to be running ripe when selected for spawning. In Australian barramundi hatcheries it is now routine practice for only the female fish to be induced by hormone treatment (Rod Garrett, pers. comm.; Darwin Aquaculture Centre spawning protocol).

Recently, advances with controlled breeding of barramundi under temperature and light controlled conditions have extended the supply of barramundi eggs and larvae to 12 months of the year. This development has seen a dramatic improvement in the reliability of the supply of eggs and larvae to barramundi farmers, especially in the more temperate areas. Environmental maturation control has also had the added effect of delaying the sex change process in the males (Thouard *et al.* pers. comm. and R. Garrett pers. comm.). The reason for the delay of the sex change is unclear and may be related to the relatively constant environmental conditions or regular, monthly spawning demands or a combination of the two (Thouard *et al.* pers. comm.). The importance of having a controlled light regime as well as controlled temperature has not been investigated. It is possible that temperature is the primary determinant of breeding condition in tropical fish (Lam 1983).

So far the main drawback to environmental breeding control is the high cost of the establishment and operation of the tanks. High volume recirculation pumps, water heaters, tank insulation and special computer controlled lighting systems make it an expensive process. Future investigations on barramundi breeding under controlled environmental conditions and the development of new hatchery technology will no doubt simplify the process and make it more cost effective.

REFERENCES

Ali, H. M., 1987. Sea bass (*Lates calcarifer*) spawning in tanks in Malaysia. In: Copland, J. W. and Grey, D. L., (eds), Management of wild and cultured sea bass/ barramundi (*Lates calcarifer*): proceedings of an international workshop held at Darwin, NT., Australia, 24-30 September, 1986. ACIAR Proceedings No. 20: 129-131.

Almendras, J. M., Duenas, C., Nacario, J., Sherwood N. M. and Crim, L. W. 1988a. Sustained hormone release. III. Use of gonadotrophin releasing hormone analogues to in induce multiple spawnings in sea bass, *Lates calcarifer*. Aquaculture, 74: 97-111.

Almendras, J. M., Duenas, C., Nacario, J., Sherwood N. M. and Crim, L. W. 1988b. Induced multiple spawning in sea bass. SEAFDEC Asian Aquaculture, 10(4): 10-11.

Amin, E. M., 1986. Induced gonadal maturation of male European eel (*Anguilla anguilla* L.) inhabiting Egyptian lakes. Arab Gulf J. Sci. Res., 4(1): 293-301.

Awaji, M. and Hanyu, I., 1988. Effects of water temperature and photoperiod on the beginning of spawning season in the orange-red type medaka. Zool. Sci., 5(5): 1059-1064.

Baggerman, B., 1980. Photoperiodic and endogenous control of the annual reproductive cycle in teleost fishes. In: Ali, M. A., (ed). Environmental Physiology of Fishes. Plenum, New York. pp. 533-567.

Ball, J. N., 1981. Hypothalamic control of the pars distalis in fishes, amphibians and reptiles. Gen. Comp. Endocrinol., 44: 135-170.

Barannikova, I. A. and Bukovskaya, O. S., 1990. Influence of luliberin on the condition of gonadotropocytes in the pituitary and the dynamics of the composition of gonadotrophins in the blood serum of stellate sturgeon, *Acipenser stellatus*. J. Ichthyol., 30(3): 34-36.

Barry, T. L., Castanos, M. T. and Fast, A. W., 1991. Induced spermiation in the male spotted scat (*Scatophagus argus*) by long-term administration of 17α-methyltestosterone followed by LHRHa. Asian Fish. Sci., 4: 137-145.

Bhattacharya, S. and Banerjee, P. P., 1990. Environmental and endocrine control of fish reproduction. In: Manna, G. K., and Jana, B. B. (eds), National Symposium on Environmental Impacts on Animals and Aquaculture, Kalyani (India); 14-16 May 1988. pp. 85-90.

Bhowmick, R. M., Kowtal, G. V., Jana, R. K. and Gupta, S. D., 1977. Experiments on second spawning of major Indian carps in the same season by hypophysation. Aquaculture, 12: 149-155.

Bieniarz, K. and Epler, P., 1977. Investigations on inducing sexual maturity in the male eel, *Anguilla anguilla* L. J. Fish Biol., 10: 555-559.

Billard, R., 1969. La spermatogénèse de *Poecilia reticulata*. II. Estimation du nombre de générations goniales et rendement de la spermatogenèse. Ann. Biol. anim. Biochim. Biophys., 9: 251-71.

Billard, R., 1976. Induction of sperm release in the goldfish by some steroids. IRCS Med. Sci. Libr. Compend., 4: 42.

Billard, R., 1977. Effects of various hormones on sperm release in the hypophysectomised goldfish. ICRS Med. Sci.: Libr. Comp., 5: 188.

Billard, R., 1978. Changes in the structure and fertilising ability of marine and freshwater fish spermatozoa diluted in media of various salinities. Aquaculture, 14: 187-198.

Billard, R., 1986. Spermatogenesis and spermatology of some teleost fish species. Reprod. Nutr, Dévelop., 26(4): 877-920.

Billard, R. 1989. Endocrinology and fish culture. Proceedings of the First International Symposium on Fish Endocrinology. Fish Physiol. and Biochem., 7(1-6): 49-58.

Billard, R. and Marcel, J., 1980. Stimulation of spermiation and induction of ovulation in pike (*Esox lucius*). Aquaculture, 21: 181-195.

Billard, R. and Richard, M., 1982. Inhibition of spermatogenesis and vitellogenesis in rainbow trout by hormonal additives in the diet. Prog. Fish-Cult., 44(1): 15-18.

Billard, R., Lakomaa, E. and Kilmstrom, J. E., 1975. Induction of sperm release in *Rana esculenta* by LH/FSH releasing hormone. IRCS Med. Sci.: Libr. Compend., 3: 17.

Billard, R., Bry, C. and Billet, C., 1981. Stress, environment and reproduction in teleost fish. In: Pickering, A. P. (ed), Stress and Fish. Academic Press, New York. pp. 1-12.

Billard, R., Fostier, A., Weil, C. and Breton, B., 1982a. Endocrine control of spermatogenesis in teleost fish. Can J. Fish Aquat. Sci., 39: 65-79.

Billard, R., Richard, M. and Rombauts, R., 1982b. Inhibition of spermatogenesis and vitellogenesis in rainbow trout by hormonal additives in the diet. Prog. Fish-Cult., 44(1):15-18.

Billard, R., Choisis, J. P. and Reinaud, P., 1983. Stimulation of spermiation in carp in response to LH-RH and D-Ala^6-LH-RH ethylamide. Aquaculture, 35: 173-176.

Billard, R., Reinaud, P., Hollobecq, M. G. and Breton, B., 1984. Advancement and synchronisation of spawning time in *Salmo gairdneri and S. trutta* following administration of LRH-A combined or not with pimozide. Aquaculture, 43: 57-66.

Billard, R., Bieniarz, K., Popek, W., Epler, P., Breton, B. and Alagarswami, K., 1987. Stimulation of gonadotropin secretion in carp by pimozide-LRH-A treatment: effects of dose and time of day. Aquaculture, 62: 161-170.

Billard, R., Bieniarz, K., Popek, W., Epler, P. and Saad, A., 1989. Observations of a possible pheremonal stimulation of milt production in carp (*Cyprinus carpio* L.). Aquaculture, 77: 387-392.

Borg, B., 1981. Effects of methyltestosterone on spermatogenesis and secondary sexual characteristics in the three-spined stickleback (*Gasterosteus aculeatus*, L.). Gen Comp. Endocrinol., 44: 177-180.

Borg, B., Paulson, G. and Peute, J., 1986. Stimulatory effects of methyltestosterone on pituitary gonadotrophic cells and testes Leydig cells of the three-spined stickleback (*Gasterosteus aculeatus*, L.) in winter. Gen Comp. Endocrinol., 62 (1): 54-61.

Breton, B. and Weil, C., 1973. Endocrinologie comparée effets du LH/FSH-RH synthétique et d'extraits hypothalamiques de carpe sur la secretion d'hormone gonadotrope *in vivo* chez la carpe (*Cyprinus carpio* L.). C. R. Acad. Sci., 277: 2061-2064.

Breton, B., Prunet, P. and Reinaud, P. 1978. Sexual differences in salmon gonadotrophin. Ann. Biol. Biochim. Biophys., 18: 759.

Bromage, N. R., 1988. Propagation and stock improvement. In: Shepherd, C. J. and Bromage, N. R., (eds). Intensive fish farming. BSP Professional Books, Oxford. pp. 103-153.

Brusle, J. and Roblin, C., 1984. Sexuality of sea bass, *Dicentrachus labrax*, controlled fish farm conditions. In: Barnabe, G. and Billard, R. (eds), Aquaculture of Sea Bass and Sparids. Proceedings of the conference, Colloque sur l'Aquaculture du Bar (Loup) et des Sparides; Sete (France), 15 March, 1983. pp. 33-43.

Carolsfeld, J., Sherwood, N. M., Kreiberg, H. and Sower, S. A., 1988. Induced sexual maturation of herring using GnRH 'quick-release' cholesterol pellets. Aquaculture, 70: 169-181.

Carrillo, M., Bromage, N., Zanuy, S., Serrano, R. and Prat, F., 1989. The effect of modifications in photoperiod on spawning time, ovarian development and egg quality in the sea bass, (*Dicentrachus labrax* L.). Aquaculture, 81(3-4): 351-365.

Castagnolli, N. and Donaldson, E. M., 1981. Induced ovulation and rearing of the pacu (*Colossoma mitrei*). Aquaculture, 25(2-3): 275-279.

Castagnolli, N., Brasil, D. F., Val Sella, M. V., Pinto, M. L. G. and Rosa, A. A. M., 1988. Induced breeding of the pacu (*Piaractus mesopotamicus = Colossoma mitrei*) and Tambaqui (*C. macropomum*) with LH-RHa and crude partially purified fish gonadotrophin. Proceedings Aquaculture International Congress and Exposition, Vancouver, British Columbia, Canada, September 6-9, 1988. p. 74.

Cedaro, R., 1992. General training physiology. In; Cedaro, R. (ed.) Triathlon: Into the Nineties. Murray Child, Sydney. pp 12-26.

Chang, C.-F., 1987. Spermatogenesis and its hormonal regulation in fish. Proceedings of the Symposium on Fish Reproduction and its Endocrine Control: Basic and Practical Aspects. Academia Sinica, Nankang, Taipei, Taiwan, ROC, June 19-20, 1987. pp. 65-81.

Chang, C.-F., Yueh, W.-S. and Lee, M.-F., 1991. Effects of LHRH-A and HCG on the steroid profiles of bisexual and mature male and female protandrous black porgy, *Acanthopagrus schlegeli*. Aquaculture, 92: 83-92.

Chang, J. P. and Peter, R. E., 1983. Effects of pimozide and Des Gly10,[D-Ala6] Luteinizing Hormone-Releasing Hormone ethylamide on serum gonadotrophin concentrations, germinal vesicle migration, and ovulation in female goldfish, *Carassius auratus*. Gen. Comp. Endocrinol. 52: 30-37.

Chang, J. P., Cook, A. F. and Peter, R. E., 1983. Influence of catecholamines on gonadotrophin secretion in goldfish, *Carassius auratus*. Gen. Comp. Endocrinol., 49: 22-31.

Chaudhuri, H., 1960. Experiments on induced spawning of Indian carps with pituitary injections. Indian J. Fish., 7: 20-48.

Chaudhuri, H., 1976. Use of hormones in induced spawnings of carps. J. Fish. Res. Board Can., 33: 940-947.

Clemens, H. P. and Grant, F. B., 1964. Gonadal hydration of carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*) after injections of pituitary extracts. Zoologica, 49: 193-210.

Clemens, H. P. and Grant, F. B., 1965. The seminal thinning response of carp (*Cyprinus carpio*) and rainbow trout (*Salmo gairdneri*) after injections of pituitary extracts. Copeia, 174-177.

Clemens, H. P. and Reed, C. A., 1967. Long term gonadal growth and maturation of goldfish (*Carassius auratus*) with pituitary injections. Copeia., 465-466.

Courtois, F., Takashima, F. and Billard, R., 1986. Stimulation of spermiation following repeated injection of carp pituitary homogenates in the carp. Bull. Jap. Soc. Fish., 52(6): 995-997.

Crim, L. W., 1982. Environmental modulation of annual and daily rhythms associated with reproduction in teleost fishes. Can. J. Fish. Aquat. Sci., 39: 17-21.

Crim, L. W. and Evans, D. M., 1979. Stimulation of pituitary gonadotrophin by testosterone in juvenile rainbow trout (*Salmo gairdneri*). Gen Comp. Endocrinol., 37: 192-196.

Crim, L. W., Peter, R. E. and Billard, R., 1976. Stimulation of gonadotropin secretion by intra ventricular injection of hypothalamic extracts in the goldfish, *Carassius auratus*. Gen Comp. Endocrinol., 30: 77-82

Crim, L. W., Sherwood, N. M. and Wilson, C. E. 1988. Sustained hormone release. II. Effectiveness of LHRH analogue (LHRHa) administration by either single time injection or cholesterol pellet implantation on plasma gonadotrophin levels in a bioassay model fish, the juvenile rainbow trout. Aquaculture, 74: 87-95

Davis, T. L. O., 1982. Maturity and sexuality in barramundi, *Lates calcarifer* (Bloch), in the Northern Territory and south-eastern Gulf of Carpentaria. Aust. J. Mar. Freshwater Res. 33: 529-45.

Davis, T. L. O., 1987. Biology of wildstock *Lates calcarifer* in Northern Australia. In: Copland, J. W. and Grey, D. L., (eds), Management of wild and cultured sea bass/ barramundi (*Lates calcarifer*): proceedings of an international workshop held at Darwin, NT., Australia, 24-30 September, 1986. ACIAR Proceedings No. 20: 22-29.

Davy, F. B. and Chouinard, A., 1981. Induced fish breeding in Southeast Asia: report of a workshop held in Singapore, 25-28 November 1980. Ottawa, Ont., IDRC. 48 pages.

Demski, L. S. and Hornby, P. J., 1982. Hormonal control of fish reproductive behaviour: brain - gonadal steroid interactions. Can. J. Fish. Aquat. Sci., 39: 36-47.

Dollerup, J. and Graver, C. M., 1985. Repeated testicular maturation and spermiation, alternating with periods of feeding and growth in silver eels, *Anguilla anguilla* (L.). Dana, 4: 19-39.

Donaldson, E. M., 1973. Reproductive endocrinology of fishes. Am. Zool., 13: 909-927.

Donaldson, E. M., Dye, H. M. and Wright, B. F., 1978. The effect of storage conditions on the biological activity of salmon gonadotrophin. Ann. Biol. anim. Bioch. Biophys., 18(4): 997-1000.

Donaldson, E. M. and Hunter, G. A., 1983. Induced final maturation, ovulation and spermiation in cultured fish. In: Hoar, W. S., Randall, D. J. and Donaldson, E. M., Fish Physiology, Volume IX, Reproduction, part B: Behaviour and Fertility Control. Academic Press, INC. London. pp. 351-403.

Donaldson, E. M., Van Der Kraak, G., Hunter, G. A., Dye, H. M., Rivier, J. and Vale, W., 1983. Teleost GnRH and analogues: effect on plasma GtH concentration and ovulation in coho salmon (*Oncoryhnchus kisutch*). XIIth Conf. Europ. Comp. Endocrinol., 1983. Sheffield, England, p. 122.

Doroshov, S. I., Clark, W. H. Jr, Lutes, P. B., Swallow, R. L., Beer, K. E., McGuire, A. B., and Cochran, M. D., 1983. Artificial propagation of the white sturgeon, *Acipenser transmontanus* Richardson. Aquaculture, 32(1-2): 93-104.

Dziuk, P. J. and Cook, B., 1966. Passage of steroids through silicone rubber. Endocrinology, 78: 208-211.

Fang, Y. and C. -S. Lee, 1989. The preliminary study on the mechanism of the action of 17α-methyltestosterone stimulatory spermatogenesis in the grey mullet (*Mugil cephalus* L.). Oceanol. Limnol. Sin/ Haiyang Yu Hazhao, 20(1): 10-14.

Fontaine, M., 1976. Hormones and the control of reproduction in aquaculture. J. Fish. Res. Board Can. 33: 922-939.

Fontaine, Y. A., Salmon, C., Fontaine-Bertrand, E., Burzawa-Gerard, E. and Donaldson, E. M., 1972. Comparison of the activities of two purified fish gonadotrophins on adenyl cyclase activity in the goldfish ovary. Can. J. Zool., 50: 1673-1676.

Fortes, R. D., 1987. Induced spawning of sea bass (*Lates calcarifer*) in the Philippines. In: Copland, J. W. and Grey, D. L., (eds), Management of wild and cultured sea bass/ barramundi (*Lates calcarifer*): proceedings of an international workshop held at Darwin, NT., Australia, 24-30 September, 1986. ACIAR Proceedings No. 20: 123-125.

Fostier, A., Le Gac, F. and Loir, M., 1987. Steroids in male reproduction. 3rd Int. Symp. Reprod. Physiol. Fish, Memorial University of Newfoundland, St Johns's, Canada, pp. 239-245.

Fujino, M., Yamazaki, I., Kobayashi, S., Fukuda, T., Shinagawa, S., Nakayama, R., White, W. F., and Rippel, R. H., 1974. Some analogues of luteinizing hormone releasing hormone having intense ovulation-inducing activity. Biochem. Biophys. Res. Commun., 57: 1248-1256.

Garcia, L. Ma. B., 1990. Advancement of sexual maturation and spawning of sea bass, *Lates calcarifer* (Bloch), using pelleted luteinizing hormone-releasing hormone analogue and 17α-methyltestosterone. Aquaculture, 86: 333-345.

Garcia, L. Ma. B., 1991. Spermiation response of mature rabbitfish, *Siganus guttatus* Bloch, to luteinizing hormone-releasing hormone analogue (LHRHa) injection. Aquaculture, 97: 291-299.

Garrett, R. N., 1987. Reproduction in Queensland barramundi (*Lates calcarifer*). In: Copland, J. W. and Grey, D. L., (eds), Management of wild and cultured sea bass/ barramundi (*Lates calcarifer*): proceedings of an international workshop held at Darwin, NT., Australia, 24-30 September, 1986. ACIAR Proceedings No. 20: 22-29.

Garrett, R. N. and Rasmussen, I. R., 1987. Induced spawning of barramundi. Australian Fisheries, 46(7): 14-17.

Garrett, R. N. and Connell, M. R. J., 1991. Induced breeding in barramundi. Austasia Aquaculture, 5(8): 10-12.

Garrett, R. N., MacKinnon, M. R. and Russell, D. J., 1987. Wild barramundi breeding and its implications for culture. Australian Fisheries, 46(7): 4-6.

Glubokov, A. I., Matloch, N. N. and Sedova, M. A., 1991. Effect of a synthetic LHRH analogue and dopamine antagonists on the maturation of bream, *Abramis brama* L. Aquaculture, 95(3-4): 373-377.

Godhino, H. P. and Godhino, A. L., 1986. Induced spawning of the pacu, *Colossoma mitrei* (Berg 1895), by hypophysation with crude carp pituitary extract. Aquaculture, 55(1): 69-73.

Goncharov, B. F., Tgumnova, L. V., Polupan, I. S. and Savelieva, E. A., 1991. Induced oocyte maturation, ovulation and spermiation in sturgeons (Acipenseridae) using synthetic analogue of gonadotrophin-releasing hormone. In: Williot, P. (ed.), Proceedings of the First International Symposium on Sturgeon, Bordeaux (Gironde, France), 3-6 October, 1989. pp. 351-364.

Grey, D. L., 1987. An overview of *Lates calcarifer* in Australia and Asia. In: Copland, J. W. and Grey, D. L., (eds), Management of wild and cultured sea bass/ barramundi (*Lates calcarifer*): proceedings of an international workshop held at Darwin, NT., Australia, 24-30 September, 1986. ACIAR Proceedings No. 20: 15-21.

Grier, H. J., Linton, J. F., Leatherland, J. R. and De Vlaming, V. L., 1980. Structural evidence for two different testicular types in teleost fishes. Am. J. Anat., 159: 331-345.

Hardy, R. W., 1988. Diet preparation. In: Halver, J. E. (ed). Fish Nutrition. Academic Press, San Diego. pp. 475-548.

Harker, K., 1992. Breeding carps with Ovaprim in India. Infofish Int., 1/92: 36-39.

Harvey, B. J. and Hoar, W. S., 1979. The theory and practice of induced breeding in fish. IDRC-TS21e. Int. Dev. Res. Cent. Ottawa, Ont. 48 pages.

Hew, C. L. and Xiong, F., 1991. Control of salmon gonadotrophin II beta gene expression. Program and Abstracts. Second International Marine Biotechnology Conference (IMBC '91). p. 94.

Hickman, C. P., Hickman, C. P., Hickman, F. M. and Roberts, L. S., 1979. Integrated Principles of Zoology, 6th Edition. C. V. Mosby Co., St Louis. 1086 pages.

Hilscher, B., Hilscher W., Bulthoff-Ohnolz, B., Kramer, U., Birke, A., Pelzer, H. and Gauss, G., 1974. Kinetics of gametogenesis. I. Comparative histological and autoradiographic studies of oocytes and transitional prospermatogonia during oogenesis and prespermatogenesis. Cell. Tiss. Res., 154: 443-470.

Hourigan, T. F., Nakamura, M., Nagahama, Y., Yamauchi, K. and Grau, E. G., 1991. Histology, ultrastructure and *in vitro* steroidogenesis of the testes of two male phenotypes of the protogynous fish, *Thalassoma duperrey* (Labridae). Gen. Comp. Endocrinol., 83(2): 193-217.

Hunter, G. A. and Donaldson, E. M., 1983. Hormonal sex control and its application to fish culture. In: Hoar, W. S., Randall, D. J. and Donaldson, E. M., Fish Physiology, Volume IX, Reproduction, part B: Behaviour and Fertility Control. Academic Press, INC. London. pp. 223-303.

Idler, D. R., Schmidt, P. J. and Biely, J., 1961. The androgenic activity of 11-ketotestosterone a steroid in salmon plasma. Can. J. Biochem. Physiol., 39: 317-320.

Jalabert, B., Breton, B., Brzuska, E., Fostier, A. and Wieniawski, J., 1977. A new tool for induced spawning: the use of 17α-hydroxy-20β-dihydroprogesterone to spawn carp at low temperature. Aquaculture, 10: 353-364.

James, P. S. B. R. and Marichamy, R., 1987. Status of sea bass (*Lates calcarifer*) culture in India. In: Copland, J. W. and Grey, D. L., (eds), Management of wild and cultured sea bass/ barramundi (*Lates calcarifer*): proceedings of an international workshop held at Darwin, NT., Australia, 24-30 September, 1986. ACIAR Proceedings No. 20: 74-79.

Jensen, G. L., Shelton, W. L. and Wilken, L. O., 1978. Use of methyltestosterone silastic implants to control sex in grass carp. In: Smitherman, R. O., Shelton, W. L. and Grover, J. H., (eds). Culture of Exotic Fishes Symposium Proceedings. Fish Cult. Sect., Am. Fish. Soc., Auburn, Ala.; p 200-219.

Juario, J. V., Quinitio, G. F., Banno, J. and Natividad, M., 1981. Effects of exogenous hormone injections on milt consistency in newly caught, wild milkfish. SEAFDEC Quart. Res. Rep., 5(4): 1-4.

Kanazawa, A., 1985. Nutritional factors in fish reproduction. In: Lee, C.-S. and Liao, I.-C. (eds). Reproduction and Culture of Milkfish. Proceedings of a workshop held at Tungkang Marine Laboratory, Taiwan, April 22-24, 1985. pp. 115-139.

Kawauchi, H., Suzuki, K., Itoh, H., Swanson, P., Naito, N., Nagahama, Y. and Nozaki, M., 1989. The duality of teleost gonadotrophins. Proceedings of the First International Symposium on Fish Endocrinology. Fish Physiol. and Biochem., 7(1-6): 29-38.

Kaya, C. M., 1973. Effects of temperature on responses of the gonads of green sunfish (*Lepomis cyanellus*) to treatment with carp pituitaries and testosterone propionate. J. Fish. Res. Board Can., 30: 905-912.

Khan, I. A., Lopez, E. and Leloup-Hatey, J., 1987. Induction of spermatogenesis and spermiation by a single injection of human chorionic gonadotrophin in intact and hypophysectomised immature European eel (L.). Gen. Comp. Endocrinol. 68: 91-103.

Kobayashi, M., Aida, K. and Stacey, N. E., 1991. Induction of testis development by implantation of 11-ketotestosterone in female goldfish. Zool. Sci., 8(2): 389-393.

Koldras, M., Bieniarz, K. and Kime, D. E., 1990. Sperm production and steroidogenesis in testes of the common carp, *Cyprinus carpio* L., at different stages of maturation. J. Fish Biol., 37(4): 635-645.

Kreiberg, H., Hunter, G. A., Donaldson, E. M., Clarke, W. C. and Baker, I., 1987. Induced ovulation and spermiation in the Pacific herring (*Clupea harengus pallasi*) using salmon pituitary preparations and a synthetic gonadotrophin-releasing hormone analogue. Aquaculture, 61(2): 155-161.

Kulikova, N. I. and Fedulina, V. N., 1987. Reaction of Black Sea golden mullet *Liza aurata* (Risso) to pituitary injection under different ambient conditions. Biol. Nauki., 12: 59-66.

Kungvankij, P., 1987. Induction of spawning of sea bass (*Lates calcarifer*) by hormone injection and environmental manipulation. In: Copland, J. W. and Grey, D. L., (eds), Management of wild and cultured sea bass/ barramundi (*Lates calcarifer*): proceedings of an international workshop held at Darwin, NT, Australia, 24-30 September, 1986. ACIAR Proceedings No. 20: 120-122.

Kungvankij, P. and Suthemechaikul, N., 1986. Mass production of seabass, *Lates calcarifer* (Bloch), by environmental manipulation. In: Maclean, J. L., Dizon, L. B. and Hosillos, L. V., (eds). The first Asian Fisheries Forum. Asian Fisheries Society, Manila, Philippines. pp. 67-70.

Kungvankij, P., Tiro, L. B. Jr., Pudadera, B. J. and Potestas, I. O., 1986. Biology and culture of sea bass (*Lates calcarifer*). Network of Aquaculture Centres in Asia (NACA) Training Manual Series No. 3. Regional Lead Centre in the Philippines (PLCP), June 1986, 70 pages.

Kuo, C.-M., 1991. Hormonal induction of maturation and spawning in female barramundi, *Lates calcarifer* (Bloch). M.Sc. Thesis, School of Applied Science, University of Tasmania. 329 pages.

Kyle, A. L., Stacey, N. E., Billard, R. and Peter, R. E., 1979. Sexual stimuli rapidly increase gonadotrophin and milt levels in goldfish. Am. Zool., 19: Abstr. 19.

Kyle, A. L., Stacey, N. E., Peter, R. E. and Billard, R., 1985. Elevations in gonadotrophin concentrations and milt volumes as a result of spawning behaviour in the goldfish. Gen. Comp. Endocrinol., 57: 10-22.

Lacanilao, F., Marte, C. L. and Lam, T. J., 1985. Problems associated with hormonal induction of gonad development in milkfish (*Chanos chanos*). In: Lofts, B. and Holmes, W. N., (eds). Current trends in Comparative Endocrinology. pp. 1247-1253.

Lake, J., 1967. Rearing experiments with five species of Australian freshwater fishes. Aust. J. Mar. Freshwater Res., 18: 137-153.

Lam, T. J., 1982. Applications of endocrinology to fish culture. Can. J. Fish. Aquat. Sci. 39: 111-137.

Lam, T. J., 1983. Environmental influences on gonadal activity in fish. In: Hoar, W. S., Randall, D. J. and Donaldson, E. M., Fish Physiology, Volume IX, Reproduction, part B: Behaviour and Fertility Control. Academic Press, INC. London. pp. 65-116.

Lam, T. J., Pandey, S. and Hoar, W. S., 1975. Induction of ovulation in the goldfish by synthetic luteinizing hormone-releasing hormone. Can. J. Zool. 53: 1139-1192.

Lee, C.-S., 1985. Environmental factors in the reproduction of milkfish. In: Lee, C.-S. and Liao, I.-C. (eds). Reproduction and Culture of Milkfish. Proceedings of a workshop held at Tungkang Marine Laboratory, Taiwan, April 22-24, 1985. pp. 99-114.

Lee, C.-S. and Weber, G. M., 1986. Effects of salinity and photoperiod on 17α-methyltestosterone-induced spermatogenesis in the grey mullet, *Mugil cephalus* L. Aquaculture, 56: 53-62.

Lee, C.-S., Tamaru, C. S. and L. W. Crim, 1985. Preparation of a luteinizing hormone-releasing hormone cholesterol pellet and its implantation in the milkfish (*Chanos chanos* Forsskal). In: Lee, C.-S. and Liao, I.-C. (eds). Reproduction and Culture of Milkfish. Proceedings of a workshop held at Tungkang Marine Laboratory, Taiwan, April 22-24, 1985. pp. 215-226.

Lee, C.-S., Tamaru, C. S., Banno J. E., Kelley, C. D., Bocek, A., and Wyban, J. A., 1986a. Induced maturation and spawning of milkfish, *Chanos chanos* Forsskal, by hormone implantation. Aquaculture, 52: 199-205.

Lee, C.-S., Tamaru, C. S., Kelley, C. D. and Banno, J. E., 1986b. Induced spawning of milkfish, *Chanos chanos* by a single application of LHRH-analogue. Aquaculture, 58: 87-98.

Lee, C.-S., Tamaru, C. S. and Kelley, C. D., 1986c. Technique for making chronic-release LHRH-a and 17α-methyltestosterone pellets for intramuscular implantation in fishes. Aquaculture, 59: 161-168.

Lee, C.-S., Weber, G. M. and Tamaru, C. S., 1986d. Effect of orally administered 17α-methyltestosterone on spermatogenesis in immature milkfish, *Chanos chanos*, Forsskal. J. Fish Biol., 29: 567-572.

Lee, C.-S., Tamaru, C. S., Banno, J. E. and Kelley, C. D., 1986e. Influence of chronic administration of LHRH-analogue and/or 17α-methyltestosterone on maturation in milkfish, *Chanos chanos*. Aquaculture, 59: 147-159.

Lee, C.-S., Tamaru, C. S., Miyamoto, G. T. and Kelley, C. D., 1987. Induced spawning of grey mullet (*Mugil cephalus*) by LHRH-a. Aquaculture, 62: 327-336.

Lee, C.-S., Tamaru, C. S. and Kelley, C. D., 1988. The cost and effectiveness of CPH, HCG and LHRH-a on the induced spawning of grey mullet, *Mugil cephalus*. Aquaculture, 73: 341-347.

Leung, L. K.-P., 1987. Cryopreservation of spermatozoa of the barramundi, *Lates calcarifer* (Teleostei: Centropomidae). Aquaculture, 64: 243-247.

Licht, P., 1974. Induction of sperm release in frogs by mammalian gonadotrophin-releasing hormone. Gen. Comp. Endocrinol. 23: 352-354.

Liley, N. R. and Stacey, N. E., 1983. Hormones, pheromones and reproductive behaviour in fish. In: Hoar, W. S., Randall, D. J. and Donaldson, E. M., Fish Physiology, Volume IX, Reproduction, part B: Behaviour and Fertility Control. Academic Press, INC. London. pp. 1-63.

Liley, N. R. and Rouger, Y., 1990. Plasma levels of gonadotrophin and 17α, 20β-dihydroxy-4-pregnen-3-one in relation to spawning behaviour of rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Biol., 37: 699-711.

Loir, M., 1986. Primary cultures of somatic testicular cells in the rainbow trout. In: Zohar, Y. and Breton, B. (eds). Reproduction in fish: basic and applied aspects in endocrinology and genetics. Tel Aviv, Israel, 10-12 November 1986. pp. 131-134.

Loir, M. and Billard, R., 1990. Hormonal control of gamete production in male teleost fish. Riv. Ital. Acquacol., 25: 43-52.

Maneewong, S., 1987. Induction of spawning of sea bass (*Lates calcarifer*) in Thailand. In: Copland, J. W. and Grey, D. L., (eds), Management of wild and cultured sea bass/ barramundi (*Lates calcarifer*): proceedings of an international workshop held at Darwin, NT., Australia, 24-30 September, 1986. ACIAR Proceedings No. 20: 116-119.

Matty, A. J., 1985. Fish Endocrinology. Croom Helm. London. 267 pages.

Miura, T., Ninzeki, M., Hirai, H. and Yamauchi, K., 1991. Introduction of final maturation by injection of chum salmon pituitary homogenate in the male Japanese huchen (*Hucho perryi*). Bull. Fac. Fish. Hokkaido Univ., 42(1): 16-25.

Moore, R. 1979. Natural sex inversion in the giant perch (*Lates calcarifer*). Aust. J. Mar. Freshwater Res. 30: 803-813.

Mourente, G. and Odriozola, J. M., 1990. Effect of broodstock diet on lipid classes and their fatty acid composition in eggs of gilthead sea bream (*Sparus aurata* L.). Fish Physiol. Biochem., 8(2): 93-101.

Nacario, J. F., 1987. Releasing hormones as an effective agent in the induction of spawning in captivity of sea bass (*Lates calcarifer*). In: Copland, J. W. and Grey, D. L., (eds), Management of wild and cultured sea bass/ barramundi (*Lates calcarifer*): proceedings of an international workshop held at Darwin, NT., Australia, 24-30 September, 1986. ACIAR Proceedings No. 20: 126-128.

Nagahama, Y., 1983. The functional morphology of teleost gonads. In: Hoar, W. S., Randall, D. J. and Donaldson, E. M., Fish Physiology, Volume IX, Reproduction, part A: Endocrine Tissues and Hormones. Academic Press, INC. London. pp. 223-275.

Nagahama, Y., 1987. Gonadotrophin action on gametogenesis and steroidogenesis in teleost gonads. Zool. Sci. 4: 209-222.

Nagahama, Y., Kagawa, H. and Young, G., 1982. Cellular sources of sex steroids in teleost gonads. Can. J. Fish. Aquat. Sci., 39: 56-64.

Nash, C. E. and Kuo, C.-M., 1976. Preliminary capture husbandry and induced breeding results with milkfish, *Chanos chanos* (Forsskal). Proc. Int. Milkfish Workshop, 1976, pp. 139-159.

Ngamvongchon, S., Kok, L. Y. and Takashima, F., 1987. Changes in endocrine profiles and spermiation response in carp after LHRH analogue injection. Bull. Jap. Soc. Fish., 53(2): 229-234.

Pankhurst, N. W. and Kime, D. E., 1991. Plasma sex steroid concentrations in male blue cod, *Parapercis colias* (Bloch and Schneider) (Pinquipedidae), sampled underwater during the spawning season. Aust. J. Mar. Freshwat. Res., 42(2): 129-137.

Partridge, B. L., Liley, N. R. and Stacey, N. E., 1976. The role of pheromones in the sexual behaviour of the goldfish. Anim. Behav., 24: 291-299.

Pearson, R. G., 1987. Barramundi breeding research - laying the foundations for industry. Australian Fisheries, 46(7): 2-3.

Pelissero, C., Cuisset, B. and Le Menn, F., 1989. The influence of sex steroids in commercial fish meals and fish diets on plasma concentration of oestrogens and vitellogenin in cultured Siberian surgeon, *Acipenser baeri*. Aquat. Living Resour., 2(3): 161-168.

Peter, R. E., 1980. Serum gonadotrophin levels in mature male goldfish in response to luteinizing hormone-releasing hormone (LH-RH) and des Gly^{10}-(D-Ala^{6})-LH-RH ethylamide. Can. J. Zool., 58: 1100-1104.

Peter, R. E., 1982. Neuroendocrine control of reproduction in teleosts. Can. J. Fish. Aquat. Sci., 39: 48-55.

Peter, R. E., 1983. The brain and neurohormones in teleost reproduction. In: Hoar, W. S., Randall, D. J. and Donaldson, E. M., Fish Physiology, Volume IX, Reproduction, part A: Endocrine Tissues and Hormones. Academic Press, INC. London. pp. 97-135.

Peter, R. E. and Crim, L. W., 1979. Reproductive endocrinology of fishes: gonadal cycles and gonadotrophin in teleosts. Annu. Rev. Physiol., 41: 323-335.

Pien, P.-C. and Liao, I.-C., 1975. Preliminary report of histological studies on the grey mullet related to hormone treatment. Aquaculture, 5: 31-39.

Pillay, T. V. R., 1990. Aquaculture: Principles and practices. Fishing News Books. London. 575 pages.

Pocsidio, G. N., 1978. Effects of testosterone proprionate, mammalian gonadotropins and growth hormone on the immature male catfish, *Clarias macrocephalus*. Fish. Res. J. Philipp., 3(2): 1-10.

Richter, C. J. J., Eding, E. H. and Roem, A. J., 1985. 17α-hydroxy-progesterone-induced breeding of the African catfish, *Clarias gariepinus* (Burchell), without priming gonadotrophin. Aquaculture, 44: 285-293.

Richter, C. J. J., Eding, E. H., Goos, H. J. Th., De Leeuw, R. D., Scott, A. P. and Van Oordt, P. G. W. J., 1987. The effect of pimozide/LHRHa and 17α-hydroxyprogesterone on plasma steroid levels and ovulation in the African catfish, *Clarias gariepinus*. Aquaculture, 63: 157-168.

Roberts, D. E., Harpster, B. V. and Henderson, G. E., 1978. Conditioning and induced spawning of the red drum (*Sciaenops ocellata*) under varied conditions of photoperiod and temperature. Proceedings of the Ninth Annual Workshop of the World Mariculture Society, Atlanta Georgia. 311-332, 1978.

Roberts, D. E. Jr, Reese, R. O., Grier, H. J. and MacGregor, R. III, 1988. Regime spawning of red drum, *Sciaenops ocellatus*: cyclical effects on serum steroids, gonadal cytology and ultrastructure. Red Drum Aquaculture. Proceedings of a Symposium on the Culture of Red Drum and Other Warm Water Fishes. Contrib. Mar. Sci., 30: 194-195.

Romer, A. S. and Parsons, T. S., 1977. The vertebrate body. W. B. Saunders Co., Philadelphia. 624 pages.

Roosen-Runge, E. C., 1962. The process of spermatogenesis in animals. Biol. Rev., Cambridge Phil. Soc. 37: 343-347.

Roosen-Runge, E. C., 1977. The process of spermatogenesis in animals. Cambridge University Press, Cambridge. 214 pages.

Rosa, A. A. M., Castagnolli, N., Zuim, S. M. F. and Manzano, J., 1988. Reversion of spermiation blockage with LH-RH agonist in *Piaractus mesopotamicus*. Proceedings Aquaculture International Congress and Exposition, Vancouver, British Columbia, Canada, September 6-9, 1988. p. 75.

Rothbard, S. and Rothbard, H., 1982. Spermiation response of carp to homologous pituitary gland. In: Richter, C. J. J. and Goos, H. J. Th., (eds), Reproductive Physiology of Fish. PUDOC, Wageningen, p. 201.

Rowland, S. J., 1988. Hormone induced spawning of the Australian freshwater Murray cod, *Maccullochella peeli* (Mitchell)(Percichthyidae). Aquaculture, 70: 371-389.

Russell, D. J., O'Brien, J. J. and Longhurst, C., 1987. Barramundi egg and larval culture. Australian Fisheries, 46(7): 26-29.

Saad, A. and Billard, R., 1987. Spermatozoa production and volume of semen collected after hormonal stimulation in the carp, *Cyprinus carpio*. Aquaculture, 65: 67-77.

Schipp, G., 1991a. Anaesthesia of barramundi broodstock. NT Department of Primary Industry and Fisheries, Fish Note No. 5, October 1991. 1 page.

Schipp, G., 1991b. Care of newly captured barramundi broodstock. NT Department of Primary Industry and Fisheries, Fish Note No. 8, October 1991. 2 pages.

Schipp, G. and Pearce, M., 1991. Diseases of barramundi broodstock. NT Department of Primary Industry and Fisheries, Fish Note No. 9, October 1991. 2 pages.

Schipp, G., Kuo, C., Baulch, G. and Wood, J., 1989. Hormone induced sperm production in male barramundi. In: Proceedings of a Symposium on Tropical Aquaculture. Australian Institute of Biology, Northern Territory Branch and the Department of Primary Industry and Fisheries, 5-6 August, 1989. Darwin. pp. 31-33.

Scott, A. P, Sheldrick, E. L. and Flint, A. P. F., 1982. Measurement of 17α, 20β-dihydroxy-4-pregnen-3-one in plasma of trout (*Salmo gairdneri* Richardson): seasonal changes and response to salmon pituitary extract. Gen. Comp. Endocrinol., 46: 444-451.

Shehadeh, Z. H., 1972. Controlled breeding of culturable species of fish - a review of progress and current problems. In: Pillay, T. V. R., ed., Coastal aquaculture in the Indo-Pacific Region, London, Fishing News (Books) Ltd., 180-194.

Shehadeh, Z. H., Madden, W. D. and Dohl, T. P., 1973. The effect of exogenous hormone treatment on spermiation and vitellogenesis in the grey mullet, *Mugil cephalus* L. J. Fish Biol., 471-478.

Sherwood, N. M., Crim, L. W., Carolsfeld, J. and Walters, S. M., 1988. Sustained hormone release. I. Characteristics of *in vitro* release of gonadotrophin-releasing hormone analogue (GnRH-a) from pellets. Aquaculture, 74: 75-86.

Shetty, V. K. and Satyanarayana Rao, G. P., 1990. Stimulation of spermatogenesis with salmon gonadotrophin and ovine luteinizing hormone in the 17β-oestradiol-treated male catfish, *Clarias batrachus* (Linn). Indian J. Anim. Sci., 60(7): 900-907.

Shireman, J. V., Colle, D. E. and Rottman, R. W., 1978. Manipulation of temperature and photo period for inducing maturation in grass carp. In: Smitherman, R., O., Shelton, W. L. and Grover, J., Eds. Symposium on culture of exotic fishes presented at Aquaculture/ Atlanta/ '78, Atlanta Georgia, 4th January 1978. pp. 156-164.

Simone, D. A., 1990. The effects of the synthetic steroid 17-alpha-methyltestosterone on the growth and organ morphology of the channel catfish, (*Ictalurus punctatus*). Aquaculture, 84: 81-93.

Sorensen, P. W. and Winn, H. E., 1984. The induction of maturation and ovulation in American eels, *Anguilla rostrata* (Le Sueur), and the relevance of chemical and visual cues to male spawning behaviour. J. Fish Biol., 25: 261-268.

Sower, S. A., Schreck, C. B. and Evenson, M., 1983. Effects of steroids and steroid antagonists on growth, gonadal development, and RNA/DNA ratios in juvenile steelhead trout. Aquaculture, 32: 243-254.

Stacey, N. E. and Goetz, F. W., 1982. Role of prostaglandins in fish reproduction. Can. J. Fish. Aquat. Sci., 39: 92-98.

Stoss, J., 1983. Fish gamete preservation and spermatozoon physiology. In: Hoar, W. S., Randall, D. J. and Donaldson, E. M., Fish Physiology, Volume IX, Reproduction, part B: Behaviour and Fertility Control. Academic Press, INC. London. pp. 305-350.

Sundararaj, B. I. and Nayyar, S. K., 1967. Effects of exogenous gonadotrophins and gonadal hormones on the testes and seminal vesicles of hypophysectomised catfish, *Heteropneustes fossilis* (Bloch). Gen. Comp. Endocrinol., 8: 403-416.

Suquet, M., Omnes, M. H., Normant, Y. and Fauvel, C., 1992. Influence of photoperiod, frequency of stripping and presence of females on sperm output in turbot, *Scophthalmus maximus* (L.). Aquaculture and Fisheries Management, 23: 217-225.

Suzuki, Y., Kobayashi, M., Nakamura, O., Aida, K. and Hanyu, I., 1990. Induced spermiation and ovulation in goldfish by oral administration of salmon gonadotrophin. In: Hirano, R. and Hanyu, I. (eds), Proceedings of the Second Asian Fisheries Forum, Tokyo, Japan, 12-22 April 1989. pp 577-580.

Takashima, F., Weil, C., Billard, R., Crim, L. W. and Fostier, A., 1984. Stimulation of spermiation by LHRH analogue in carp. Bull. Jap. Soc. Sci. Fish., 50(8): 1323-1329.

Tamaru, C. S., Lee, C.-S., Kelley, C. D., Banno, J. E., Ha, P. Y., Aida, K. and Hanyu, I., 1988. Characterising the stage of maturity most receptive to an acute LHRH-analogue therapy for inducing milkfish (*Chanos chanos*) to spawn. Aquaculture, 74: 147-163.

Tamaru, C. S., Lee, C.-S., Ha, P. Y., Ginoza, J. M., Aida, K. and Hanyu, I., 1990. Release rates of steroids from silastic tube implants in vitro and in vivo with juvenile milkfish, *Chanos chanos*. Aquaculture, 87: 91-101.

Terner, C. 1986. Evaluation of salmonid sperm activity for cryopreservation. Prog. Fish. Cult., 48: 230-232.

Thomas, P. and Boyd, N. W., 1989. Dietary administration of an LHRH analogue induces spawning of spotted seatrout (*Cynoscion nebulosus*). Aquaculture, 80: 363-370.

Thorgaard, G. H., 1983. Chromosome set manipulation and sex control in fish. In: Hoar, W. S., Randall, D. J. and Donaldson, E. M., Fish Physiology, Volume IX, Reproduction, part B: Behaviour and Fertility Control. Academic Press, INC. London. pp. 405-434.

Thorgaard, G. H., 1986. Ploidy manipulation and performance. Aquaculture, 57: 57-64.

Trendall, J., 1990. Reduce your chances of being a victim of cannibalism. Barramundi Aquaculture, 1(4): 1-3.

Ueda, H., Young, G., Crim, L. W., Kambegawa, A. and Nagahama, Y., 1983. 17α, 20β-dihydroxy-4-pregnen-3-one: plasma levels during sexual maturation and *in vitro* production by the testes of amago salmon (*Oncorhynchus rhodurus*) and rainbow trout (*Salmo gairdneri*). Gen. Comp. Endocrinol., 51: 106-112.

Ueda, H., Kambegawa, A. and Nagahama, Y., 1985. Involvement of gonadotrophin and steroid hormones in spermiation in the amago salmon, *Oncorhynchus rhodurus*, and the goldfish, *Carassius auratus*. Gen. Comp. Endocrinol., 59: 24-30.

Ueda, H., Hiroi, O., Yamauchi, K., Hara, A., Kagawa, H., Adachi, S. and Nagahama, Y., 1991. Changes in serum steroid hormone levels and *in vitro* steroid hormone production in the testes of male chum salmon, *Oncorhynchus keta* during spawning migration. Bull. Jap. Soc. Sci. Fish., 57(6): 1111-1114.

Van Der Kraak, G., Pankhurst, N. W., Peter, R. E. and Lin H. R., 1989. Lack of antigenicity of human chorionic gonadotrophin in silver carp (*Hypophthalmichthys molitrix*) and goldfish (*Carassius auratus*). Aquaculture, 78: 81-86.

Van Oordt, P. G. W. J. and Peute, J., 1983. The cellular origin of pituitary gonadotrophins in teleosts. In: In: Hoar, W. S., Randall, D. J. and Donaldson, E. M., Fish Physiology, Volume IX, Reproduction, part A: Endocrine Tissues and Hormones. Academic Press, INC. London. pp. 137-186.

Van Weerd, J. H. and Richter, C. J. J., 1991. Sex pheromones and ovarian development in teleost fish. Comp. Biochem. Physiol., A, 100A(3): 517-527.

Varadaraj, K., 1990. Production of monosex male *Oreochromis mossambicus* (Peters) by administering 19-norethisterone acetate. Aquaculture and. Fisheries Management, 21(1): 133-135.

Von Ihering, R., 1937. A method for inducing fish to spawn. Prog. Fish Cult. (US), 34: 15-16.

Watanabe, T., Arakawa, T., Kitajima, C. and Fujita, S. 1984a. Effect of nutritional quality of broodstock diets on reproduction in Red Sea bream. Bull. Jap. Soc. Sci. Fish., 50(3): 495-501.

Watanabe, T., Itoh, A., Kitajima, C. and Fujita, S. 1984b. Effect of dietary protein levels on reproduction of Red Sea bream. Bull. Jap. Soc. Sci. Fish., 50(6): 1015-1022.

Weber, G. M. and Lee, C.-S., 1985. Effects of 17α-methyl-testosterone on spermatogenesis and spermiation in the grey mullet, *Mugil cephalus* L. J. Fish Biol., 26: 77-84.

Weil, C. and Crim, L. W., 1983. Administration of LHRH analogues in various ways: effect on the advancement of spermiation in prespawning landlocked salmon, *Salmo salar*. Aquaculture, 35: 103-115.

Weil, C. and Crim, L. W., 1985. Manipulation of the hypothalamo-pituitary gonadal axis in the juvenile rainbow trout: influence of pituitary transplantation and LHRH analogue treatment. Reprod. Nutr. Dévelop., 25(5): 895-907.

Wongsomnuk, S., and Manevonk, S., 1973. Results of experiments on artificial breeding and larval rearing of the sea-bass *Lates calcarifer* Bloch. Contr. Songkhla Marine Fisheries Station, 5. 22 pages.

Yamamoto, K., 1973. Endocrinological studies related to artificial propagation of fish. In: Motoda, S. (ed.), Propagation of marine resources of the Pacific Ocean. Tokai University, Tokyo. pp. 11-29.

Yamauchi, K., 1990. Studies of gonadal steroids involved in final maturation in the Japanese eel, *Anguilla japonica*, a review. Int. Rev. Gesamt. Hydrobiol., 75(6): 849-860.

Yamazaki, F., 1976. Application of hormones in fish culture. J. Fish. Res. Board. Can., 33: 948-958.

Yamazaki, F., 1983. Sex control and manipulation in fish. Aquaculture, 33: 329-354.

Yamazaki, F., and Donaldson, E. M., 1969. Involvement of gonadotrophin and steroid hormones in the spermiation of the goldfish (*Carassius auratus*). Gen. Comp. Endocrinol., 12: 491-497.

Zhao, W. and Wright, R. S., 1988. A study on sex steroids of male Atlantic salmon, *Salmo salar*, *in vivo* and *in vitro*. Oceanol. Limnol. Sin., 19(6): 532-538.

Zohar, Y., 1986. Gonadotrophin releasing hormone in spawning induction in teleosts: basic and applied considerations. In: Zohar, Y. and Breton, B. (eds). Reproduction in fish: basic and applied aspects in endocrinology and genetics. Tel Aviv, Israel, 10-12 November 1986. pp. 47-62.

Zohar, Y., 1989. Fish reproduction: its physiology and artificial manipulation. In: Shilo, M. and Sarig, S. (eds), Fish Culture in Warm Water Systems: Problems and Trends. CRC Press Inc. Boca Raton, Florida. pp. 65-119.

Zohar, Y. and Billard, R., 1984. Annual and daily changes in plasma gonadotrophin and sex steroids in relation to teleost gonad cycles. Trans. Am. Fish. Soc., 113(4): 444-451.

APPENDIX 1. Summary of Hormone Induced Spermatogenesis and Spermiation of Male Fish Using Various Reproductive Hormones.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Amago salmon	*Oncorhynchus rhodurus*	SPH	1 mg kg^{-1}	IP injection (saline)	Single injection	Induced spermiation	Ueda *et al.* 1985.
Amago salmon	*Oncorhynchus rhodurus*	17α-20βP	1 mg kg^{-1}	IP injection (saline)	Two injections 24 hours apart	Induced spermiation (less than SPH)	Ueda *et al.* 1985.
Amago salmon	*Oncorhynchus rhodurus*	Testosterone	1 mg kg^{-1}	IP injection (saline)	Two injections 24 hours apart	No effect	Ueda *et al.* 1985.
Amago salmon	*Oncorhynchus rhodurus*	11-KT	1 mg kg^{-1}	IP injection (saline)	Two injections 24 hours apart	No effect	Ueda *et al.* 1985.
Atlantic Salmon	*Salmo salar*	LHRHa (D-ala^6)	125 µg kg^{-1}	IP injection (saline)	every 2 days for 12 days	Increased spermiation	Weil and Crim 1983.
Atlantic Salmon	*Salmo salar*	LHRHa (D-ala^6)	375 µg kg^{-1}	IP injection (propylene glycol)	every 7 days for 12 days	Increased spermiation	Weil and Crim 1983.
Atlantic Salmon	*Salmo salar*	LHRHa (D-ala^6)	1.6 mg kg^{-1}	IP implant (silastic)	once	Increased spermiation	Weil and Crim 1983.
Atlantic Salmon	*Salmo salar*	LHRHa (D-2 naphthylalanine)	25 µg kg^{-1}	IP implant (cholesterol)	once	Increased spermiation	Weil and Crim 1983.
Barramundi	*Lates calcarifer*	LHRHa Salmon GnRHa	10 µg kg^{-1}	IM Injection	Once	Results not quantified	Almendras *et al.* 1988a.
Barramundi	*Lates calcarifer*	LHRHa (D-ala^6)	100 µg kg^{-1}	IM Implant (Cholesterol)	One implant per month for three months	Increased spermiation early in the spawning season	Garcia 1990.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Barramundi	*Lates calcarifer*	17-MT	100 µg kg^{-1}	IM Implant (Cholesterol)	One implant per month for three months	Increased spermiation early in the spawning season	Garcia 1990.
Barramundi	*Lates calcarifer*	17-MT + LHRHa (D-ala^6)	100 µg IM kg^{-1} (each)	IM Implant (Cholesterol)	One implant per month for three months	Increased spermiation early in the spawning season	Garcia 1990.
Barramundi	*Lates calcarifer*	LHRHa (D-ala^6)	10 µg kg^{-1}	IM Injection	2 injections, 1st dose T_0 2nd dose T_{24}	Results not quantified. Female treatments complicate data interpretation	Nacario 1987.
Barramundi	*Lates calcarifer*	LHRHa (D-ala^6)	50 µg kg^{-1}	IM Implant (Cholesterol)	Once	Results not quantified	Nacario 1987.
Barramundi	*Lates calcarifer*	HCG	500 IU kg^{-1} 1000 IU kg^{-1}	IM injection (saline)	1st dose T_0 2nd dose T_{24}	Nil response	Schipp un pub. data.
Barramundi	*Lates calcarifer*	CPE + LHRHa (D-ala^6) followed by CPE	4.5 mg kg^{-1} 20-30 µg kg^{-1} 8.0 mg kg^{-1}	IM injection (saline) IM implant IM injection (saline)	Daily for four days Once Once	Spermiation response after 24 hours lasted 1-2 days. Large increase in sperm volume	Schipp *et al* 1989.
Barramundi	*Lates calcarifer*	iCPE	8.0 mg kg^{-1}	IM injection (saline)	Two injections, T_0 and T_{24}	Not as consistent as normal CPE in stimulating spermiation	Schipp, present study.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Barramundi	*Lates calcarifer*	LHRHa (D-ala^6) + pimozide	25 µg kg^{-1} 50 µg kg^{-1} 100 µg kg^{-1} each + 1 mg kg^{-1} pim	IM injection (saline)	Single injection	moderate increase in sperm production. No indication that pimozide effective.	Schipp, present study.
Barramundi	*Lates calcarifer*	Ovaprim SC	0.1 ml kg^{-1}	IM injection (oil)	Two injections, T_0 and T_{24}	Moderate increase in spermiation	Schipp, present study.
Barramundi	*Lates calcarifer*	17α–20β	1.0 mg kg^{-1}	IM injection (saline)	Two injections, T_0 and T_{24}	Increased spermiation	Schipp, present study.
Barramundi	*Lates calcarifer*	Sustanon '250'	1.0 ml fish^{-1} 0.5 ml fish^{-1}	IM injection	Two injections, T_0 and T_{24}	No response	Schipp, present study.
Barramundi	*Lates calcarifer*	17-MT	100 mg kg^{-1} 200 mg kg^{-1} 400 mg kg^{-1}	IM implant (cholesterol)	Checked weekly for 4 weeks	No effect	Schipp, present study.
Barramundi	*Lates calcarifer*	17-MT	12.5 mg kg^{-1}	Oral	Daily for 28 days	No effect	Schipp, present study.
Barramundi	*Lates calcarifer*	17-MT 19-NE	10 mg pellet^{-1}	IM pellet (silicone)	Examined monthly for three months	No effect	Schipp, present study.
Barramundi	*Lates calcarifer*	LHRHa (D-ala^6) + 17-MT	25 µg kg^{-1} 50 µg kg^{-1} 100 µg kg^{-1} 100 µg kg^{-1}	IM implant (cholesterol)	Examined after 28 days	25 + 100 dose moderately stimulated spermatogenesis during spawning season. Other doses not effective	Schipp, present study.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Barramundi	*Lates calcarifer*	LHRHa (D-ala^6)	25 µg kg^{-1} 50 µg kg^{-1}	IM implant (cholesterol)	Examined after 28 days	Both doses not effective at stimulated. spermatogenesis during non-spawning season. 25 µg dose effective during spawning season	Schipp, present study.
Bream	*Abramis brama*	LHRHa (D-ala^6) + either eglonil o metoclopramide	4 µg kg^{-1} 4 mg kg^{-1} 4 mg kg^{-1}	IM injection (saline)	Once	Increased spermiation in 100% of fish 20-30 hours after treatment	Glubokov *et al.* 1991.
Catfish	*Clarias batrachus*	Ovine LH SG-G100	5.2 mg kg^{-1} 5.2 mg kg^{-1}	IP injection (0.3% saline)	Every two days for 16 days	SG-G100 more effective than Ovine LH in stimulating spermatogenesis	Shetty and Satyanarayana Rao 1990.
Catfish	*Clarias macrocephalus*	Ovine LH	50 µg 100 µg	IP injection (saline)	Daily for 21 Days	Stimulated spermatogenesis	Pocsidio 1978.
Catfish	*Clarias macrocephalus*	Ovine FSH	50 µg 100 µg	IP injection (saline)	Daily for 21 Days	No effect	Pocsidio 1978.
Catfish	*Clarias macrocephalus*	HCG	50 USP	IP injection (saline)	Daily for 21 Days	Stimulated spermatogenesis	Pocsidio 1978.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Catfish	*Heteropneustes fossilis*	Testosterone propionate (TP)	0.5 mg $fish^{-1}$ 2.5 mg $fish^{-1}$	IM injection (olive oil)	Daily for 30 days	Both doses induced spermatogenesis and secretory activity in the testes	Sundararaj and Nayyar 1967.
Catfish	*Heteropneustes fossilis*	Oestradiol benzoate	20 µg $fish^{-1}$	IM (olive oil)	Daily for 30 days	No effect	Sundararaj and Nayyar 1967.
Catfish	*Heteropneustes fossilis*	HCG	1 IU $fish^{-1}$ 2 IU $fish^{-1}$ 5 IU $fish^{-1}$	IM (saline)	Daily for 30 days	Resumption of spermatogenesis and secretion	Sundararaj and Nayyar 1967.
Catfish	*Heteropneustes fossilis*	FSH	50 µg $fish^{-1}$ 100 µg $fish^{-1}$	IM (saline)	Daily for 30 days	Slight increase in testis weight	Sundararaj and Nayyar 1967.
Catfish	*Heteropneustes fossilis*	HCG and FSH	50 µg + 1 iu $fish^{-1}$	IM (saline)	Daily for 30 days	Better response than HCG alone	Sundararaj and Nayyar 1967.
Common carp	*Cyprinus carpio*	LHRH LHRHa (D-ala^6)	10 µg kg^{-1} 50 µg kg^{-1}	IP injection	Daily for 4 days	Spermiation stimulated	Billard *et al.* 1983.
Common Carp	*Cyprinus carpio*	Pimozide + LHRHa (D-trp^6)	1 mg kg^{-1} or 10 mg kg^{-1} 0.1 µg kg^{-1} or 1.0 µg kg^{-1} or 10.0 µg kg^{-1}	IP injection (saline)	Pimozide injected at T_0 and LHRHa at T_6 Milt volume checked on Days 1 and 5 following treatment	Dose dependent response to LHRHa	Billard *et al.* 1987.
Common Carp	*Cyprinus carpio*	Pimozide + LHRHa (D-trp^6)	1.0 mg kg^{-1} 10.0 µg kg^{-1}	IP injection + IP silicone implant	Treated once at either 6 am or 8 pm.	Response dependent on time of injection and implantation	Billard *et al.* 1987.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Common Carp	*Cyprinus carpio*	CPH	1.0 mg kg^{-1}	Injection (site not specified)	Once	Large increase in m Milt volume in 24 hours	Billard *et al.* 1989.
Common Carp	*Cyprinus carpio*	CPH	3.3 mg kg^{-1}	Injection (site not specified)	Two injections, 0.3 mg T_0, 3.0 mg T_{12}	Large increase in milt volume	Billard *et al.* 1989.
Common carp	*Cyprinus carpio*	CPH	0.22 mg kg^{-1} 2.20 mg kg^{-1} 22.0 mg kg^{-1}	IP injection (distilled water)	Injected once and checked after 24 hours	Dose dependent spermiation response	Clemens and Grant 1965.
Common carp	*Cyprinus carpio*	HCG	440 Units kg^{-1}	IP injection (not stated)	Injected once and checked after 24 hours	Spermiation induced but not as reliable as GtH	Clemens and Grant 1965.
Common Carp	*Cyprinus carpio*	CPH HCG	3 mg kg^{-1} 1000-2000 IU kg^{-1}		Injected once then checked 24 hrs later	CPH stimulated spermiation, HCG - no effect	Courtois *et al.* 1986.
Common Carp	*Cyprinus carpio*	LHRHa (D-ala^6)	30 µg kg^{-1}	Injection	Injected once	Volume of milt increased after 12 hours and remained high for 24 hours	Ngamvongchon *et al.* 1987. (abstract only)
Common Carp	*Cyprinus carpio*	CPE	2.0 mg kg^{-1}	IP injection (saline)	Once (checked 5 times in 48 hours)	Increase in milt volume after 12 hours. (response temperature dependent)	Saad and Billard 1987.
Common Carp	*Cyprinus carpio*	CPE	1.0 mg kg^{-1} 2.0 mg kg^{-1} 4.0 mg kg^{-1}	IP injection (saline)	Once, checked daily	Dose dependent response. Maximum volume after 24 hours	Saad and Billard 1987.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Common Carp	*Cyprinus carpio*	CPE	2.0 mg kg^{-1}	IP injection (saline)	Once a week for 9 months	Stimulated spermiation over a long period but not spermatogenesis	Saad and Billard 1987.
Common Carp	*Cyprinus carpio*	11 KT	1.0 mg kg^{-1} 2.0 mg kg^{-1}	IP injection (saline)	Once	No response	Saad and Billard 1987.
Common Carp	*Cyprinus carpio*	17α-20βP	1.0 mg kg^{-1} 2.0 mg kg^{-1}	IP injection (saline)	Once	No response	Saad and Billard 1987.
Eel, Silver	*Anguilla anguilla*	HCG	500 IU kg^{-1}	IM (saline)	One injection on Day_0 and another Day_7	Induced spermiation	Dollerup and Graver 1985.
Eel, Silver	*Anguilla anguilla*	HCG	250 IU kg^{-1}	IM (saline)	One injection	Caused an increase in spermatogenesis lasting for 3 months	Khan *et al.* 1987.
Goldfish	*Carassius auratus*	PPCG	10 mg kg^{-1} 20 mg kg^{-1} 60 mg kg^{-1} 120 mg kg^{-1}	Injection (site and solvent not specified)	Injected every 3 days for 88 days	Highest dose initiated spermatogenesis and spermiation after 42 days.	Clemens and Reed 1967.
Goldfish	*Carassius auratus*	17αP	1 mg kg^{-1}	IP injection (saline)	Two injections 24 hours apart	Spermiation induced (less than SPH)	Ueda *et al.* 1985.
Goldfish	*Carassius auratus*	SPH	1 mg kg^{-1}	IP injection (saline)	Two injections 24 hours apart	Strong spermiation response	Ueda *et al.* 1985.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Goldfish	*Carassius auratus*	17α-20βP	1 mg kg^{-1}	IP injection (saline)	Two injections 24 hours apart	Spermiation induced (less than SPH)	Ueda *et al.* 1985.
Goldfish	*Carassius auratus*	11-KT	1 mg kg^{-1}	IP injection (saline)	Two injections 24 hours apart	2 out of 7 fish spermiated	Ueda *et al.* 1985.
Goldfish	*Carassius auratus*	Testosterone	1 mg kg^{-1}	IP injection (saline)	Two injections 24 hours apart	1 out of 9 fish spermiated	Ueda *et al.* 1985.
Goldfish	*Carassius auratus*	SPH	2.0 ml kg^{-1}	Oral intubation	one dose	Increased plasma levels of T and 17α-20β after 6 hours and milt supply after 24 hours	Suzuki *et al.* 1990.
Goldfish	*Carassius auratus*	17-MT	1 ppm 10 ppm 30 ppm	Oral	Daily, duration not specified	Initiated courtship behaviour	Yamazaki 1976.
Goldfish	*Carassius auratus*	17-MT	10 mg kg^{-1} 50 mg kg^{-1} 100 mg kg^{-1} 300 mg kg^{-1} 500 mg kg^{-1} 1 000 mg kg^{-1}	IP injection (peanut oil)	Once, checked after 24 hours	Dose dependent response. 100% of fish spermiated at 1 000 mg kg^{-1}	Yamazaki and Donaldson 1969.
Goldfish	*Carassius auratus*	DHD	10 mg kg^{-1} 100 mg kg^{-1} 500 mg kg^{-1} 1 000 mg kg^{-1}	IP injection (peanut oil)	Once, checked after 24 hours	Dose dependent response. 100% of fish spermiated at 1 000 mg kg^{-1}	Yamazaki and Donaldson 1969.
Goldfish	*Carassius auratus*	11-KT	10 mg kg^{-1} 50 mg kg^{-1} 100 mg kg^{-1} 200 mg kg^{-1}	IP injection (peanut oil)	Once, checked after 24 hours	Dose dependent response. 80% of fish spermiated at 200 mg kg^{-1}	Yamazaki and Donaldson 1969.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Goldfish	*Carassius auratus*	SPH	0.2 mg $fish^{-1}$	Injection (site and solvent not specified)	Injected three times per week for 3 weeks	Spermiation was restored after 3 weeks	Yamazaki and Donaldson 1969
Green Sunfish	*Lepomis cyanellus*	Testosterone propionate (TP)	1.0 mg $fish^{-1}$	IP (corn oil)	Every second day	Increase in GSI values after 4 weeks. Temp. dependent	Kaya 1973.
Green Sunfish	*Lepomis cyanellus*	CPH	2.0 mg $fish^{-1}$	IP (saline)	Every second day	Increase in GSI values after 4 weeks. Temp. dependent	Kaya 1973.
Grey Mullet	*Mugil cephalus*	17-MT	1 mg kg^{-1}	IP (peanut oil)	Every two days during pre spawning season	Spermatogenesis and spermiation stimulated. Peanut oil caused peritoneal granuloma	Shehadeh *et al* 1973.
Grey Mullet	*Mugil cephalus*	17-MT	50 mg kg^{-1}	IP (saline)	Every two days during spawning season.	Spermatogenesis and spermiation stimulated	Shehadeh *et al* 1973.
Grey Mullet	*Mugil cephalus*	HCG	200 IU kg^{-1}	IM (saline)	Daily during spawning season	Traces of milt after 4 weeks	Shehadeh *et al* 1973.
Grey Mullet	*Mugil cephalus*	17-MT	12.5 mg kg^{-1} 25.0 mg kg^{-1}	Oral	Once per day	Spermatogenesis induced within one month and maintained for 55 weeks	Weber and Lee 1985; Lee and Weber 1986.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Japanese Huchen	*Hucho perryi*	SPH		Injection	Once	Increased plasma levels of 17α-20β and sperm motility	Miura *et al.* 1991. (abstract only)
Milkfish	*Chanos chanos*	HCG	5 000 IU kg^{-1}	Injection (site and solvent not specified)	Once	Increase in spermiation lasting two days	Juario *et al.* 1981.
Milkfish	*Chanos chanos*	Durandon Forte '250'	1 ml $fish^{-1}$	Injection (site not specified) (arachis oil)	Once	Prolonged spermiation response lasting 7 days	Juario *et al.* 1981.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Milkfish	*Chanos chanos*	SPH	10 mg kg^{-1}	IM or IP implant	Treated once only, checked after 3-4 weeks.	No advancement of spermatogenesis or spermiation for any of the treatments	Lacanilao *et al.* 1985.
		SPH + T_4	10 mg kg^{-1} + 10 ppm	IM + oral			
		SPH + T_4	10 + 1 mg kg^{-1}	IM or IP implant			
		SG-G100	10 mg kg^{-1}	IP implant			
		SG-G100 + HCG	10 mg kg^{-1} + 1250 IU kg^{-1}	IP implant			
		HCG	2 500 IU kg^{-1}	IP implant			
		Durandon Forte + HCG	1 ml $fish^{-1}$ + 500 IU kg^{-1} + 1 000 IU kg^{-1}	IM injection			
Milkfish	*Chanos chanos*	17-MT and LHRHa ($D\text{-}ala^6$)	200 µg LHRHa 250 µg MT	LHRHa in Cholesterol pellet. 17-MT in silastic pellet.	Implanted once, checked monthly	Increase in spermiation within one month. The combination of the two hormones was better than LHRHa alone	Lee *et al.* 1986a.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Milkfish	*Chanos chanos*	17-MT	25.0 mg kg^{-1} 12.5 mg kg^{-1} 6.5 mg kg^{-1}	Oral	Daily for 12 weeks	All doses advanced spermatogenesis but not spermiation. 12.5 mg most effective.	Lee *et al.* 1986d.
Milkfish	*Chanos chanos*	17-MT (liquid)	250µg $fish^{-1}$	IM (pellet)	Implanted twice in three months	No difference in maturation rate compared to controls	Lee *et al.* 1986e.
Milkfish	*Chanos chanos*	17-MT (crystalline)	10 mg $fish^{-1}$	IM (pellet)	Implanted once	No difference in maturation compared to controls	Lee *et al.* 1986e.
Milkfish	*Chanos chanos*	17-MT (liquid) + LHRHa (D-ala^6)	250 µg + 200 µg $fish^{-1}$	IM (pellet)	17-MT Implanted twice in three months and LHRHa every month	90% of fish matured within one month of treatment	Lee *et al.* 1986e.
Milkfish	*Chanos chanos*	17-MT (crystalline) + LHRHa (D-ala^6)	10 mg + 200 µg $fish^{-1}$	IM (pellet)	17-MT implanted once, LHRHa implanted monthly	Maturation rate increased after 2 months	Lee *et al.* 1986e.
Milkfish	*Chanos chanos*	LHRHa (D-ala^6)	20-65 µg kg^{-1}	IM injection	Once	Treatment with hormone improved spawning success	Tamaru *et al.* 1988.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Murray Cod	*Maccullochella peeli*	HCG	250 IU kg^{-1} 500 IU kg^{-1} 1 000 IU kg^{-1} 2 000 IU kg^{-1}	IP injection (distilled water)	Single injection checked after 47 hours	Significant increase in sperm volume for doses above 500 IU	Rowland 1988.
Northern Pike	*Esox lucius*	LHRHa (D-trp^6)	0.1 µg kg^{-1} 1.0 µg kg^{-1} 10.0 µg kg^{-1}	IC injection (saline)	3 injections 8 hours apart	No result	Billard and Marcel 1980.
Northern Pike	*Esox lucius*	PPSG	5 µg kg^{-1} 10 µg kg^{-1} 20 µg kg^{-1} 100 µg kg^{-1}	IP injection (saline)	One injection, examined after 2 days	3-11 fold increase in sperm production	Billard and Marcel 1980.
Northern Pike	*Esox lucius*	Fresh Pike Pituitary	1.2 mg kg^{-1} 14.0 mg kg^{-1}	IP injection (saline)	One injection, examined after 2 days	3-7 fold increase in sperm production	Billard and Marcel 1980.
Northern Pike	*Esox lucius*	CPE	0.5 mg kg^{-1} 1.0 mg kg^{-1} 3.0 mg kg^{-1}	IP injection (saline)	One injection, examined after two days	3-6 fold increase in sperm production	Billard and Marcel 1980.
Pacific Herring	*Clupea harengus pallasi*	mLHRHa	170-220 µg kg^{-1} 800-1000 µg kg^{-1}	IP pellet	Implanted once	Advanced maturation	Carolsfeld *et al.* 1988.
Pacific Herring	*Clupea harengus pallasi*	sLHRHa	800-1000 µg kg^{-1}	IP pellet	Implanted once	No effect	Carolsfeld *et al.* 1988.
Pacific Herring	*Clupea harengus pallasi*	LHRHa PPSG SPH	0.02 mg kg^{-1} 0.2 mg kg^{-1} 0.1 mg kg^{-1} 1.0 mg kg^{-1} 10.0 mg kg^{-1}	IP (0.65% saline)	Injected once	All treatments stimulated spermiation. Dose dependent response for LHRHa	Kreiberg *et al.* 1987.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Pacu	*Colossoma mitrei* (=*Piaractus mesopotamicus*)	SG-G100 and SPH	0.2 mg kg^{-1} SG-G100 + 14 mg kg^{-1} SPH	IM Injection (0.05% saline)	SG-G100 at T_0, SPH at T_{24}	Spermiation induced but male also in close contact with ovulating female	Castagnolli and Donaldson 1981.
Pacu	*Colossoma mitrei* (=*Piaractus mesopotamicus*)	LHRHa (D-ala^6) SPH CPE Semi purified *Semaprochilodus* sp) Pituitary extract		Injection		All treatments increased sperm production	Castagnolli *et al.* 1988.
Pacu	*Colossoma mitrei* (= *Piaractus mesopotamicus*)	CPE	0.5 mg kg^{-1}	IM (distilled water)	Once only, at the same time the female received her second injection	Induced spermiation but not quantified	Godhino and Godhino 1986.
Pacu	*Piaractus mesopotamicus*	LHRHa (D-ala^6)		Injection	Daily for 5 days	Increased spermiation	Rosa *et al.* 1988. (abstract only)
Rabbitfish	*Siganus guttatus*	LHRHa (D-Ala^6)	20 µg kg^{-1}	IM injection (saline)	One injection. Milt stripped at T_0, T_{24}, and T_{48}.	Thinning of milt maximal after 24 hours	Garcia 1991.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Rainbow Trout	*Oncorhynchus mykiss*	TP 11 KT	Not stated	Silastic pellet implanted into testes		Both hormones of equal efficiency in stimulating spermiation (8 times better than controls)	Billard *et al.* 1982.
Rainbow Trout	*Oncorhynchus mykiss*	Trout GtH	1 mg kg^{-1}	Injection (site and solvent not specified)	Injected 3 times per week for unknown period	Stimulated testis growth and sperm release	Billard *et al.* 1982.
Rainbow Trout	*Oncorhynchus mykiss*	17-MT	50 µg kg^{-1} 500 µg kg^{-1} (both per kg o feed)	Oral	Daily for 7 months	Inhibited spermatogenesis	Billard *et al.* 1982a.
Rainbow Trout	*Oncorhynchus mykiss*	Big Mouth Buffalo (*Lectiobus cyprinellus*) pituitary extrac	0.04 mg kg^{-1} 0.44 mg kg^{-1} 4.40 mg kg^{-1}	IP injection (distilled water)	Injected once and checked after 24 hours	Dose dependent spermiation response	Clemens and Grant 1965.
Rainbow Trout	Oncorhynchus mykiss	17-MT	1.0 mg kg^{-1} 5.0 mg kg^{-1} 15.0 mg kg^{-1} 35.0 mg kg^{-1}	Oral	Fed once per day	Spermatogenesis advanced in immature fish	Sower *et al.* 1983.
Rainbow Trout	Oncorhynchus mykiss	17-MT + flutamide	5.0 mg + 20 mg kg^{-1}	Oral	Fed once per day	Spermatogenesis advanced in immature fish	Sower *et al.* 1983.
Rainbow Trout	Oncorhynchus mykiss	Flutamide	20 mg kg^{-1}	Oral	Fed once per day	Spermatogenesis advanced in immature fish	Sower *et al.* 1983.
Rainbow Trout	Oncorhynchus mykiss	Testolactone	10 mg kg^{-1}	Oral	Fed once per day	Spermatogenesis advanced in immature fish	Sower *et al.* 1983.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Rainbow Trout	Oncorhynchus mykiss	Methyl-androstenolone	10 mg kg^{-1}	Oral	Fed once per day	Spermatogenesis advanced in immature fish	Sower *et al.* 1983.
Rainbow Trout	*Oncorhynchus mykiss*	PPSG	4-5 mg kg^{-1}	IP injection (saline)	3 times per week for 3 weeks	Advanced maturation	Yamazaki 1976.
Rainbow Trout	*Oncorhynchus mykiss*	17-MT	5 ppm	Oral	Daily for 1 month	Advanced maturation	Yamazaki 1976.
Rainbow Trout	*Oncorhynchus mykiss*	Diethyl-stilbestrol	5 ppm	Oral	Daily for 1 month	Advanced maturation when combined with SGtH	Yamazaki 1976.
Spotted Scat	*Scatophagus argus*	LHRHa (D-ala^6)	22-100 µg kg^{-1}	IM pellet	Implanted once	No effect	Barry *et al.* 1991.
Spotted Scat	*Scatophagus argus*	17-MT	2.2-10 mg kg^{-1}	IM pellet	Implanted once	No effect	Barry *et al.* 1991.
Spotted Scat	*Scatophagus argus*	LHRHa + 17-MT	22-100 µg kg^{-1} 2.2-100 µg kg^{-1}	IM pellet	Implanted once	No effect	Barry *et al.* 1991.
Spotted Scat	*Scatophagus argus*	HCG	5 000 IU kg^{-1} 10 000 IU kg^{-1}	IM injection (saline)	T_0 - 0.3 of dose T_{12} - 0.7 of dose	Decreased spermiation	Barry *et al.* 1991.
Spotted Scat	*Scatophagus argus*	LHRHa (D-ala^6)	66-142 µg kg^{-1}	IM pellet	Implanted once, checked every 8 hours	No difference compared to control fish	Barry *et al.* 1991.

APPENDIX 1.

Common Name	Scientific Name	Hormone(s)	Dose	Method (solvent)	Frequency/ Duration	Result	Author
Spotted Scat	*Scatophagus argus*	17-MT (LHRHa)	10 mg kg^{-1} feed	Oral	Daily for 45 days, then implanted with 35 µg kg^{-1} LHRHa	No observable effect until implanted with LHRHa, then a significant spermiation response	Barry *et al.* 1991.
Spotted Scat	*Scatophagus argus*	17-MT (LHRHa)	25 µg $fish^{-1}$	Silastic pellet	Implanted once, after 45 days implanted with 35 µg kg^{-1} LHRHa	No observable effect until implanted with LHRHa, then a significant spermiation response	Barry *et al.* 1991.
Sturgeon, stellate	*Acipenser stellatus*	Luliberin		Injection	Either one or two injections	Stimulated spermiation	Barannikova and Bukovskaya 1990. (abstract only)
Sturgeon, Stellate, Russian and Beluga	*Acipenser spp.*	SPE LHRHa		Injection		Increased spermiation	Goncharov *et al.* 1991. (abstract only)
Sturgeon, white	*Acipenser transmontanus*	Sturgeon and carp pituitary glands	0.5 - 1.0 mg kg^{-1} of dried pituitaries	IM (distilled water)	Injected once, when required	Stimulated spermiation	Doroshov *et al.* 1983.
Three Spined Stickle-back	*Gasterosteus aculeatus*	17-MT	35 µg L^{-1}	Added to tank water (ethanol)	50% of water and hormone renewed every 2 days	Inhibited spermatogenesis	Borg 1981.

Abbreviations for Appendix 1

11-KT	=	11-Ketotestosterone
17-MT	=	17α methyltestosterone
17α-20βP	=	17α, 20ß dihydroxy-4-pregnen-3-one
17α-OH PRG	=	17α Hydroxy progesterone
CPE	=	Carp pituitary extract
CPH	=	Carp pituitary homogenate
DHD	=	Dehydroepiandrosterone
Durandon Forte '250'	=	Commercial preparation of testosterone propionate, testosterone phenylpropionate, testosterone isocaproate and testosterone decanoate
FSH	=	Follicle stimulating hormone
GtH	=	Gonadotrophin
HCG	=	Human Chorionic Gonadotrophin
LH	=	Luteinising hormone
LHRHa	=	Luteinising Hormone Releasing Hormone analogue
mLHRHa	=	Mammalian LHRHa
sLHRHa	=	Salmon LHRHa
Pellet	=	Unless otherwise stated, refers to cholesterol based pellets.
PPSG	=	Partially purified salmon gonadotrophin
Saline	=	0.9% saline, unless otherwise stated
SG-G100	=	Salmon Pituitary extract concentrated via a sephadex column
SPH	=	Salmon pituitary homogenate
T_4	=	L-Thyroxine
TP	=	Testosterone propionate

APPENDIX 2

METHOD OF MANUFACTURE OF CHOLESTEROL PELLETS

The following method for the manufacture of cholesterol pellets is based on Lee *et al.* (1985) with modifications developed at the Darwin Aquaculture Centre.

Preparation of the Cholesterol/ Hormone Mixture

Three types of cholesterol/ hormone pellets were made:

1. Cholesterol and LHRHa
2. Cholesterol and 17α-methyltestosterone (17-MT)
3. Cholesterol and LHRHa + 17-MT

Synthetic LHRH analogue (Des Gly^{10}-[D-ala^{6}]-LHRH ethylamide) was purchased from Peptide Technologies in Sydney, NSW. Cholesterol and 17-MT were purchased from the Sigma Chemical Company in the USA. Catalogue numbers C8503 and M7252 respectively.

Calculation of Pellet Weights

Prior to the commencement of these trials, tests were carried out to determine the average weight of pellets produced from each of the two moulds and the average wastage or loss of cholesterol mixture during the manufacturing process. The main wastage was from the cholesterol mixture adhering to the mixing bowl. The wastage figure was taken into account and compensated for each time pellets were made. Approximately 20% of the pellet mixture was wasted during the manufacturing process.

Pellet Mixture

The pellet was prepared by mixing the required weight of cholesterol with the hormone. The cholesterol was weighed on a Denver analytical balance to the nearest 0.1 milligram.

In the case of LHRHa, the hormone was first dissolved in 1 ml of 80% ethanol to give a hormone concentration of 1 µg/

µl, the required volume was then extracted using a micro pipette and mixed thoroughly with the cholesterol.

17-MT was weighed dry and then mixed with the cholesterol powder. A few drops of 80% ethanol were added to assist mixing.

Once mixed, the cholesterol/ hormone mixture was left to air dry in a laminar flow cabinet for 1-2 hours before being packed into the hormone mould. Unlike Lee *et al.* (1985), cocoa butter was not used, as a binder was found to be unnecessary. Pellets manufactured using only cholesterol and hormone, without the addition of cellulose, are termed slow release pellets (Sherwood *et al.* 1988) and can effectively release hormone for over 30 days.

The Moulds

The pellet moulds consist of two small sheets of plexiglass measuring approximately 15 cm x 9 cm. One sheet was drilled with several holes of one millimetre diameter and the other left undrilled (Figure 9a).

Pellet Manufacture

To make a pellet, the drilled sheet was placed directly on top of the other and both were supported on a bench top. The cholesterol hormone mixture was then placed on the top sheet and pressed into the holes using a piece of 1 mm stainless rod. Firm pressure was used during the packing process to ensure a solid pellet (Figure 9b).

When sufficient pellets were packed into the mould the top plexiglass sheet was raised on two pieces of wood and the completed pellets forced out using the 1 mm stainless rod (Figure 9c).

During these trials, two thicknesses of plexiglass were used, 4 mm and 6 mm producing pellets of two different lengths and weights.

Example

Aim:

To implant 4 fish with LHRHa/ cholesterol pellets containing 25 µg LHRHa per kilogram body weight.

Calculations:

Weight of male fish: 4.0, 5.0, 6.0, 6.0 kg.

Therefore the fish receive: 100, 125, 150 and 150 µg LHRHa respectively. It is easier to manufacture the pellets in two concentrations than to make individual pellets for the fish. In this example the two concentrations chosen are 100 µg LHRHa/ pellet and 25 µg LHRHa/ pellet.

The calculations for the cholesterol and hormone weights required are shown in Table 24.

Table 24. Example of quantities of cholesterol and LHRHa hormone needed to manufacture pellets for implantation.

Fish Weight (kg)	No. Pellets containing 100 µg LHRHa	µg LHRHa	mg Cholesterol	No. Pellets containing 25 µg LHRHa	µg LHRHa	mg Cholesterol
4.0	1	100	9.23	-		
5.0	1	100	9.23	1	25	9.23
6.0	1	100	9.23	2	50	19.46
6.0	1	100	9.23	2	50	18.46
Ttl= 22.0	4	400	36.92	5	125	46.15
	+20% wastage	480	44.3		150	55.4

The pellet mix is then prepared in two batches, 480 µg LHRHa + 44.3 mg of cholesterol and 150 µg LHRHa + 55.4 mg of cholesterol and is then mixed, dried and manufactured according to the instructions given above.

Storage

Once manufactured, the pellets were stored at -18°C until required. Pellets were always manufactured close to the time of implantation and were never stored for more than one week.

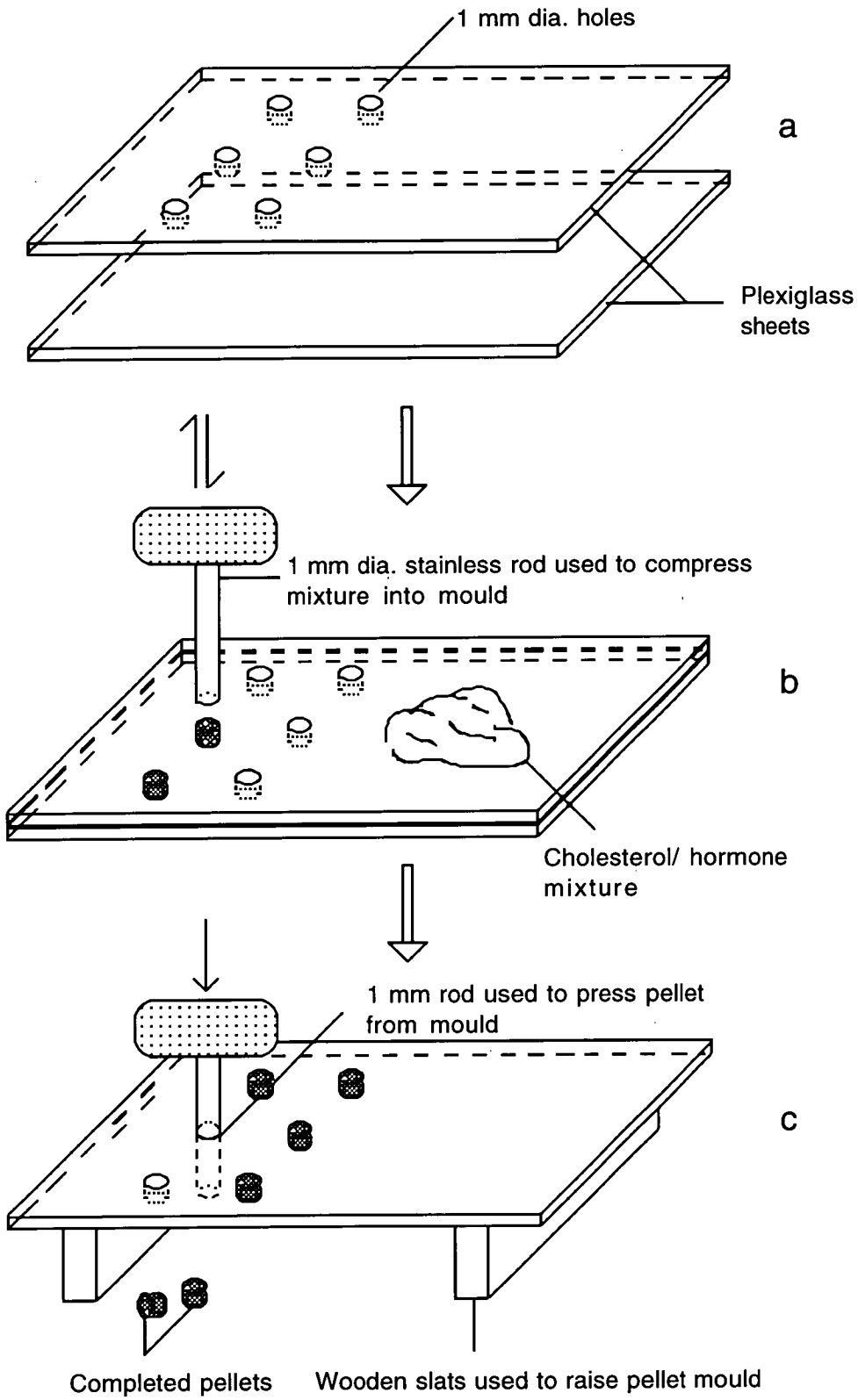

Figure 9. Method of manufacture of cholesterol/ hormone pellets.

APPENDIX 3.

SUMMARY OF RESULTS FROM FOUR YEARS OF BARRAMUNDI SPAWNINGS AT THE DARWIN AQUACULTURE CENTRE.

1989/ 90 SPAWNING SEASON

January 1990

Table 25. Spawning results for January 30 and 31, 1990.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
889	6.4	S	CPE	064	10.0	1.7×10^6 90%	2×10^6 80%
095	6.5	S	CPE				
890	5.2	S	CPE	098	6.8	0.8×10^6 90%	1.5×10^6 85%
097	7.7	S	CPE				

S = sperm in cannula sample. CPE = carp pituitary extract, 8 mg kg^{-1}, 2 injections, 24 hours apart.

April 1990

Table 26. Spawning results from April 6 and 7, 1990.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
885	6.2	S	CPE	851	11.0	1.2×10^6 80%	0.3×10^6 80%
895	6.5	S	CPE				
889	6.5	S	CPE				
088	6.8	S	CPE	853	15.4	6.5×10^6 75%	0.5×10^6 70%
074	5.5	S	CPE				

S = sperm in cannula sample, CPE = carp pituitary extract, T_0 - 2 mg kg^{-1}, T_{24} - 8 mg kg^{-1}.

1990/ 91 SPAWNING SEASON

September 1990

Table 27. Spawning results for September 24 and 25, 1990.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
078	4.4	S	nil	065	11.2	2.5×10^6 <1%	no spawn
067	3.7	S	nil				
072	4.8	S	nil				
071	3.4	S	25L + 1P				
058	5.4	S	25L + 1P	091*	10.2	3.0×10^6 75%	2.2×10^6 60%
889	7.6	S	25L + 1P	097*	8.4		
895	7.2	S	25L + 1P				
890	6.7	S	25L + 1P				
095	7.2	S	25L + 1P				

S = sperm in cannula sample, 25L + 1P = 25 µg LHRHa kg^{-1} + 1 mg pimozide kg^{-1}, 2 injections, 24 hours apart. *Two females combined in the one tank

October 1990

Table 28. Spawning results from October 6 and 7, 1990.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
889	7.6	S	nil	861	10.6	2×10^6 50%	no spawn
058	5.4	S	nil				
856	3.4	S	25L+1P	092	10.8	no spawn	no spawn
095	7.0	S	25L+1P				
895	7.2	S	25L+1P	888	10.2	1×10^6 60%	0.3×10^6 60%
071	3.4	S	25L+1P				
890	6.7	S	25L	879	10.0	no spawn	no spawn
078	4.4	S	25L				

S = sperm in cannula sample, 25L = 25 µg LHRHa kg^{-1}, 25L + 1P = 25 µg LHRHa kg^{-1} + 1 mg pimozide kg^{-1}.

November 1990

Table 29. Spawning results for November 10, 11 and 12, 1990.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised	Day 3 Egg No./% Fertilised
889		S	nil	851	11.6	3.4×10^6 40%	5.1×10^6 100%	3.7×10^6 90%
088		S	nil	077	12.6	2.9×10^6 60%	7.0×10^6 90%	6.0×10^6 93%
856		S	nil					
078		S	nil	099	14.2	2.3×10^6 0%	7.4×10^6 100%	2.8×10^6 90%
864		S	nil	064	12.5	6.3×10^6 95%	6.1×10^6 90%	
074		S	nil	855	15.6	13.4×10^6 90%	8.4×10^6 95%	
095		S	nil	880	15.3	1.2×10^6 90%	4.7×10^6 50%	
2136		S	nil					
063		S	nil	854	15.9	9.5×10^6 66%	11.6×10^6 93%	
061		S	nil					
890		S	nil	052	14.0	7.5×10^6 65%	4.7×10^6 93%	
2135		S	nil					
2138		S	nil	881	17.8	11.4×10^6 90%	7.5×10^6 80%	5.1×10^6 90%
058		S	nil	079	058	6.8×10^6 20%	6.1×10^6 70%	5.0×10^6 75%

S = sperm in cannula sample.

December 1990

Table 30. Spawning results for December 12 and 13, 1990.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised	Day 3 Egg No./% Fertilised
889	7.8	S	nil	097	9.0	4.4×10^6 60%	2.8×10^6 90%	1.6×10^6 80%
895	7.5	S	nil					
074	8.0	S	nil	883	12.2	no spawn	no spawn	no spawn
095	8.2	S	nil					
088	7.9	S	CPE	098	7.8	5×10^6 0%	no spawn	no spawn
073	7.2	S	CPE					
078	6.2	S	CPE	057	11.0	1.7×10^6 65%	2.8×10^6 80%	no spawn
2136	6.4	S	CPE					
856	5.0	S	25 L	894	9.0	2.0×10^6 80%	no spawn	no spawn
890	7.0	S	25 L					
2137	7.2	S	25 L	879	10.4	2.2×10^6 20%	2.3×10^6 80%	no spawn
063	6.0	S	25 L					
072	6.0	S	50 L	065	11.8	2.4×10^6 100%	1.4×10^6 25%	no spawn
061	7.8	S	50 L					

S = sperm in cannula sample, CPE = Carp pituitary extract, 8 mg kg^{-1} in two saline injections, 24 hours apart. 25 L = 25 µg LHRHa kg^{-1} in two saline injections, twenty four hours apart. 50 L = 50 µg LHRHa kg^{-1} in two saline injections, twenty four hours apart.

January 1991

Table 31. Spawning results for January 27 and 28, 1991.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
058	8.0	S	50 L	863	16.2	5.1×10^6 98%	4.4×10^6 78%
078	6.4	S	50 L				
895	7.8	F	50 L				
137	8.0	S	50 L	132	15.8	7.2×10^6 44%	4.5×10^6 87%
150	6.0	F	50 L				
889	8.0	F	50 L				
061	7.8	S	50 L	064	13.2	6.7×10^6 0%	1.3×10^6 0%
149	5.8	F	50 L				
095	8.0	S	50 L				

S = sperm in cannula sample, F = fluid in cannula sample, 50 L = 50 µg LHRHa kg^{-1}.

1991/ 92 SPAWNING SEASON

August 1991

Table 32. Spawning results for August 22 and 23, 1991.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
2112	9.4	2	nil				
2149	8.2	S	nil	090	13.0	$3x10^6$	$4x10^6$
072	9.5	S	nil	880	19.0	3%	70%
2137	10.0	S	nil				

S = sperm in cannula sample, 2 = thick fluid sperm expressed by hand pressure.
All fish in the one, 45,000 litre spawning tank.

September 1991

Table 33. Spawning results for September 4 and 5, 1991.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
2137	10.0	S	CPE				
095	9.0	S	CPE	2132	16.5	0.7×10^6 40%	7.5×10^6 95%
2149	8.2	S	CPE	880	19.0	5×10^6	4.5×10^6
2121	8.4	S	CPE			60%	100%

S = sperm in cannula sample, 2 = thick fluid sperm expressed by hand pressure,
CPE = 8 mg kg^{-1}, single injection.

September 1991

Table 34. Spawning results for September 10 and 11, 1991.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
2135	10.2	2	CPE	2134	19.0	8×10^6 90%	no spawn
895	8.8	S	CPE				
078	7.4	S	CPE				
				074	11.0	2×10^6 80%	no spawn
2112	9.4	S	CPE				
2113	8.4	S	CPE				

S = sperm in cannula sample, 2 = thick fluid sperm expressed by hand pressure.
CPE = carp pituitary extract, 8 mg kg^{-1}, single injection.

October 1991

Table 35. Spawning results for October 29 and 30, 1991.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
2130	CPE	S	CPE	132	17.2	6×10^6 60%	3.5×10^6 90%
2149	CPE	S	CPE				
2110	CPE	S	CPE				
2115	nil	S	nil	880	20.0	6×10^6 70%	3.5×10^6 40%
2120	nil	S	nil				
2146	nil	S	nil				

S = sperm in cannula sample, CPE = carp pituitary extract, 8 mg kg^{-1}, single injection.

November 1991

Table 36. Spawning results for November 6 and 7, 1991.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
095	8.8	S	CPE	2136	9.6	2×10^6 90%	no spawn
2121	9.0	S	CPE				
895	8.6	S	CPE				
2113	9.0	S	nil	890	10.4	1.8×10^6 <1%	no spawn
2146	6.0	S	nil				
2143	9.2	S	nil				

S = sperm in cannula sample, CPE = carp pituitary extract, 8 mg kg^{-1}, single injection.

December 1991

Table 37. Spawning results for December 4 and 5, 1991.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
2121	9.2	S	CPE	2109	9.8	0.3×10^6 90%	no spawn
095	8.8	S	CPE				
2137	10.0	S	CPE				
2110	8.2	S	nil	2116	10.6	no spawn	no spawn
2130	5.4	S	nil				
2145	6.2	S	nil				

S = sperm in cannula sample, CPE = carp pituitary extract, 8 mg kg^{-1}, single injection.

February 1992

Table 38. Spawning details for February 7 and 8, 1992.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
2121	9.6	S	CPE	2109	9.8	0.6×10^6 0%	
2195	6.4	S	CPE				
2110	9.0	S	nil	2111	11.2	2.5×10^6 60%	1.0×10^6 100%
095	8.8	S	nil				

S = sperm in cannula sample, CPE = carp pituitary extract, 8 mg kg^{-1}, single injection.

1992/ 93 SPAWNING SEASON

October 1992

Table 39. Spawning results from October 8 and 9, 1992.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
newly caught #1	10.6	S	25 L	2175	15.6	$<1 \times 10^6$ <1%	$2 \times 10^{6*}$
newly caught #2	12.0	S	nil				
2168	8.0	S	25 L	2172	13.8	4.0×10^6 70%	1.5×10^6 75%

S = sperm in cannula sample, 25 L = 25 µg LHRHa kg^{-1}, * = salinity dropped to 9 ppt because female was sick.

November 1992

Table 40. Spawning results for November 8 and 9, 1992.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
398	5.0	S	nil	2172	13.8	6×10^6 <10%*	1.5×10^6 90%***
2168	9.9	S	nil				
2166	11.0	S	nil				
392	5.0	S	nil	2173	14.6	5.5×10^6 <10%**	1.5×10^6 <10%**
2171	8.1	S	nil				

S = sperm in cannula sample, * = low fertilisation rate was due to poor egg quality, not to the failure of the males. ** = failure most likely due to males as egg quality appeared good. *** = small male was pushed out of the way by the larger males and not allowed to participate in the spawning.

November 1992

Table 41. Spawning results for November 13 and 14, 1992.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
2168	9.9	S	nil	2169	11.6	3×10^6 90%	2×10^6 90%
2167	8.5	S	nil				
2166	10.9	S	nil				
2195	7.5	S	nil	2170	10.9	3×10^6 90%	4×10^6 95%
2171	7.8	S	nil				
707	6.5	S	nil				

S = sperm in cannula sample.

December 1992

Table 42. Spawning results for December 15 and 16, 1992.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
2167	8.6	S	nil	No Tag	14.0	$<0.2 \times 10^6$ 0%	no spawn
no tag	12.0	S	nil				
2168	10.2	S	nil	2169	13.0	6×10^6 90%	1.5×10^6 90%
2195	7.5	S	nil				

S = sperm in cannula sample.

January 1993

Table 43. Spawning results from January 19 and 20, 1993.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
2195	7.6	S	nil	2155	14.1	3.0×10^6 90%	1.0×10^6 90%
2165	12.0	S	nil				
2151	10.3	S	nil	2169	13.2	4.0×10^6 95%	2.0×10^6 95%
2152	9.0	S	nil				

S = sperm in cannula sample.

February 1993

Table 44. Spawning results from February 23 and 24, 1993.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
2195	7.6	S	nil	2174	16.0	1.5×10^6 0%	no spawn
2152	9.0	S	nil				

S = sperm in cannula sample.

March 1993

Table 45. Spawning results for March, 6 and 7, 1993.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
no tag	13.0	S	Ovaprim	no tag	15.6	3×10^6 0%*	$<1 \times 10^6$ 0%*
392	6.3	S	Ovaprim				
398	5.8	S	Ovaprim	2172	14.6	2.5×10^6 0%*	2.8×10^6 50%
227	6.0	S	Ovaprim				
226	5.4	S	Ovaprim				

Ovaprim = 0.1 ml kg^{-1}, single injection.

March 1993

Table 46. Spawning results for March 30 and 31, 1993.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
2152	10.0	S	Ovaprim	2174	15.8	1.5×10^6 60%	0.2×10^6 50%
398	6.8	S	Ovaprim				
226	5.4	S	Ovaprim	2172	14.1	4×10^6 0%*	no spawn
227	6.0	S	Ovaprim				

Ovaprim = 0.1 ml kg^{-1}, single injection. * = failure due to poor egg quality.

April 1993

Table 47. Spawning details for April 20 and 21, 1993.

Male No.	Weight	Male Reprod. Condition prior to spawning	Male Treatment	Female No.	Weight	Day 1 Egg No./% Fertilised	Day 2 Egg No./% Fertilised
2195	8.4	S	Ovaprim	2172	14.0	2×10^6 60%	1×10^6 90%
392	7.0	S					
2154	9.2	S	Ovaprim	2174	15.5	0.5×10^6 0%*	no spawn
398	7.1	S					

S = sperm in cannula sample, Ovaprim = 0.1 ml kg^{-1}, single injection.