

The Effects of Nutrition on Reproduction in the Eastern Rainbowfish,  
*Melanotaenia splendida splendida*

Thesis submitted by  
Amanda Catherine BADGER BSc (Dalhousie University)  
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## Abstract

Fish broodstock raised in an aquaculture setting must be provided with a diet not only adequate to meet the demands of their basic metabolic functions, but also to support reproduction. The frequency, ration, and types of nutrients provided within a diet are all very important to the performance of broodstock.

The eastern rainbowfish, *Melanotaenia splendida splendida*, can be easily reared and readily spawn in captivity. This species has many characteristics that make it a good model species for reproductive experiments and is also commercially important to the aquarium trade. Studies were carried out to investigate the effects of nutrition on reproductive performance of this species.

Firstly, experiments were carried out to establish the basic reproductive parameters of *M. splendida splendida*. Periods of starvation were then carried out to gauge which parameters would be the more effective indicators for reproductive performance of the fish broodstock. It was determined that egg number, survival to eyed embryo, hatching rate, and unfed larval life were the parameters that would indicate reproductive success of this fish.

The next two studies were carried out to evaluate feeding frequency and feeding ration on reproductive performance in this species. Different feeding regimes were designed with feeding frequencies at every one, two, three, four and five days and feeding rations of 100%, 50%, 25%, and 12.5% satiation. The results showed that the reproductive parameters significantly declined when fish were fed less than every day, and less than 100% satiation.

The effects of energy, protein and lipid content on reproduction were then assessed. Diets containing 17 MJ Kg<sup>-1</sup>, 14 MJ Kg<sup>-1</sup> and 11MJ Kg<sup>-1</sup> were made and tested. The 17 MJ KG<sup>-1</sup> energy diet resulted in the highest egg number as well as the

longest unfed larval life. The protein experiment examined the effects of three protein levels, 35%, 43% and 50%, on reproductive parameters. The results showed that the 35% protein level gave reduced reproductive success ( $p < 0.05$ ). The 43% and 50% level diets gave better reproductive success but between the treatments had no significant differences in reproductive parameters ( $p > 0.05$ ). The 43% protein diet was recommended for subsequent experiments as it resulted in the longest unfed larval life and it would theoretically cost less to provide and would likely reduce water quality problems. Three diets containing 20%, 12% and 9% lipid were evaluated. The 20% lipid diet caused deformities in larvae, and led to reduced fertilisation and hatching rates. The 12% lipid diet gave the best overall results ( $p < 0.05$ ).

Three essential fatty acids, arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), were respectively supplemented into three diets, a fourth diet was supplemented with all three fatty acids and a fifth had no fatty acid supplementation and was used as a control. Egg number, survival to eyed embryo and hatching rate were not significantly different in any of the four diets containing no or only one fatty acid ( $p > 0.05$ ), however, supplementation of the diet with all three fatty acids resulted in significantly longer unfed larval life ( $p < 0.05$ ).

In summary, the results of the current study showed that a diet containing 17 MJ KG<sup>-1</sup> energy, 43% protein, 12% lipid and supplemented with all three fatty acids at the levels tested would maximise the level of reproduction in *M. splendida splendida*.

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## **1. Introduction**

## 1.1 General Introduction

Reproductive success is vital for sustaining an aquaculture system. One main factor affecting maturation, fecundity, and survival of larvae in fish is female condition. Female condition is largely affected by their nutrition, therefore a proper diet is essential to the reproductive performance of broodstock. Nutrition, which is determined by diet, has direct and indirect effects on maturation (Encina and Granado-Lorencio, 1997), egg production (Kazakov, 1981), and the viability of eggs and larvae (Kjorsvik *et al.*, 1990).

Good nutrition enhances fish growth and allows broodstock to reach a larger size sooner. Larger fish have been shown to produce more young (Smith *et al.*, 1979; Wroblewski *et al.*, 1999) and within a species larger fish tend to produce larger eggs (Hislop *et al.*, 1978; Knox *et al.*, 1988) and/or more eggs (Bagenal, 1969a; Encina Granado-Lorencio, 1997). Larger egg size confers a larger size larvae at hatching and a better chance of future survival (Bagenal, 1969b). The quality of broodstock nutrition also affects the survival of young. Protein (Smith *et al.*, 1979), lipid (Fernandez-Palacios *et al.*, 1997), vitamins (Kjorsvik *et al.*, 1990), and inorganic components (Kjorsvik *et al.*, 1990) are all important aspects of broodstock nutrition.

In an intensive indoor aquaculture setting, fish rely entirely on feed given to meet their nutritive requirements. It is therefore important to determine what nutrients are required and in what quantity they should be provided to enable the breeding of high quality stock.

## 1.2 Rainbowfish

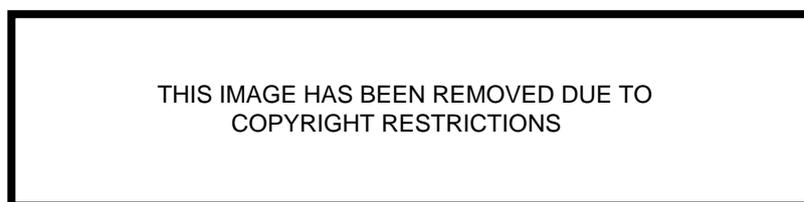
Rainbowfish belong to the order Atheriniform and the family Melanotaeniidae (Grant, 1987). This family consists of eight genera (Merrick and Schmida, 1984)

including the genus *Melanotaenia* containing the species used in these experiments. Rainbowfish are found in rivers, streams and lakes throughout Australia and Papua New Guinea. In Australia this genus is represented by at least 25 different species (Allen and Cross, 1978). The sub-species used in this study was *M. splendida splendida* (Fig 1.1), also known as the eastern rainbowfish. There are three other subspecies described to this species, *M. splendida australis*, *M. splendida inornata* and *M. splendida tatei*. The species *M. splendida splendida* was chosen as it is endemic to rivers and streams in North Queensland where James Cook University is located. During breeding, males of this species become brilliantly coloured, their fin rays turning a bright red or yellow colour (Milton and Arthington, 1984). Females spawn year round on aquatic vegetation when water temperature ranges from 26-28 °C (Allen and Cross, 1978). During spawning, females attach the eggs to vegetation by filaments in groups of 1-10 eggs (Fig 1.2). These eggs can take six to seven days to hatch (Milton and Arthington, 1984).

Fish of the genera *Melanotaenia* and *Pseudomugil* both in the family Melanotaeniidae are apparently some of the most sought after species for home aquaria (Don Booth, Personal Communication). In the retail year of 2001/2002 total



**Figure 1.1:** Pair of eastern rainbowfishes (*Melanotaenia splendida splendida*) from Harvey Creek near Cairns. The female is above the male [from Merrick and Schmida (1984)].



**Figure 1.2:** An egg from the genus *Melanotaenia* attached to a water plant by filaments [from Merrick and Schmida (1984)].

Australian sales of exotic aquarium fish was worth \$5.4 million dollars. Less than \$0.3 million coming from native fish species (Walker, 2004). Over the past few years, the desirability of native fish species has increased, as has the amount of money people are willing to pay for these fish. Currently rainbowfish for the aquarium trade are mostly wild caught (Don Booth, Personal Communication). This will eventually put a strain on the populations in the areas as their colouration makes them valuable. The rise in value of these fish, however, is encouraging local breeders to dedicate more resources to culturing native species (Walker, 2004). Most people that breed rainbowfish still use commercially available diets or diets that have been developed for other fish species such as barramundi (Bruce Atkins, Personal Communication).

Many fish cultured for food generally have a very long life cycle and do not reproduce frequently with the exception of Tilapias. Many of these fish are single spawners producing eggs once or twice a year, and as such experiments on the effects of nutrition on egg quality in these species are difficult. The length of time needed to see the effects on egg quality might be very long, up to a year or more. Also in larger species having enough space to hold several breeding fish for each treatment and their replicates is not feasible. For this reason very few thorough systematic studies on feeding condition and the effect of nutrition on reproductive success have been done on large freshwater species. A model species that requires minimum space is a good alternative. Undertaking experiments on a species that breeds frequently and readily will allow a better understanding of nutritive effects on reproductive performance of fish and allow diets to be produced and hypotheses tested. While nutritional requirements are not universal among fish species useful information can be obtained from such studies.

### 1.3 General Reproductive Biology of Fish

Fish reproduction is the process by which genetic material is passed on to the offspring. Understanding this important part of the life cycle, such as the age or time of maturity or processes that regulate reproduction, is critical to successfully breed fish in captivity. For some fish species, reproductive maturity may be at a certain length or weight. When a fish has fully developed gonads and can produce viable gametes it is defined as reproductively mature.

A separate male and female exist in most fish species, although hermaphroditism does occur (Brule *et al.*, 2000; Whittington *et al.*, 2000). Some fish may also undergo sex change at a certain stage of life (Brule *et al.*, 2000).

The gonads of vertebrates, including fish, arise in the dorsolateral lining of the peritoneal cavity, on each side of the dorsal mesentery (Hoar, 1969). Each gonad has a double origin developing from two distinct cellular proliferations. The laterally located cortex becomes the ovary while the medulla becomes the testes (Yamamoto, 1969).

Oogenesis is the process by which eggs are produced in females and spermatogenesis the process by which milt is produced in males. Ovulation is the final release of the oocyte from the follicle (Moyle and Cech, 2000). Prior to ovulation, vitellogenesis occurs and this is the process by which the liver mobilises vitellogenin, a lipoprotein, and transports it to the developing oocyte. In the oocyte, vitellogenin is stored in the yolk vesicle/granules as a food source for the developing embryo. The formation of the vitelline membrane is the final stage of vitellogenesis (Mommensen and Walsh, 1988). Spermatogenesis is the development of spermatocytes, the predecessors of spermatozoa (Moyle and Cech, 2000). Spermiation and ovulation result in the release of gametes. Spermatogenesis and vitellogenesis, as well as spermiation and ovulation are all hormonally controlled and are affected by nutrition.

In most fish species, the ova are released into the peritoneal cavity and move outside the fish through the Mullerian ducts (Hoar, 1969). The vas deferens are the ducts by which sperms exit the testes. Spawning strategies vary between species. Some fish are mass spawners (Kiflawi *et al.*, 1998) in which many females and males aggregate in the water column releasing their gametes together. Mass spawners in general produce many small eggs and rely on the number produced to ensure fertilisation and survival of the species. Other fish are more selective with the female choosing a particular male that she will allow to fertilise her eggs. These fish tend to produce fewer but larger eggs; the size instead of number ensuring their survival (De Gaudemar *et al.*, 2000).

In a hatchery the natural spawning process may be allowed to take place. This, however, can be costly and labour intensive. Hormone induction of spawning and egg stripping are other means by which fertilisation can be implemented in an aquaculture setting. The fertilised eggs are then incubated to hatching and the larvae reared.

Many factors can influence the development of gonads and therefore the development of oocytes and spermatozoa. Two major factors impacting the development of gametes as well as age or size of first maturity are nutrition and environmental parameters that are mediated through the nervous and endocrine systems thereby hormones play an active role.

#### **1.4 Reproductive Endocrinology**

Hormones play an essential role in fish reproduction amongst other life processes. They determine what sex a fish will be and regulate vitellogenesis and spawning. Through appropriate administration of hormone treatments fish can be induced to spawn. This enables better control of husbandry of the fish and may ensure the availability of product year round where possible. Hormones can also be used

during the labile period of larvae to induce single sex populations (Athauda, 2000). The role of hormones on reproduction needs to be better understood to enable their use for better control of breeding and husbandry.

#### 1.4.1 GnRH

Gonadotropin-releasing hormone (GnRH) is produced in the hypothalamus in response to some type of environmental stimulus. Eleven forms of GnRH have been identified to date: Tunicate I and II, lamprey I and III, catfish, dogfish, salmon, seabream, chicken I and II, and mammalian (FSH-RH) or lutenising hormone releasing hormone (LH-RH). GnRH is released from the hypothalamus and then transported to the pituitary by hypothalamic fibres. In the pituitary, GnRH stimulates the production of other hormones such as gonadotropin (GtH) I and II (Kawauchi *et al.*, 1989; Elizur *et al.*, 1995; Lo *et al.*, 1995; Melamed *et al.*, 1995; Yaron *et al.*, 1995; Pati and Habibi, 1998, Roelants *et al.*, 2000).

Roelants *et al.* (2000) showed that oral delivery of sGnRH (salmon GnRH) resulted in a significantly higher mean GtH II plasma level in the common carp, *Cyprinus carpio*. In salmonoids GtH I secretion was responsive to GnRH stimulation at early stages of gonadal development (Kawauchi *et al.*, 1989). Elizur *et al.* (1995) and Lo *et al.* (1995) showed a correlation between cGnRH II (Chicken II GnRH) and GtH I and II release in the gilthead seabream, *Sparus aurata*, and in goldfish, *Carassius auratus*. An increase in GtH level was also evident in immature black carp, *Mylopharyngodon piceus* and *C. auratus* after exposure to sGnRH (Lo *et al.*, 1995; Yaron *et al.*, 1995). Tilapia, *Preochromis niloticus*, also showed elevated levels of GtH II 12-24 hours after an injection of sGnRH (Melamed *et al.*, 1995).

GnRH also plays a role in gonadal maturation and spawning. Gothilf *et al.* (1997) showed that all three forms of GnRH present in female *S. aurata* peaked prior to final oocyte maturation (FOM). Progress for the induction of spawning using GnRH compounds has been made with many species including barramundi *Lates calcarifer* (Harvey *et al.*, 1985), common sole, *Solea solea* (Ramos, 1986), sable fish *Anoplopoma fimbria*, (Solar *et al.*, 1997). In males it has also been implicated in the onset of spermiation (Roelants *et al.*, 2000).

#### 1.4.2 GtH I

GtH I is produced in the pituitary in response to the release of GnRH. It is also known as follicle stimulating hormone or the vitellogenic gonadotropin (Gen *et al.*, 2000). The release of this hormone was shown to coincide with the release of GnRH in coho salmon, *Oncorhynchus kisutch* (Swanson *et al.*, 1991), African catfish, *Clarias gariepinus* (Schulz *et al.*, 1995a), and rainbow trout, *O. mykiss* (Chyb *et al.*, 1999). GtH I has been shown to be higher in reproductively developing fish and is important to gametogenesis (Elizur *et al.*, 1995; Meiri *et al.*, 1995; Weil *et al.*, 1995). In males, GtH I is associated with being a regulator of spermatogenesis and spermatogonial multiplication (Gomez *et al.*, 1999; Gen *et al.*, 2000). In females it is implicated in the early phases of the initiation of vitellogenesis (Schultz *et al.*, 1995b; Gothilf *et al.*, 1997; Gomez *et al.*, 1999; Gen *et al.*, 2000). In previtellogenic *O. mykiss*, GtH I levels were higher and predominant when compared to GtH II levels (Weil *et al.*, 1995). Elizur *et al.* (1995) showed that in *S. aurata* GtH I levels peaked near the beginning of the spawning season. Meiri *et al.* (1995) found a trend showing that GtH I levels declined 12 hours before spawning in *S. aurata*.

### 1.4.3 GtH II

GtH II, like GtH I, is produced in the pituitary also in response to the release of GnRH. It is also known as lutenising hormone or the maturational gonadotropin (Gen *et al.*, 2000). This hormone is linked to the final maturation of gametes: spermiation in males (Roelants *et al.*, 2000; Gomez *et al.*, 1999; Weil *et al.*, 1995) and ovulation in females (Schultz *et al.*, 1995b; Weil *et al.*, 1995; Meiri *et al.*, 1995; Gothilf *et al.*, 1997; Swanson *et al.*, 1991; Gomez *et al.*, 1999). Studies have shown an increase in GtH II just prior to spawning in *O. mykiss* (Gomez *et al.*, 1999), salmonoids (Suzuki *et al.*, 1988), red seabream, *Pagrus major* (Gen *et al.*, 2000), and *S. aurata* (Elizur *et al.*, 1995).

In males GtH II was shown to peak just prior to spermiation in *O. mykiss* (Weil *et al.*, 1995; Gomez *et al.*, 1999) and in *C. carpio* (Roelants *et al.*, 2000). In females GtH II levels were correlated with final oocyte maturation and ovulation in *C. carpio* (Schulz *et al.*, 1995b), *S. aurata* (Meiri *et al.*, 1995; Gothilf *et al.*, 1997), *O. kisutch* (Swanson *et al.*, 1991) and *O. mykiss* (Gomez *et al.*, 1999).

GtH I and II have some effect on steroid hormone production in salmon (Yan *et al.*, 1991; Planas and Swanson, 1995) and *C. carpio* (Barry *et al.*, 1990a). All three studies showed an increase in sex steroid production in the gonads after a peak in GtH blood plasma levels.

### 1.4.4 Steroid Hormones

Steroid hormones such as testosterone, estrogen, and progesterone, also have a key role in the development of gonads and in reproduction. They determine the sex of a fish by influencing the development of gonadal tissue (Goetz *et al.*, 1979; Pandian and

Sheela, 1995). Steroids also play a role in vitellogenesis and ovulation (Sumpter *et al.*, 1985; Dye *et al.*, 1986; Hyllner *et al.*, 1991; Shimizu *et al.*, 1999; Prat *et al.*, 1999) as well as in spermiation (Scott and Baynes 1982; Dye *et al.*, 1986; Prat *et al.*, 1999).

In females, steroids affect vitellogenesis. In *O. mykiss*, estradiol and testosterone levels peaked two months prior to spawning (Scott and Baynes 1982; Sumpter *et al.*, 1985) indicating their role in vitellogenesis. Scott and Baynes (1982) also noted that estradiol levels remained high until after ovulation. Estrogen was also seen to cause the production of vitelline in Sakhalin taimen, *Hucho perryi* (Shimizu *et al.*, 1999), sea bass *Dicentrarchus labrax* (Prat *et al.*, 1999) and pink salmon, *O. gorcusha* (Dye *et al.*, 1986). Estrogen has also been found in the testes of male fish (Loir 1990; Loomis and Thomas, 1999) although its role in male reproduction is still unclear.

In males  $17\alpha$ ,  $20\beta$  -progesterone has been shown to be responsible for the final maturation of sperm and has been implicated in spermiation (Miura *et al.*, 1992). It has also been shown to increase after spermiation in males and ovulation in females. The roles of testosterone and 11-keto testosterone, however, are more widely studied. Testosterone has been shown to peak during spermiation in salmonids (Dye *et al.*, 1989; Scott and Sumpter, 1989). 11-keto testosterone has also been found to peak during this period (Scott and Sumpter, 1989; Prat *et al.*, 1999). However, both tend to decline at spermiation (Dye *et al.*, 1986; Barry *et al.*, 1990b; Prat *et al.*, 1999). This would indicate that progesterone might have a greater impact on spermiation than does testosterone.

Hormonal induction of sex reversal has also been reported by Goetz *et al.* (1979) and Pandian and Sheela (1995). This is possible because fish gonads tend to remain labile from 10-40 days after hatching (Pandian and Sheela 1995). Female *O. kisutch* produce testicular tissue when exposed to  $17\beta$  -methyltestosterone while male *O.*

*kisutch* produce ovaries and oocytes when exposed to estradiol-17. Athauda (2000) also demonstrated the ability to make more than 90% of larval Nile tilapia, *Oreochromis niloticus* male with the induction of male steroid hormones administered under ultrasound. Naturally sex-inverting species have also been shown to respond to hormonal stimulus. *L. calcarifer* (Forrester, 2000) and black porgy, *Acanthopagrus schlegeli* (Chang *et al.*, 1995a; Chang *et al.*, 1995b; Chang and Lin, 1998) undergo sex reversal with the administration of oestradiol-17.

It is clear that many hormones play a vital role in the reproduction of fish. They regulate development and differentiation of gonads, as well as ovulation and spermiation.

### **1.5 Female Condition**

The condition of broodstock females affects their reproductive success. In the wild, food resources can be limited during certain times of the year and this can affect the weight and body composition of female fish. Encina *et al.* (1997) showed that in wild barbel, *Barbus sclateri*, seasonal abundance of food affected the maturation of the gonad and the number of eggs produced. In a study on herring, *Clupea harengus*, Laine and Rajasilta (1999) showed those females with a high condition factor and fat content showed low early stage egg mortality and high hatching and survival success of larvae. Nutrition plays a major role in fish size. If a fish is not fed a full ration or the proper nutrients are not supplied the fish will grow at a slower rate. This either leads to a longer time to maturation or when the fish is mature leads to fewer eggs being produced (Encina *et al.*, 1997).

The size of the female plays a role in fecundity. In many fish species such as Atlantic salmon, *Salmo salar* (Kazakov, 1981), brown trout, *S. trutta* (Bagenal, 1969b), Atlantic cod, *Gadus morhua* (Karlsen *et al.*, 1995), three-spined stickleback,

*Gasterosteus aculatus* (Wooton, 1973), and haddock, *Melanogrammus aeglefinus* (Hislop *et al.*, 1978), the weight of the female significantly affects the number and size of the eggs produced. Female condition and therefore egg production is related to food level. Fish fed on larger rations tend to grow faster and produce more eggs than fish fed on smaller rations (Bagenal, 1969b; Wooton, 1973; Hislop *et al.*, 1978; Karlsen *et al.*, 1995). For *G. aculatus*, egg production was almost linearly related to nutrition (Wooton, 1973) this study also showed that fish fed more had a shorter spawning interval and spawned more times. Food level has an effect on liver size especially during spawning periods (Karlsen *et al.*, 1995). Liver size is important as vitellogenin is produced in the liver using the stores of proteins and lipids. *S. trutta* L. had larger ovaries and had more stored eggs per gram of ovary when fed on higher rations (Bagenal, 1969b; Wroblewski *et al.*, 1999). From these studies it has been demonstrated that food has a significant effect on female condition and egg production.

Female size also influences egg size, a larger female normally producing larger eggs. This was seen in studies on *M. aeglefinus* by Hislop *et al.* (1978) and on rainbow trout, *S. gairdneri* by Knox *et al.* (1988) where fish with larger ration sizes produced larger eggs. This is particularly important for young lecithotropic fish, like salmon, which rely on nutrients supplied by the yolk sac until they are able to feed on their own, therefore it is imperative for the egg to contain the essential nutrients. Larger eggs also produce larger larvae (Kazakov, 1981). Larger larvae tend to survive longer without exogenous food than smaller larvae hatched from smaller eggs (Kjorsvik *et al.*, 1990). This allows a longer transition period from yolk sac absorption (lecithotrophy) to exogenous feeding.

Good nutrition for broodstock, more so for females, is essential, and the nutrient type and amounts must be closely monitored. Appropriate nutrition levels not only enhances female condition but in doing so, increases reproductive success. Nutrients

such as lipids and fatty acids, proteins, as well as vitamins all affect the broodstock and the quality of the gametes that they produce which determines the survival of eggs and larvae.

## **1.6 Lipids/Fatty Acids**

Lipids, like proteins, are major constituents of fish eggs. The absence or presence of a lipid globule, or multiple globules in an egg often determines its viability. It is important to feed fish the proper level of lipid as too little will affect reproductive success while too much may effect the viability of young. Fernandez-Palacios *et al.* (1997) showed that fish fed too little lipid did not spawn as many eggs and that fish fed defatted squid had fewer abnormal larvae. The hatchability of the eggs in fish that spawned was lower ( $p < 0.05$ ). Springate *et al.* (1985) also showed that too much lipid in a diet caused deformities in eyes of embryos and this decreased hatching success.

### 1.6.1 Effect of lipids on Reproduction

Lipid content of the diet can effect fish growth and egg quality as shown by Eskelinen (1989), Rajasilta (1992), MacFarlane *et al.* (1993), and Naves *et al.* (1997). A fat supplemented semi-moist feed supported the best growth in *S. salar* broodstock (Eskelinen, 1989). MacFarlane *et al.* (1993) showed that a diet deficient in lipid led to a decrease in liver or mesenteric fat tissue, and decreased ovary size. A smaller ovary would produce fewer eggs leading to a reduction in young produced by the broodstock. Naves *et al.* (1997) demonstrated that viability and survival to hatching were higher in fish fed high quality lipid.

### 1.6.2 Effects of Fatty Acids on Reproduction

A nutritionally important group of lipids are the fatty acids (De Silva and Anderson, 1995). Dietary lipids should provide fatty acids that fish can not synthesise themselves, but which are required for the maintenance of cellular functions (Halver, 1989). These are known as essential fatty acids (EFA), and it is important that they be included in broodstock and larval diets (Halver, 1989). Most EFA are long chain highly unsaturated fatty acids (HUFA) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Halver, 1989). Polyunsaturated fatty acids (PUFA) are also important, however, more studies must be done on this lipid group to determine their importance in fish diets.

Fatty acids are important components of egg membranes, helping maintain their structure and function (Sargent *et al.*, 1995). Marine fish, however, lack the ability to produce their own HUFAs (Sargent *et al.*, 1995). Studies have shown that fatty acids have an effect on the condition of females (Izquiereo *et al.*, 1992; Furuita *et al.*, 2000). As described earlier, female condition has an important impact on egg number, size, and quality. Both Furuita *et al.* (2000) and Izquiereo *et al.* (1992) showed that Japanese flounder, *Paralichthys olivaceus* broodstock fed a diet high in n-3 HUFA had an increased growth rate in terms of body length and weight.

Fatty acids appear to have a significant impact on spawning performance, number of eggs, and the viability of eggs produced by broodstock females (Fernandez-Palacios *et al.*, 1995; Bruce *et al.*, 1999; Furuita *et al.*, 2000). Bruce *et al.*, (1999) showed that spawning performance, percent viability, percent survival at 48 h, and hatching rate of *D. labrax* was increased when the diet was supplemented with 27.3% docosahexaenoic acid (DHA, 22:6 n-3) and 5.4% eicosapentaenoic acid (EPA, 20:5 n-3). Fernandez-Palacios *et al.* (1995) showed a relationship between dietary n-3 HUFA level and the percentage of morphologically normal eggs in *S. aurata*. They also

demonstrated that the percent of unfertilised eggs was reduced in those groups fed diets with increasing levels of n-3 HUFA. Furuita *et al.* (2000) also illustrated that the number of viable eggs per spawn as well as the percentage of normal larvae produced increased with increasing level of n-3 HUFA. These studies suggest that in order to maximise reproduction in relation to egg number and viability of eggs it is essential to ensure that broodstock females be supplied with enough of the essential fatty acids in their diets.

### 1.6.3 Effects of Fatty Acid on Larvae Development

Fatty acids are also essential to the development of larvae. Many aspects of larval development have been shown to be effected by the amount and type of fatty acid in larval diets (Navarro *et al.*, 1988; Dhert *et al.*, 1990; Lemm and Lemarie, 1991; Castel *et al.*, 1994; Estevez *et al.*, 1999; Furuita *et al.*, 1998; Furuita *et al.*, 1999). Growth of larvae was shown to be significantly effected by fatty acids in their diet. *P. major* showed improved growth when fed 22:6 n-3 (Kanazawa, 1997) while in *S. maximus*, 20:4 n-6 supported the best growth. Van Ballaer *et al.* (1985) found that growth was best in larvae fed n-3 HUFA enriched food. Mourente and Tocher (1992) and Furuita *et al.* (1998) showed that 22:6 n-3 increased brain growth in developing larvae. The larval stage of a fish is one with several critical stages that may show high mortality, such as when first feeding occurs, or during the inflation of the air bladder. It is because of these difficulties that a larva must overcome that is it vital to ensure that they have enough of the essential fatty acids in their diet. Ensuring that they have all the required nutrients will enable them to grow better and may enhance their chances of surviving these difficult stages.

Before larvae start to feed they obtain all their nutrients, including fatty acids from stores within the egg. It is important, therefore to make sure that the broodstock is

supplied with adequate amounts of fatty acids so that it can accumulate an appropriate amount in the eggs. Very few studies have been conducted looking at this aspect of pre-reproductive nutrition. However, this could in part be due to the logistics of keeping enough broodstock to be able to do replicate studies, or obtaining eggs in which to do analyses.

Since lipids and fatty acids play such a major role in broodstock management and survival of young it is necessary to supply the proper amounts in the feed. Too little or too much will affect the quality and number of eggs produced.

## **1.7 Protein**

Protein plays an important role in female reproductive performance. Dahlgren (1980) showed that elevated feed protein levels increased ovarian weight, width, and length. This suggested that females with a higher protein intake were better able to mobilise protein for reproductive purposes. Protein is also a contributing factor for maturity in fish. Gunasekera *et al.* (1995) observed that fish maintained on extremely low protein levels did not reach puberty. As protein levels increased so did the number of fish observed to reach maturity.

Protein levels in feed have been attributed to aiding in the onset of vitellogenesis. Fish fed a low level of protein undergo vitellogenesis at a much slower rate and later in life than do fish on a high protein diet (Washburn *et al.*, 1990; Gunasekera *et al.*, 1995). A high protein diet has been shown to increase fecundity in some fish species. *O. mykiss* maintained on a high protein diet produced significantly more eggs than those on a low protein diet (Smith *et al.*, 1979). The weight of eyed eggs may also be effected; high protein giving heavier eggs (Smith *et al.*, 1979).

The source and quality of protein also effects reproductive success. This will be discussed in the following section of natural vs. artificial feed section.

## 1.8 Natural vs. Artificial Feed

There is an ongoing debate about which is better for a fish, natural feed or an artificial diet. One advantage of an artificial diet opposed to natural feeds is that the introduction of disease to a culture system can be kept at a minimum. It is controversial, however, at the current level of knowledge and manufacturing technology whether an artificial diet can supply the proper nutrition to broodstock. Appleford (1996) looked at the digestibility of different ingredients of artificial diets and discovered that some feeds are more digestible and therefore provide more nutrients to fish than others. To be able to maximise feed nutrition it is necessary to know what components of a diet fish will be able to utilize best.

Watanabe *et al.*, (1985) showed that reproductive success was increased with the provision of raw krill before spawning in replacement of diets containing corn oil. The researchers Watanabe *et al.*, (1995) suggested that higher levels of n-3 HUFA in the krill could be the reason for greater breeding success. It has also been suggested that a supplement of natural foods on top of a normal feeding regime would increase reproductive success when artificial diets were used (Santiago *et al.*, 1985). The main problem with artificial diets probably lay with a lack of understanding of the proper nutritional requirements for the target species. A natural food supplement therefore may provide the essential nutrients lacking in many artificial diets thereby improving reproductive success.

Some ingredients for artificial feeds may provide better nutrition for fish than others. For instance lupin seed has been suggested as a better source of protein in artificial diets than soybean meal as fish fed a diet with lupin seed meal had a significantly higher body protein composition than those fed soybean meal (Robaina *et*

*al.*, 1995). Thus, when feeding broodstock it is important to take into consideration the nutritive requirements of the targeted fish and select the ingredients that best fits the demands of the broodstock being cultured, as dietary requirements are not universal.

### **1.9 Effects of Nutrition on Hormones**

There is strong evidence that nutrition affects many aspects of reproduction from maturation to the hatchability of eggs and subsequent survival of larvae. Evidence also exists that nutrition has an effect on hormones as well, although very few studies have been conducted in this area (Horne *et al.*, 1991; Naves *et al.*, 1998). As a variety of hormones control reproduction, it is important to gain an understanding of the overall role of nutritional factors on reproductive hormonal influence.

Horne *et al.* (1991) showed that diet can enhance  $17\alpha$ -estradiol levels in largemouth bass, *Micropterus salmoides*. In *D. labrax*, a low protein, high carbohydrate diet effected the release but not the synthesis of GnRH. Naves *et al.* (1998) showed that in *D. labrax*, fish fed diets with higher lipid and n-3 PUFA had higher  $17\alpha$ -estradiol and GtH II levels than the control group. Cerda *et al.* (1995) demonstrated that  $17\alpha$ -estradiol and testosterone levels in *D. labrax* were elevated in groups fed a diet higher in protein and lipid than two commercial diets.

These few studies suggested that nutrition was very important to the hormonal levels of broodstock. Information, however, is lacking in this area. It is necessary for this aspect of nutrition to be studied further so that its implication on breeding fish in captivity is better understood.

## 1.10 Summary

Good nutrition for broodstock is vital if fish are to be raised in a culture setting. When fish are maintained in enclosures, they rely almost entirely on feed given to them to supply the nutrients that they need. It is essential that all nutritive requirements are met or the reproductive success of those individuals may be affected. Nutrition influences the condition of females, the number and quality of eggs produced and subsequent survival of larvae. Nutrition affects many aspects of reproduction and there are many components of a diet, including lipids, proteins, vitamins, and minerals, which may influence the outcome of fish reproduction.

As *Melanotaenia splendida splendida* is easily bred and reared in captivity they make a good model species for reproductive experiments. The aim of this thesis is firstly to use *M splendida splendida* as a model species to assess effects of feeding frequency, feeding ration, and various nutrients such as energy, protein, lipid and fatty acids on reproductive performance. Secondly this experiment aims to develop a diet for *M. splendida splendida* which maximises reproductive output for this species.

## **2. General materials and methods**

## 2.1 Fish Capture

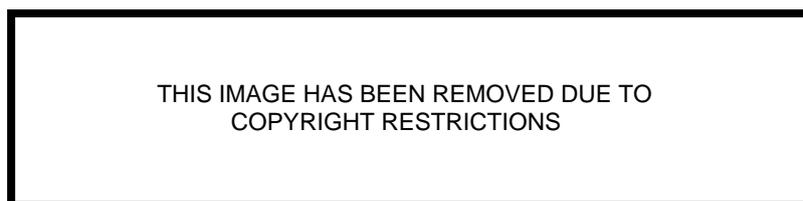
The fish used in this experiment were captured from freshwater streams around Townsville (19°16'S; 146°49'E) and Walkamin (17°08'S; 145°26'E) in northern Queensland, Australia (Fig 2.1). Forty-eight fish in total were taken from Walkamin stream (Fig 2.2) using a dip net and an electro fisher. The electro fisher was handled by Terry Valence an employee at the Walkamin Department of Primary Industries. Over the period of two and a half years a total of 24 fish were captured in the Ross River (Fig 2.3), 32 were taken from Danishmen Creek in High Range (Fig 2.3) and 113 were taken from Stony Creek in Townsville (Fig 2.3). During the years 2001 and 2002 there was a drought in this area and many of the smaller streams and rivers dried up. This made sampling fish problematic. Many methods of fish capture were used to obtain fish during this time. At night *M. splendida splendida* tend to rest near the surface of the water. Between 8 pm and 1 pm at Aplins weir of the Ross River, Townsville, fish were captured by shining a torch onto the water to spot the fish before scooping them out using a large dip net. Live traps were used to catch the fish from Danishmen and Stony Creeks. These traps were baited using either canned cat food or rancid pork fat. One trap was a fully enclosed rectangular trap that was put in the water and left for at least half an hour, at the end of this time the trap was removed and the fish sorted. The other live trap consisted of two metal hoops of approximately 30 cm supporting mosquito netting. Fishing sinkers were attached to the bottom hoop to make sure that it sank, and pork fat tied in the middle. The upper hoop was connected to two pieces of rope. This was put into the water and when fish appeared to be at ease eating the pork fat the upper hoop was raised using the rope and the fish trapped inside. Fish deemed to be of mature size were sorted and held in a 70L-transport container, which was aerated, until transported to James Cook University research facilities.



**Figure 2.1:** A map showing an overview of the sampling sites (●). It shows where they are in relation to each other and the area in Australia where they are located is marked out in black on the main map (■).



**Figure 2.2:** A map showing Walkamin, and the Department of Primary Industries research station. The black dots indicate the streams where rainbowfish were collected. The ●'s mark the location of collection sites.



**Figure 2.3:** A map showing locations of Stony and Danishmen Creeks. The black dots indicate where fish were collected along the creeks. The ●'s mark the location of collection sites.

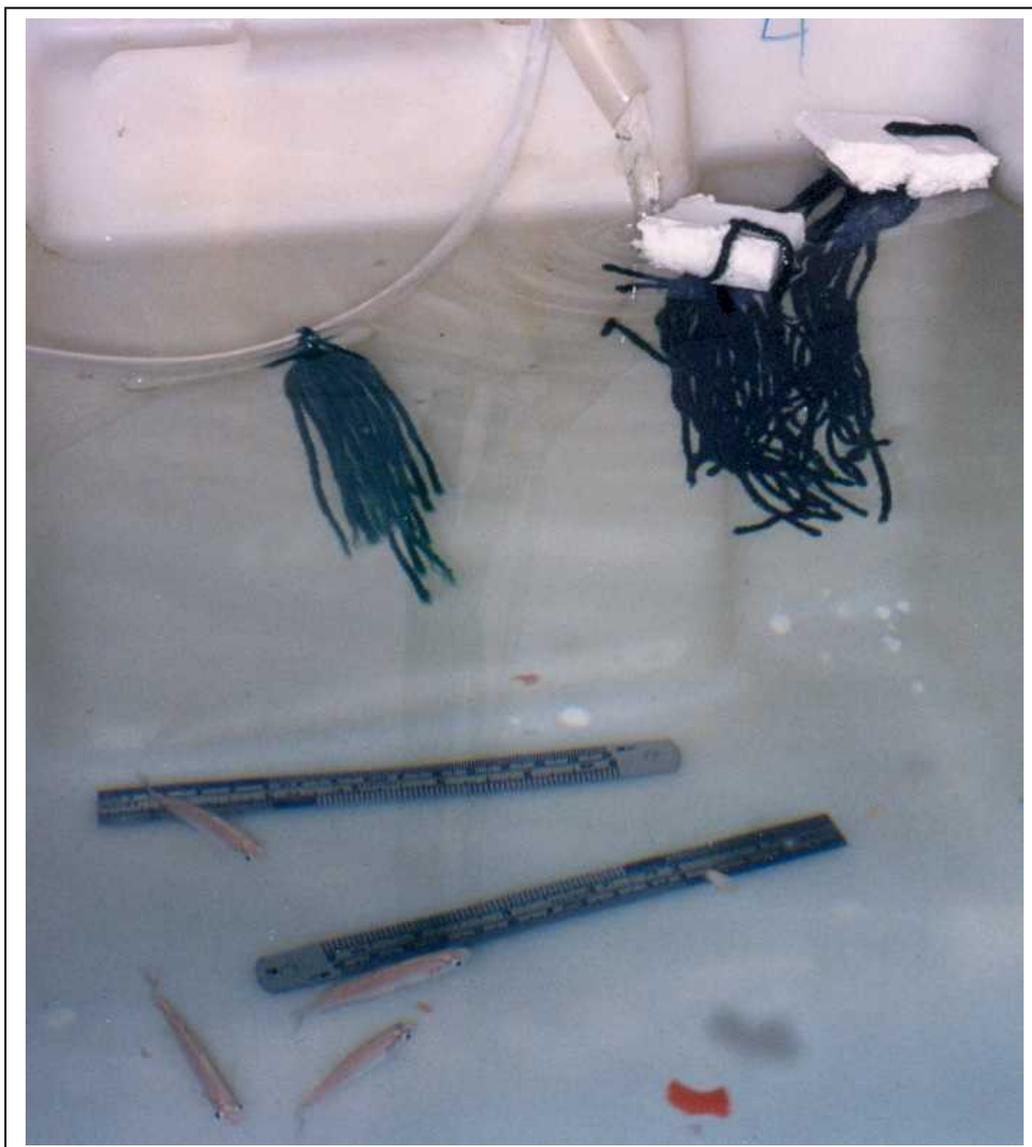
## 2.2 Fish Holding

Upon the fish being transported to the Marine and Aquaculture Research Facility Unit (MARFU) of James Cook University, Townsville campus, the fish were sorted and fish of similar size were placed into a tank (see Table 3.1). Four fish were held per tank of which two were female and two were male fish. Fish sexes were distinguished by the fins, the males having much more elaborate fins with more colouration than the females (Fig 1.1). The tanks were 70L (l 59 cm x w 37 cm x h 38 cm) rectangular plastic bins and were provided with approximately 50L hr<sup>-1</sup> of de-chlorinated freshwater from a recirculating aquarium system. The water temperature was maintained at 26°C (± 2°C) with a photoperiod of L14 h:D10 h. Each tank had its own aeration stone ensuring adequate oxygen for all fish. Breeding mops consisting of approximately 20 strands of knitting cotton were placed in each of the tanks as a spawning substrate (Fig 2.4).

Tanks were cleaned on a daily basis during the experimental period with any excess food or faeces siphoned out after feeding. A 25% water exchange was performed on a weekly basis and clean freshwater used to refill the system. A clear viewing window in each tank allowed fish to be monitored to ensure that they quickly returned to their normal behaviour after cleaning and did not remain stressed.

Breeding mops were checked daily and if eggs were found, the mop was removed and replaced with a new one. For all experiments, eggs were counted with the aid of a dissecting microscope. Then survival to eyed embryo, hatching rate, and the time that a larva survived under starvation from hatching hereafter referred to as “unfed larval life” were determined for each clutch.

Eggs and larvae were held in small food containers l 10 cm x w 5 cm x h 4 cm. These containers had 2 cm<sup>2</sup> holes cut in the bottom over which a mesh of 2µm was glued. Water was slowly circulated through these tanks at approximately 1L h<sup>-1</sup>. When



**Figure 2.4:** A picture showing the breeding mops made out of knitting cotton that provided a spawning substrate for fish to attach their eggs.

larvae hatched they were removed from the egg container and placed into a larvae holding container (Fig 2.5), larvae hatching on the same day from the same clutch being kept together. They were then monitored and the time from hatching until death recorded.

Between experiments fish were fed Nutrafin® a commercially available complete tropical fish flake ( $14\text{MJ Kg}^{-1}$ , 43% protein, 6% lipid) to ensure that the treatments prior to the experiment would not effect the fish on their next diet trial. Fish were allowed to acclimatise to this food until all fish began spawning again. This period of acclimatisation took approximately three weeks. This time period is longer than was shown to be necessary in the reproductive parameters and the effects of starvation experiments (Chapter 3) but extra time was allocated to ensure that the effects from the earlier treatments would be negligible.

The largest and smallest fish from the experiments in Chapter 3 were given away to the School of Tropical Environmental Science at James Cook University, for use in experiments there. The fish between the size range of 5.2 cm and 6.5 cm were kept for future experiments. Over the period of this study several groups of fish needed to be replaced as they had grown too large, and the size of the fish would have started to confound the effects of the diets. When fish grew too large replacement fish of an appropriate length were captured.

This project was conducted under approval number A604\_00 of the James Cook University animal ethics committee.



**Figure 2.5:** The eggs and larvae holding containers used during the experiments.

### 2.3 Measuring Fish

Fish used during all experiments were measured using the fork length. This length being measured from the nose of the fish to the fork of its tail. This measurement is used instead of standard length when it is difficult to determine the end of the vertebral column (Froese and Pauly, 2003). Standard length is generally used because during preservation of a fish, often parts of the caudal fin are lost due to alcohol degeneration. To determine standard length a fish is laid flat and the tail is bent upward while the caudal peduncle is being held until a fold appears and this is the point to the measurement is made (Froese and Pauly, 2003). This can be very stressful on small live fish, such as *M. splendida splendida*, and as such, the fork length was used for the current experiments as *M. splendida splendida* has a very distinctive forked tail. The fish were kept in small groups and no apparent fighting occurred leaving the tails of the fish undamaged through the entirety of all experiments.

### **3. Basic reproductive parameters and effects of starvation on such parameters**

### 3.1 General Introduction

Nutrition may have an effect on the size of females, which plays a role in fecundity. In many fish species such as, *S. salar* (Kazakov, 1981), *S. trutta* (Bagenal, 1969b), *G. morhua* (Karlsen *et al.*, 1995), *Gasterosteus aculeatus* (Wootton, 1973) and *M. aeglefinus* (Hislop *et al.*, 1978), larger females produced more and/or larger eggs than smaller females. Larger eggs may facilitate better survival of the larvae for many fish species (Kazakov, 1981). Kjorsvik *et al.* (1990) showed that larger larvae survive longer without food than smaller larvae.

It has been noted that mass spawners tend to produce many small eggs (Kiflawi *et al.*, 1998), while selective spawners tend to produce larger eggs (De Gaudemar *et al.*, 2000). Whether a fish allocates energy to fewer larger eggs or many smaller eggs may also help determine how best to breed a certain species of fish in captivity. For those fish species that allocate energy to larger instead of more eggs, egg size may be considered as a more important criteria to judge reproductive success, while for other species egg number might be the case.

Large numbers of small eggs or a few large sized eggs alone, however, is not the only good measure of reproductive success. Survival to eyed embryo and hatching rate of those eggs should also be considered. A fish might produce many eggs or large eggs but only a small percentage of these eggs might be fertilised or they might have a low hatching rate.

Unfed larval life is another factor that must be considered. Many fish are lecithotropic, they live off the yolk sac provided within an egg for a certain period of time after hatching. This means that there is a certain period of time where it is not necessary for a larval fish to obtain their nutrition from exogenous sources. Other larval fish, however, don't have a yolk sack when they hatch, and must feed on food found in

the water column almost immediately upon hatching. If an egg does not hold adequate nutrients, larvae hatching from that egg would have a shorter period of unfed larval life than a larvae hatching from an egg with a good supply of nutrients. Therefore, length of the period before exogenous feeding, unfed larval life, can be an important criteria by which to measure the amount of nutrients female broodstock deposit into eggs.

The first part of this experiment was to establish the baseline data for reproductive success in *M. splendida splendida*. All fish were reared under identical feeding conditions to determine the effect of fish size on egg number, egg diameter, hatching rate, survival to eyed embryo, and unfed larval life. Egg diameter was also examined to see if there was any effect on survival to eyed embryo, hatching rate and unfed larval life.

Two extremes for food availability may exist in natural systems. There is an abundance of food or rarity (in the extreme case causing starvation). If food availability is going to affect a reproductive parameter it is assumed that it would be more obvious in the most extreme case resulting in starvation. Therefore, the second part of the experiment sought to determine the effects of starvation on a number of reproductive parameters in order to determine which were effective measures of reproductive success, for use in the later experiments.

## 3.2 Methodology

### 3.2.1 Baseline reproductive parameters

Fish used in this experiment ranged in size from 3.7 cm to 11.5 cm. These sizes were used to determine the effect that fish size had on egg diameter and egg number. Four fish were held in each 70 L tank, and no more than 1 cm separated the largest fish from the smallest fish in each tank. There were four tanks containing small fish, the average size of the four fish in each of these tanks being: tank 3, 4.20 cm  $\pm$  0.5 cm, tank 8, 4.17 cm  $\pm$  0.3 cm, tank 9, 4.2 cm  $\pm$  0.5 cm, and tank 11, 4.2 cm  $\pm$  0.3 cm. The other tanks contained fish with a variety of size ranges up to 10.9  $\pm$  0.9 cm (Table 3.1). During this experiment all fish were fed a commercially available complete tropical fish flake (Nutrafin®). The fish in each tank were fed to satiation once daily for three months from March 2001 through May 2001. All eggs for each group of fish were counted and placed in containers for hatching. Hatching time, survival to eyed embryo, hatching rate and unfed larval life for each clutch was recorded. Survival to eyed embryo was considered successful if eyed larvae appeared by the end of day three at 26°C( $\pm$ 2°C). If the egg turned milky before the appearance of the eyed larvae within the egg, that egg was only counted towards determining survival to eyed embryo. Only those eggs that survived to eyed embryo stage were used to determine hatching rate. Hatching rate was determined as the number of eggs that survived to eyed embryo stage that subsequently hatched. Survival to eyed embryo was calculated as:

$$\% \text{ eyed eggs} = \frac{\text{Number of eyed eggs}}{\text{Number of eggs laid}} \times 100$$

Hatching rate was calculated as

$$\% \text{ Hatching} = \frac{\text{Number of larvae hatched}}{\text{Number of eggs which survived to eyed embryo stage}} \times 100$$

Unfed larval life was determined as the time from hatching to death without the larvae eating any exogenous foods.

Random clutches of eggs from each tank were also sorted according to diameter using a graticule on a dissecting microscope and placed in size appropriate containers to hatch. Hatching time, survival to eyed embryo, hatching rate and unfed larval life were examined for each egg diameter.

### 3.2.2 Effects of starvation

When sufficient data to determine the base line variations of reproductive parameters had been obtained after three months, three groups of fish which had previously exhibited reliable spawning were selected to test the effects of starvation of broodstock on the reproductive parameters. Fish used during the starvation trial were the same fish previously used to determine baseline reproductive parameters. As such it is possible that the prior experiment influenced the outcome in the starvation trial. The fish were starved until all fish from each group stopped spawning, which was determined to be a period of nine days and over this period the changes in reproduction monitored. At the end of the nine-day starvation period fish were again fed to satiation for a period of nine days. This process was repeated four times for two tanks and three times for one tank (one of the fish in this tank died during the period of re-feeding). This data was pooled to get the overall effect of starvation on reproduction. During the period of feeding which followed periods of starvation all fish commenced spawning before being starved again.

Statistical analyses were performed using SPSS. Independent t-tests were used for egg diameter data, ANOVA was used to compare egg number, survival to eyed

embryo and hatching rate using tank as treatments and Sheffe's test for *post hoc* comparison of means, descriptives were used for fish sizes, and the pair wise comparison statistic for unfed larval life. The percentage data were arc-sine transformed before statistical analyses were conducted. Bivariate correlation was used to determine if egg diameter had any effect on hatching rate or larval survival.

### 3.3 Results

#### 3.3.1 Baseline reproductive parameters

Among the smallest fish ( $4.2\text{cm} \pm 0.3\text{cm}$ ) held in tank eight and tank eleven no eggs were produced. Fish in tank three ( $4.2\text{cm} \pm 0.5\text{cm}$ ) produced eggs on one occasion at which only three eggs were laid. Fish in tank nine ( $4.2\text{cm} \pm 0.5\text{cm}$ ) produced eggs on two occasions, four eggs being produced in the first clutch and two being laid in the second clutch. None of the eggs from either group of fish survived to eyed embryo stage. The remaining fish bred periodically throughout the experiment with fish in tanks one ( $9.1\text{cm} \pm 0.9\text{cm}$ ), five ( $4.9\text{cm} \pm 0.7\text{cm}$ ) and six ( $10.9\text{cm} \pm 0.9\text{cm}$ ) proving the most reliable (Table 3.1). The largest fish from tanks six ( $10.9\text{cm} \pm 0.9\text{cm}$ ) and one ( $9.1\text{cm} \pm 0.9\text{cm}$ ) had the most number of spawns producing eggs on 68 and 25 different occasions, respectively.

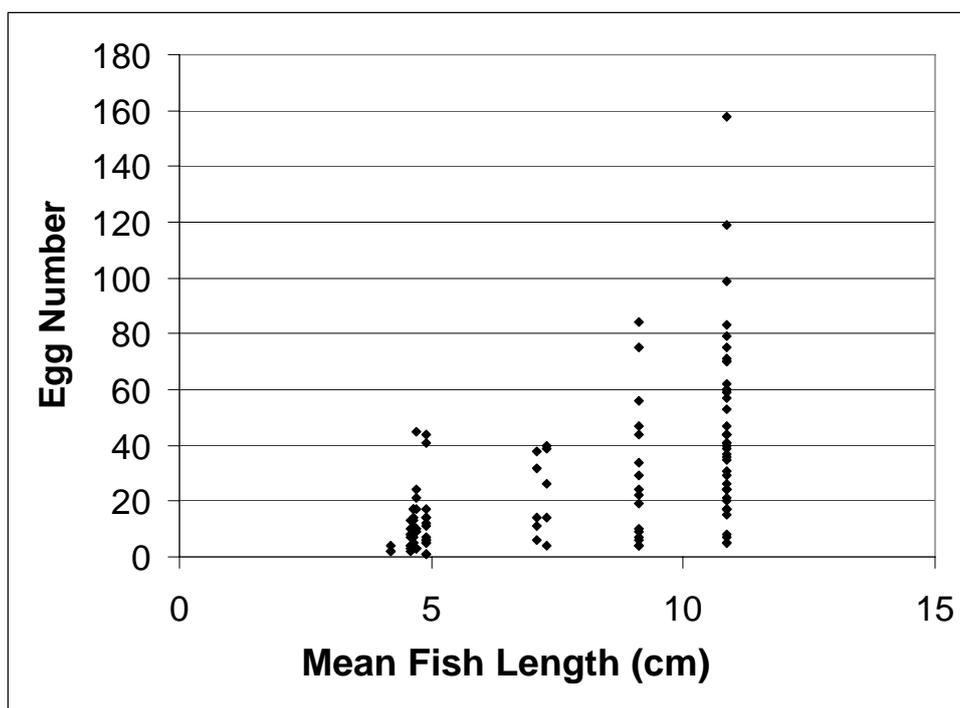
Mean fish size had an effect on the number of eggs laid in each clutch (Fig 3.1) as well as the number of clutches laid by the fish over the experimental period (Table 3.1). Total egg production over time was much higher for larger fish, egg production decreasing as fish size decreased (Fig 3.2). Fish in tank six, having the largest average fish size (10.86 cm), had an average clutch size of  $45.6 \pm 20.2$  eggs per clutch and the largest clutch for these fish was 158 eggs. Tank 1 contained the next largest average fish size (9.13 cm). Average clutch size for fish in tank 1 was  $29.6 \pm 10.2$  eggs per clutch. Tank four and seven contained the smallest fish (4.6 cm) that spawned regularly. Average clutch size for fish in tank four was  $6.5 \pm 3.7$  eggs per clutch, while average clutch size for fish in tank seven was  $9.6 \pm 3.1$  eggs per clutch. Clutch size increased with increased average fish size (Fig 3.1). Clutch size varied within any given tank, however, the upper limit of the number of eggs laid changed while the lower limit tended to remain constant for each fish size.

**Table 3.1**

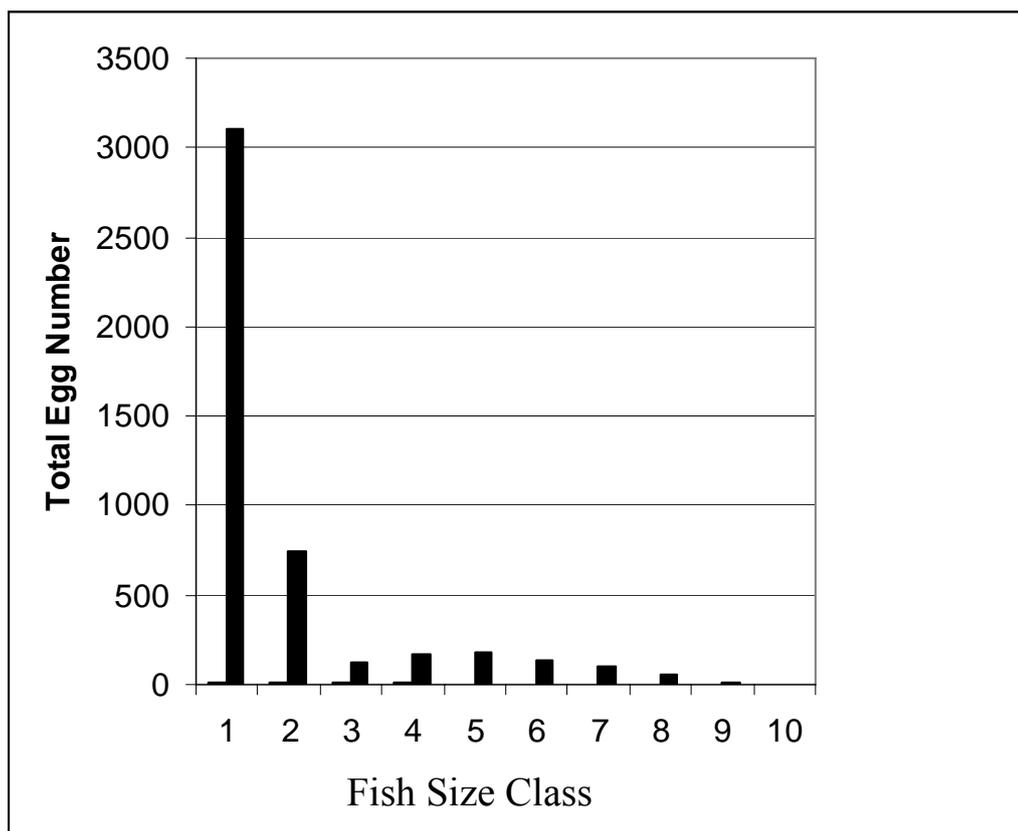
Mean *M. splendida splendida* length (cm) per tank and total number of spawns and spawning average for each group. Values with the same superscripts indicate treatment groups that were not significantly different (Scheffe's test,  $p < 0.05$ ).

<b>Mean Fish Size</b>	<b>Number of Spawns</b>	<b>Mean No. Eggs per Spawn</b>	<b>Mean Egg diameter (mm)</b>	<b>Tank Number</b>
10.9 ± 0.9	68	45.6 ± 20.2 <sup>a</sup>	0.95 ± 0.1	6
9.1 ± 0.9	25	29.6 ± 10.2 <sup>b</sup>	0.94 ± 0.1	1
7.3 ± 0.8	5	24.7 ± 9.4 <sup>bc</sup>	0.96 ± 0.1	2
7.1 ± 0.6	8	21.1 ± 8.7 <sup>c</sup>	0.94 ± 0.1	12
4.9 ± 0.7	18	10.2 ± 3.4 <sup>d</sup>	0.95 ± 0.1	5
4.7 ± 0.7	9	15.3 ± 6.5 <sup>cd</sup>	0.95 ± 0.1	10
4.6 ± 0.6	10	9.6 ± 3.4 <sup>d</sup>	0.94 ± 0.1	7
4.6 ± 0.6	9	6.5 ± 3.7 <sup>de</sup>	0.95 ± 0.1	4
4.2 ± 0.5	2	3.0 ± 1.0 <sup>e</sup>	N.A.	9
4.2 ± 0.5	1	3.0 ± 0.0 <sup>e</sup>	NA	3
4.2 ± 0.3	0	N.A.	N.A.	11
4.2 ± 0.3	0	N.A.	N.A.	8

Mean fish size was calculated from all four fish in the tank. ± values are s.d. The sample size for all tanks was four (two males and two females)

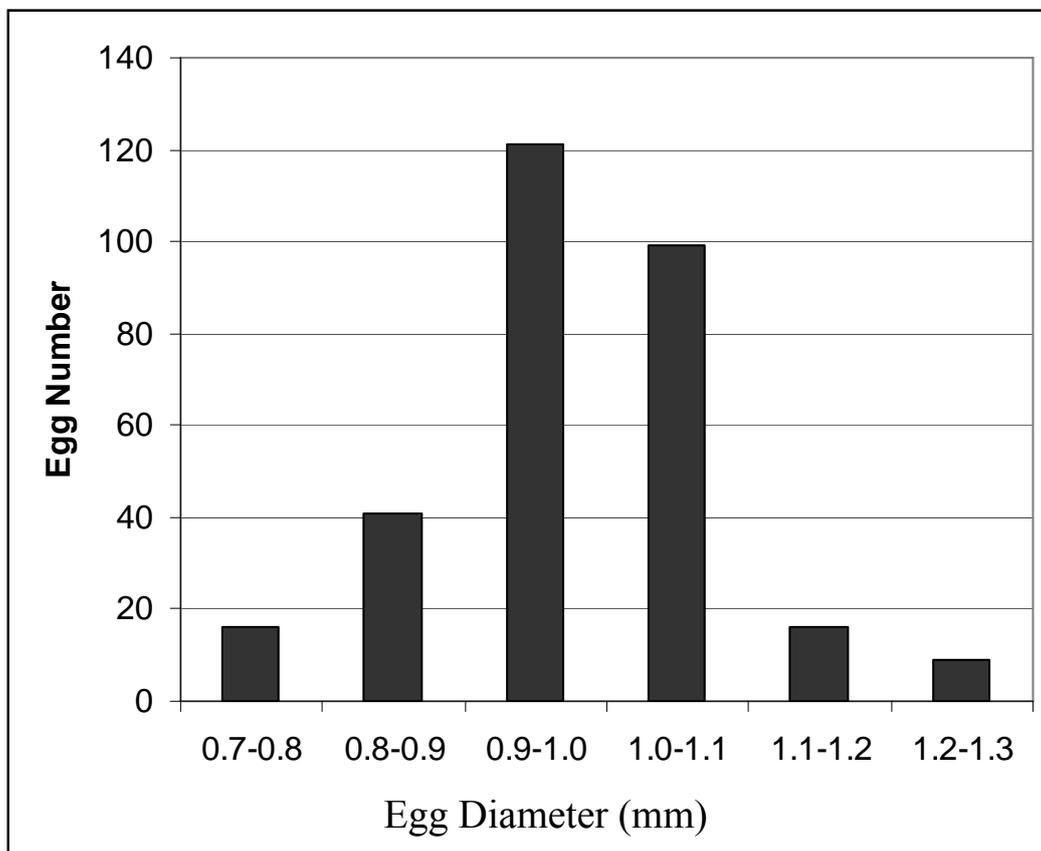


**Figure 3.1:** Number of eggs laid over a three month period for each size class of *M. splendida splendida* fed a daily ration. Each data point represents one clutch. Fish length is calculated from the mean of all four fish in the tank

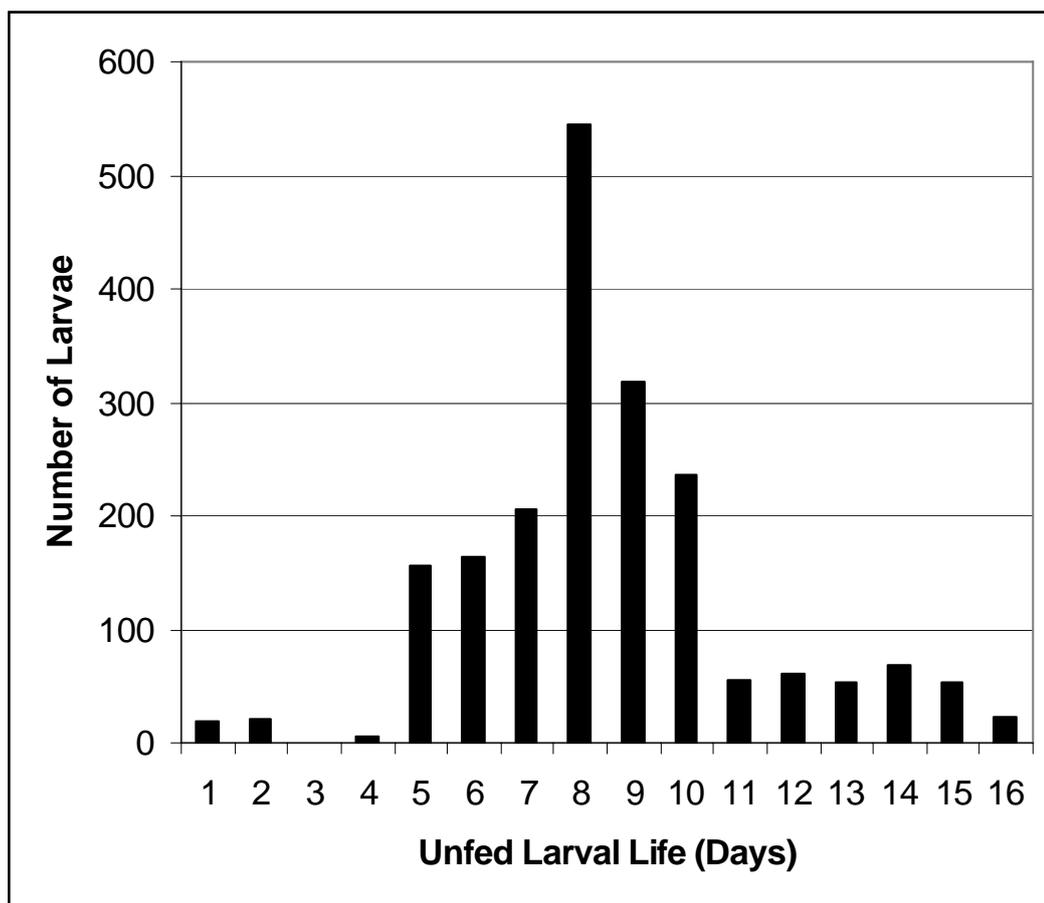


**Figure 3.2:** Total number of eggs laid over a three month period in relation to fish size class. It ranges from the largest fish (1) to the smallest fish (9 and 10) as listed in Table 3.1.

Egg diameter did not vary significantly with fish size (bivariate correlation  $p > 0.05$ ), because of this data from all fish sizes was pooled for the egg diameter data. Eggs ranged in size from 0.7 to 1.2 mm (Fig. 3.3) and the mean egg diameter was  $0.95 \text{ mm} \pm 0.19 \text{ mm}$ . The majority of eggs produced were between the size range of 0.9 – 1.1 mm (75.6%), with very few 0.7-0.8 mm (3.4%) 0.8-0.9 mm, 1.1-1.2 mm, and 1.2-1.3 mm (1.3%) eggs being produced. Egg diameter also did not affect hatching rate (bivariate correlation  $p > 0.05$ ) or larval survival (bivariate correlation  $p > 0.05$ ). Ninety-seven percent of all eggs that survived to eyed embryo stage hatched regardless of their size. Eggs that were survived to eyed embryo and did not hatch were evenly distributed through the different sizes of eggs (1 egg from 0.8-0.9 mm 5 eggs from 0.9-1.0 mm) 3 eggs from (1.0-1.1 mm and one egg from 1.1-1.2 mm), so no apparent effect of egg diameter was seen on survival to eyed embryo. Unfed larval life ranged from 1-16 days (Fig 3.4). Larvae hatching from 0.7 mm eggs had a survival time between 3-14 days and larvae hatching from 1.2 mm eggs had a survival time of 2-13 days. In all egg diameters, eyeing occurred between day two and three, and hatching occurred after six to eight days.



**Figure 3.3:** Frequency distribution of egg diameter (mm) in *M. splendida splendida* ranging in size from 4.7 to 12.5 cm pooled from all fish sizes over a three month period. Fish were fed once daily to satiation.

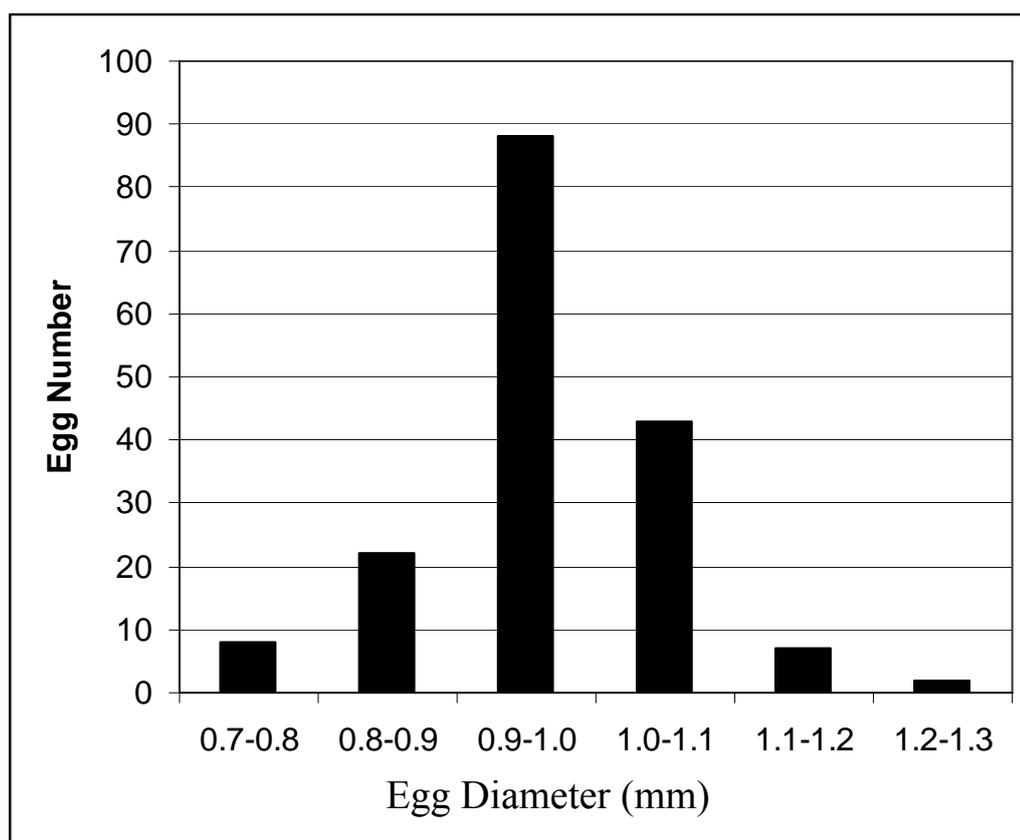


**Figure 3.4:** Unfed larval life of *M. splendida splendida* hatched from all egg diameters produced by fed fish over a three month period.

### 3.3.2 Effects of starvation

Starvation did not have any effect on egg diameter. As in fed fish, the size distribution of eggs laid by starved fish ranged from 0.7 mm to 1.2 mm with most eggs being 0.9 mm (Fig 3.5). Egg hatch rate and survival to eyed embryo dropped dramatically in eggs of starved fish. Survival to eyed embryo of eggs from fed fish was 96.6%, while in eggs from starved fish it decreased to 67.5% on day 4 of starvation and to 27% by day six of starvation. Hatch rate of those eggs that were fertilised decreased from 97% to 45.5% by day four of starvation and to 5.7% by day six of starvation. The mean survival period was eight days for larvae from fed fish, whereas the unfed larval life was also shortened to a mean of 6 days for larvae from starved fish ( $p < 0.05$ ). Eyeing time of eggs was not different from that of eggs from fed fish, eyeing occurring between days two and three.

Starvation had dramatic effects on egg production (Fig 3.6). The number of eggs laid during starvation was expressed in percentages to allow data between fish of different sizes to be standardised. Egg number decreased dramatically in those fish being starved (Fig 3.6). After day nine of starvation, no eggs were produced from the fish in any of the tanks undergoing starvation trials. The largest fish in the starvation trial ( $10.9\text{cm} \pm 0.9\text{cm}$ ) started laying eggs immediately upon re-feeding (day 1), some fish starting to reproduce as soon as food was given and by day three all groups of fish had resumed egg laying (Fig 3.6).

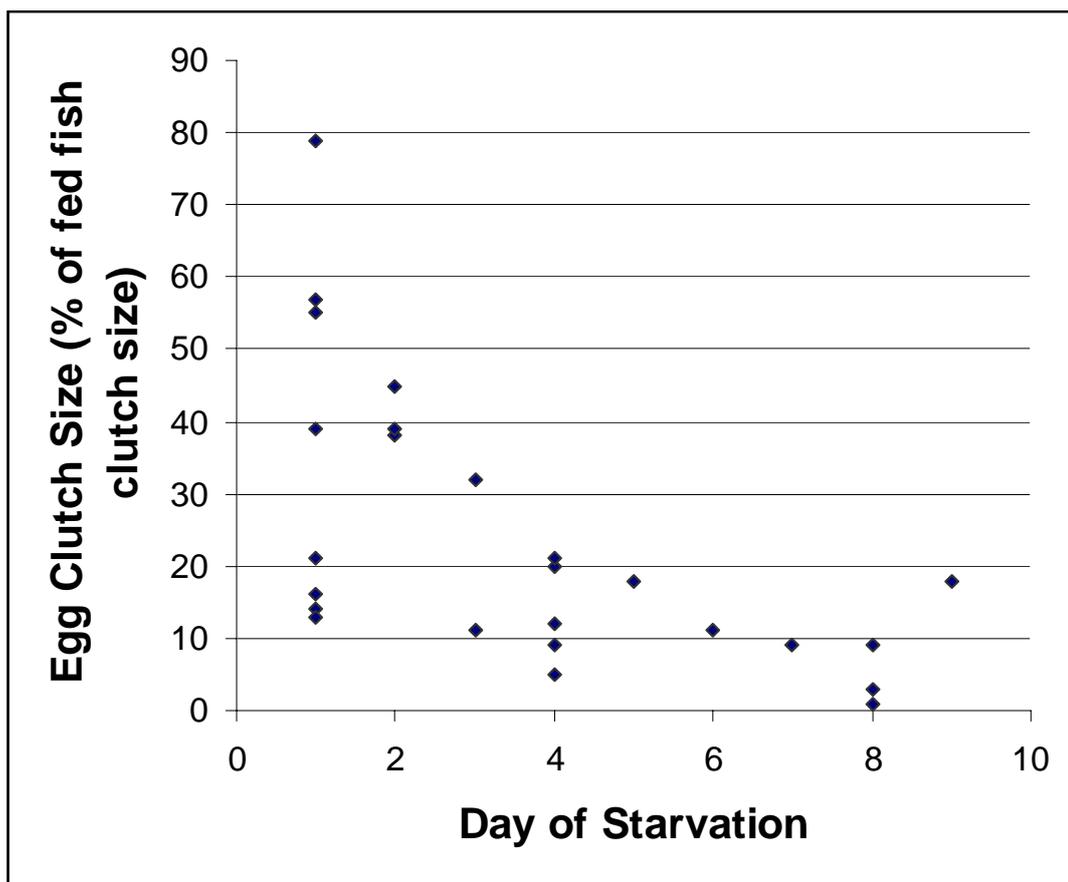


**Figure 3.5:** Frequency distribution of egg diameter (mm) in starved *M. splendida splendida* pooled from all tanks in each starvation period.

**Table 3.2**

The effects of feeding and starvation on survival to eyed embryo, hatching rate, and unfed larval life.

<b>Treatment</b>	<b>Fertilisation Rate %</b>	<b>Hatching Rate %</b>	<b>Unfed Larval Life (Days)</b>
<b>Fed Broodstock</b>	96.6 ± 0.9	97.0 ± 0.6	8 ± 1.2
<b>Starved Day 4</b>	67.5 ± 1.9	45.5 ± 0.9	6 ± 1.1
<b>Starved Day 6</b>	27.0 ± 2.9	5.7 ± 4.9	6 ± 0.8



**Figure 3.6:** Effects of starvation and re-feeding on egg production by *M. splendida splendida*. Egg production is expressed as a percent of the average clutch size for that group of fish when fed. Data is pooled from all three tanks of fish that underwent periods of starvation.

### 3.4 Discussion

In the rainbowfish *M. splendida splendida*, fish size had an impact on both time of reproductive maturity and egg number. In the first experiment it was shown that the smallest fish (4.2 cm) do not reproduce. This is probably due to the fact that they had not yet reached a size where they were reproductively mature. Both Kazakov (1981) and Karlsen *et al.* (1995) have shown that female size is important for reproductive maturity. In the two instances where eggs were found with groups of small fish, the largest fish in each tank was a female (4.7 and 4.8 cm) and it is most that the eggs laid in these groups came from this females in each group. These eggs, however, were not fertilised and this may indicate that the males in the tank were not yet reproductively mature and therefore produced no milt for fertilisation. A study by Pusey *et al.*, (2001) also indicates that in the genus *Melanotaenia* gonad development is related to body size. From this study it would suggest that a length approaching 4.7 cm is the length of first maturity for *M. splendida splendida*.

Unlike *Gobiomorphus breviceps* (McDowall and Eldon, 1997) and catfish of the family *Ariidae* (Coates, 1988) in which egg size increases with fish length to a point, in *M. splendida splendida* egg diameter did not increase with increased fish length. The average egg diameter found in this study, approximately 9.5 mm was, however, slightly smaller than those reported by Pusey *et al.* (2001) in which the average egg diameter in three fish of the genus *Menalotaenia* were reported to be between 1.10 and 1.24 mm. Egg number, however, did increase with fish length as also reported in tilapia, *Tilapia zillii* (Coward and Bromage, 1999) and *S. salar* (Thorpe *et al.*, 1984). Starvation, which is an extreme in food availability, had no effect on egg diameter. Fish have a limited amount of resources that they can allocate to reproduction. It is therefore reasonable to assume that in *M. splendida splendida* there will be a trade-off between egg diameter and egg number as has been seen in other species, such as *Gymnocephalus cornus*

(Devine *et al.*, 2000), *Oncorhynchus nerka* (Quinn *et al.*, 1995) and *S. salar* (Thorpe *et al.*, 1984). In *O. nerka* as egg weight increased egg number decreased (Quinn *et al.*, 1995), whereas in *G. cornus* (Devine *et al.*, 2000) and *S. salar* (Thorpe *et al.*, 1984) as egg number increased egg size decreased. The first experiment of this study indicated that as *M. splendida splendida* grow, they invest the resources allocated for reproduction into increased numbers of eggs of similar size instead of fewer larger eggs. The starvation trials also showed that in periods of low food availability, fish still produce the same size eggs as those fish being fed frequently. Unfed larval life was also not seen to differ significantly with egg diameter. This implied that nutrient content of the eggs was not directly linked with egg diameter. This was also supported by the unfed larval life of larvae from eggs of those fish starved. These larvae having a significantly shorter life than those larvae hatching from eggs from fed females, but no significant size difference between eggs being seen.

Two strategies appear to have been adopted in order to maximise reproduction in fish in response to variable nutrition. The first is to increase the number of eggs produced with improved nutrition condition as seen in *Barbus sclateri* (Encina and Granado-Lorencio, 1997), *G. morhua* (Karlsen *et al.*, 1995), *S. trutta* (Bagenal, 1969b), *G. aculeatus* (Wootton, 1973), *M. aeglefinus* (Hislop *et al.*, 1978), *C. harengus* (Devine *et al.*, 2000), *Pomatoschistus minutus* (Kvarnemo, 1997). The second is to increase the size of eggs produced as nutrition improves as seen in *Pimephales promelas* (Devine *et al.*, 2000), *S. salar* (Kazakov, 1981), *S. gairdneri* (Knox *et al.*, 1988) and *Sebastes flavidus* (MacFarlane *et al.*, 1993). Whilst this phenomenon is widely reported, no explanation has been offered in the literature as to the benefits of one strategy over the other. Benefits may be proposed for each of these strategies. If egg size increases, larval size has been seen to increase (Kazakov, 1981; Lobon-Cervia, 2000) and larger larvae have been shown to have a longer unfed larval life (Kjorsvik *et al.*, 1990). This

would be beneficial in areas where food availability is limited or cyclical and larval food supply is unpredictable, as well as in circumstances where larger larvae would prove less susceptible to predation. The strategy of increasing the number of eggs would be preferred when the benefits of larger eggs are not accrued. Such would be the case when food supply is relatively stable, and larval size has relatively low impact on levels of predation. Under such situations, increased fitness would come from producing many similar size larvae as opposed to fewer larger larvae. *M splendida splendida* appear to have adopted the second of these two strategies with the females allocating nutrients to produce many eggs of a small size instead of fewer large eggs.

#### **4. Effect of feeding frequency and feeding ration on reproductive parameters**

#### 4.1 General introduction

Female condition greatly affects reproduction and this can be affected both by the type and quality of the nutrients present in the diet as well as the amount of food available. In the wild, food availability fluctuates throughout the year and this fluctuation in food abundance can have major impacts on reproductive ability. In *Barbus sclateri*, the seasonal abundance of food affected both the maturation of gonads and the number of eggs produced (Encina and Granado-Lorencio, 1997). In *Engraulis ringens*, peak spawning was reported to occur in areas where food abundance was high (Castro *et al.*, 2000). Also in *Abudefduf abdominalis* spawning activity increased during periods of food influx (Tyler and Stanton, 1995). Female *Clupea harengus* with a high condition factor, as determined by fat content, showed low egg mortality, high hatching rates and high larval survival (Laine and Rajasilta, 1999).

Feeding frequency and feeding ration are important because fish respond differently to different amounts of food availability. In *Claries gariepinus*, fish fed twice a day showed better growth and food conversion ratios than those fish that were fed three times a day (Pantazis and Neofitou, 2003). In *Limanda ferruginea* feeding frequency affected food consumption, fish that were fed less frequently ate more than fish fed more than twice a day (Dwyer *et al.*, 2002). For *Chanos chanos*, fish fed 4% of their body weight per day produced more eggs and had a higher mean number of eggs per spawn than those fish fed only 2% of their body weight per day (Emata *et al.*, 1996). These two variables also interacted with each other. The effect of ration size on growth in *Amphiprion percula* depended on the frequency of feeding (Johnston *et al.*, 2003). In this experiment, it was illustrated that fish fed once a day on a ration above 12% body weight did not have an improved growth rate, but at two feedings a day fish fed to at

least 6% body weight had good growth (Johnston *et al.*, 2003). Since fish size is often related to reproductive performance (Kazakov, 1981; Karlsen, 1995) achieving good growth may help maximise reproduction.

It is obvious that food availability and abundance have important effects on reproduction. However, it is not always the case that more food is better (Collins and Anderson, 1999). Therefore determining exactly how much food and how often to feed a fish in order to maximise reproductive capacity is important for optimising reproductive output. Apart from impacting directly on reproduction, over feeding can cause water quality problems such as high ammonia, nitrate, nitrite and phosphates in an aquaculture setting which can compromise fish health and create significant problems.

There are two ways whereby food intake can be varied experimentally. These are by varying feeding frequency and by varying feeding ration. The first experiment in this chapter sought to determine the effect of feeding frequency under the same feed ration on reproduction. Different feeding frequencies were used to determine the effects on egg number, survival to eyed embryo, hatching rate, and unfed larval life. The second experiment in this chapter aimed to evaluate the effects of different rations but the same frequency had on these reproductive parameters.

## **4.2 Methodology**

### **4.2.1 Feeding frequency experiment**

In this experiment, fish which ranged in size from 5.2-6.3 cm were randomly chosen from the 19 tanks that contained four breeding fish (two males and two females), and allocated to each of five treatments. In treatment I, three tanks of fish were used while four tanks of fish were used for all other treatments. In the work described in Chapter 3 it was clear that in *M. splendida splendida*, larger fish produce more eggs. However, capturing fish larger than 6 cm proved to be very difficult. In order to keep experimental groups as similar as possible it was necessary to have fish of approximately the same size in each treatment group, and fish of 5-6 cm were the largest fish that could be reliably captured. This experiment was also conducted during a period of severe drought, which significantly impacted on the availability of fish, which was why only 19 tanks of fish were used. In treatment I fish were fed daily, treatment II they were fed every 2<sup>nd</sup> day, treatment III every 3<sup>rd</sup> day, treatment IV every 4<sup>th</sup> day and treatment V every 5<sup>th</sup> day. All fish in all treatments were fed to satiation on feeding days, with excess food siphoned out with the faeces. This part of the experiment lasted over a period of thirty days and all groups of fish were spawning normally prior to the commencement of the experiment.

Statistical analyses were performed using SPSS. ANOVA (Tukey's HSD) was used for egg number, spawn number, average spawning interval, survival to eyed embryo, and hatching rate, and the pair wise comparison statistic was used for unfed larval life. The percentage data was arc-sine transformed prior to analysis.

### **4.2.2 Feeding ration experiment**

This experiment aimed to examine the effect of varying feeding ration on the selected reproductive parameters. Fish were randomly selected from the 20 tanks that

contained four breeding fish (two males and two females) which ranged in size from 5.3-6.4 cm and allocated to each of four treatments.

Prior to commencement of this experiment, the amount of food consumed by the fish in each of twenty tanks was recorded over a period of one month. This was done by weighing out set amounts of food and slowly feeding each tank. When fish stopped feeding the remaining amount of food was weighed, and total amount of food consumed was recorded. At the end of this time the average amount of food consumed per day for each group of fish was determined. From this the average amount of food required to feed the fish to satiation was obtained and four treatments with varying percent of satiation determined. The treatments were 100%, 50%, 25% and 12.5% of satiation and this percent of food was given on a daily basis. Five tanks of fish were allocated to each of the four treatments. This experiment was conducted over a period of 30 days. Total number of spawns and spawning interval were also determined at the end of the experiment.

Statistical analyses were performed using SPSS. ANOVA (Tukey's HSD) was used for egg number, number of spawns, average spawning interval, survival to eyed embryo and hatching rate and the pair wise comparison statistic was used for unfed larval life. The percentage data was arc-sine transformed prior to analysis.

## 4.3 Results

### 4.3.1 Feeding frequency experiment

Table 4.1 shows the results from this experiment. Fish fed every day produced significantly more eggs than fish fed every second day ( $p < 0.05$ ) which produced more eggs than the fish in the other three treatments ( $p < 0.05$ ). Fish fed every 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> day did not differ in the numbers of eggs laid (Table 4.1) ( $p < 0.05$ ). Average total number of eggs produced showed a clear trend of decline with increased feeding interval. It declined from 152 eggs in those fish fed every day, to 99 eggs in fish fed every 2<sup>nd</sup> day, 26 eggs for fish fed every 3<sup>rd</sup> day, 21 eggs for fish fed every 4<sup>th</sup> day and 19 eggs for fish fed every 5<sup>th</sup> day.

The number of spawns was also affected by feeding, with the highest number of spawns occurring in fish fed daily ( $p < 0.05$ ) and the fewest in fish once fed every five days (Table 4.1). This was reflected in spawning interval ( $p < 0.05$ ) where fish fed daily had significantly shorter spawning intervals than fish fed every 2<sup>nd</sup> and 3<sup>rd</sup> day (Table 4.1). Spawning intervals for fish on treatments IV and V were not determined, as fish did not spawn more than twice during the whole experiment. Spawning was last observed in fish fed in treatment IV on day five and in treatment V on day nine, because of this average time between spawns would not have been an accurate representation for these two treatments.

The high survival to eyed embryo rates in the first two treatment groups were not significantly different (Table 4.1) ( $p > 0.05$ ) but higher than those in the other treatments. Treatment three was different from all other groups while four and five were not significantly different from each other but they did differ from the first three treatments having the lowest rate of fertilisation,  $76.1\% \pm 0.1\%$  (Table 4.1). Hatching rate also declined from fish fed daily, to fish fed once every five days (Table 4.1). The

hatching rates of treatment I and II were not different ( $p < 0.05$ ), while that in treatments III, IV and V were different ( $p < 0.05$ ) with treatment V having the lowest hatching rate ( $72.6 \% \pm 0.1\%$ ) (Table 4.1).

Feeding frequency significantly affected unfed larval life, with the longest survival in larvae from broodstock in treatments I, II and III (Table 4.1) ( $p < 0.05$ ) but significantly longer than those in treatments IV and V ( $p < 0.05$ ). Treatment four had a significantly shorter unfed larval life than the first three treatments, while treatment five had the shortest unfed larval life of  $6.48 \pm 2.06$  days (Table 4.1).

**Table 4.1**

Effects of feeding frequency on egg number, number of spawns, spawning interval, survival to eyed embryo, hatching rate, and unfed larval life in *M. splendida splendida*.

<b>Treatment</b>	<b>Feeding</b>	<b>Total Egg Number</b>	<b>No. of Spawns</b>	<b>Ave. Spawn Interval (Days)</b>	<b>Survival to Eyed Embryo %</b>	<b>Hatching Rate %</b>	<b>Unfed Larval Life (Days)</b>
<b>1 (n = 3)</b>	<b>Daily</b>	152 <sup>a</sup> ±	14 <sup>a</sup> ± 0.0	2.14 <sup>a</sup> ±	98.2 <sup>a</sup> ±	98.5 <sup>a</sup> ± 0.0	8.3 <sup>a</sup> ±
		11.2		0.0	0.0		1.2
<b>2 (n = 4)</b>	<b>Second Day</b>	99 <sup>b</sup> ± 8.5	7.25 <sup>b</sup> ±	4.14 <sup>b</sup> ±	98.2 <sup>a</sup> ±	98.2 <sup>a</sup> ± 0.1	8.5 <sup>a</sup> ±
			0.5	0.3	0.2		1.5
<b>3 (n = 4)</b>	<b>Third Day</b>	26 <sup>c</sup> ± 5.1	6.5 <sup>b</sup> ±	4.64 <sup>b</sup> ±	95.1 <sup>b</sup> ±	94.5 <sup>b</sup> ± 0.1	8.5 <sup>a</sup> ±
			0.6	0.4	0.0		1.7
<b>4 (n = 4)</b>	<b>Fourth Day</b>	21 <sup>c</sup> ± 4.1	3.5 <sup>c</sup> ± 0.6	N.D	75.2 <sup>c</sup> ±	82.5 <sup>c</sup> ± 0.1	7.6 <sup>b</sup> ±
					0.1		2.2
<b>5 (n = 4)</b>	<b>Fifth Day</b>	19 <sup>c</sup> ± 6.8	2.25 <sup>c</sup> ±	N.D	76.1 <sup>c</sup> ±	72.6 <sup>d</sup> ±	6.5 <sup>c</sup> ±
			0.5		0.1	0.1	2.1

The n value refers to the number of tanks of fish (two males and two females per tank) allocated to each treatment. Significant differences for egg number, number of spawns, spawning interval, survival to eyed embryo, and hatching rate were determined using ANOVA (Tukey's HSD  $\alpha = 0.05$ ) while the pairwise comparison statistic was used for unfed larval life ( $\alpha = 0.05$ ). Values are mean  $\pm$  s.e; same letters indicate homogenous subsets; N.D. not determined. Arc-sine transformations were performed on the percentage data.

### 4.3.2 Feeding ration experiment

Table 4.2 shows the results of this experiment. There were significant differences between fecundity of fish on each of the four treatment rations. Fish fed to 100% satiation produced more eggs than any other treatment group (146) ( $p < 0.05$ ) while fish from treatment IV (12.5%) produced the lowest number at just 15 eggs (Table 4.2).

The total number of spawns per treatment group and average spawning interval was affected by ration size. Fish from treatment I had more spawns as well as a shorter spawning interval than fish in the other treatment groups (Table 4.2) ( $p < 0.05$ ). Treatments II and III did not appear to differ in either spawning interval or number of spawns ( $p < 0.05$ ), while treatment IV spawned the most infrequently (Table 4.2). Average spawning interval was not determined for fish in treatment IV as they failed to spawn past day ten of the experiment, because of this average spawning interval would not have been a good representation of spawning performance (Table 4.2).

The survival to eyed embryo from treatments I and II were similar (98.2% and 98.0%) ( $p > 0.05$ ) but they were significantly higher than the other two treatment groups (Table 4.2) ( $p < 0.05$ ). Treatment III values were significantly different from treatment IV being fed at 12.5% satiation ( $p < 0.05$ ) which had the lowest survival to eyed embryo of  $80.8\% \pm 0.5\%$  (Table 4.2).

The hatching rate was also affected by ration size, with fish fed larger rations producing the best hatching rates (93.8 – 99.3%). Treatment IV had the lowest overall hatching rate  $6(8.3\% \pm 8.2\%)$  (Table 4.2).

The unfed larval life was also affected by broodstock ration size. Larvae from treatments I and II had the longest unfed larval life,  $9.2 \pm 2.8$  days and  $9.3 \pm 2.6$  days, respectively, which were significantly longer than the larvae in treatment IV ( $p < 0.05$ ),

however, the results were significantly different to treatment I, II and III. Treatment four had the shortest unfed larval life of  $6.4 \pm 2.2$  days (Table 4.2).

**Table 4.2**

Effects of feeding ration on egg number, number of spawns, spawning interval, survival to eyed embryo, hatching rate and unfed larval life in *M. splendida splendida*.

<b>Treatment</b>	<b>Feeding</b>	<b>Total Egg Number</b>	<b>No. of Spawns</b>	<b>Ave. Spawning interval (Days)</b>	<b>Survival to Eyed Embryo %</b>	<b>Hatching Rate %</b>	<b>Unfed Larval Life (Days)</b>
<b>1 (n = 5)</b>	<b>100%</b>	146 <sup>a</sup> ± 10.0	15.2 <sup>a</sup> ± 0.8	1.9 <sup>a</sup> ± 0.1	98.2 <sup>a</sup> ± 0.6	99.3 <sup>a</sup> ± 0.6	9.2 <sup>a</sup> ± 2.8
<b>2 (n = 5)</b>	<b>50%</b>	72 <sup>b</sup> ± 6.1	7 <sup>b</sup> ± 0.7	4.3 <sup>b</sup> ± 0.4	98.0 <sup>a</sup> ± 0.7	98.2 <sup>ab</sup> ± 0.1	9.3 <sup>a</sup> ± 2.6
<b>3 (n = 5)</b>	<b>25%</b>	50 <sup>c</sup> ± 5.4	6.2 <sup>b</sup> ± 0.4	4.8 <sup>b</sup> ± 0.3	92.1 <sup>b</sup> ± 0.5	93.8 <sup>b</sup> ± 3.9	8.4 <sup>a</sup> ± 2.4
<b>4 (n = 5)</b>	<b>12.5%</b>	15 <sup>d</sup> ± 4.3	2.4 <sup>c</sup> ± 0.5	N.D.	80.9 <sup>c</sup> ± 0.5	68.3 <sup>c</sup> ± 8.2	6.4 <sup>b</sup> ± 2.2

The n value refers to the number of tanks of fish (two males and two females per tank) allocated to each treatment. Significance factors for egg number, number of spawns, spawning interval, survival to eyed embryo and hatching rate were determined using ANOVA (Tukey's HSD  $\alpha = 0.05$ ), while the pairwise comparison statistic was used for unfed larval life ( $\alpha = 0.05$ ). Values are mean  $\pm$  S.E; same letters indicate homogenous subsets; N.D., not determined. Arc-sine transformations were performed on the percentage data.

## 4.4 Discussion

### 4.4.1 Discussion of general findings

In the wild, food sources may be limited due to a variety of reasons such as environmental degradation. During times of low food availability fish must allocate resources to meet basic metabolic functions rather than reproduction (Seims and Sikes, 1998). Earlier studies on *S. trutta* (Bagenal, 1969b), *Gadus morhua* (McDowall and Eldon, 1997) and *Gasterosteus aculatus* (Wooton, 1973; Ali and Wooton, 1999) have shown that during periods of low food availability, a decrease and then cessation of reproduction occurs. This was also demonstrated in *M. splendida splendida*. Egg number decreased significantly with a decrease in both food frequency (Table 4.1) and food abundance (Table 4.2). The lowest feeding frequency and feeding ration had a dramatic effect on reproduction firstly with a decrease in number of eggs produced and reached a complete cessation in reproduction after only a few days. Egg quality also declined during periods of lower food availability. Eggs that had been laid after the onset of reduced feeding frequency (Table 4.1) or feeding ration (Table 4.2) had correspondingly lower fertilisation and hatching rates. Lowered survival to eyed embryo may have been due to at least one of two factors: a) the males were affected and in so doing produced less milt or sperm that was not as viable or with a lower motility rate and/or b) the viability of the eggs was affected, either the structure could have been effected making fertilisation difficult or impossible, or there were inadequate nutrients to support any embryonic development. These two effects may have acted alone or in concert. The eggs that were fertilised also had a decrease in hatching success.

These findings are important as it is necessary to know how often and how much to feed a fish in broodstock management. In the wild fish will adjust its food consumption to satisfy their energy requirements (De Silva and Anderson, 1995) and if

the amount of food available to the fish decreased it will increase its food intake during feeding (Gamage, 2001; Pirhonen and Forsman, 1998). In captivity, fish rely entirely on the amount of feed given to satisfy all of its energy requirements. This means that a dominant fish may be able to consume more feed than smaller fish which are held in the same aquarium. Feeding too little will result in decreased reproduction, while feeding excessively will increase the cost of holding fish, as well as increase the probability of having poor water quality. This experiment demonstrated that to maximise reproductive parameters in *M. splendida splendida* the fish should be fed to 100% satiation on a daily basis.

#### 4.4.2 Feeding ration vs feeding frequency

Although the data were obtained in separate experiments, which may make direct comparison subjective, interesting differences were observed between these two studies. There is also the confounding effect of compensatory feeding by animals fed to satiety at lower frequencies as has been described in numerous other studies of fish feeding (De Silva and Anderson, 1995). However, the daily feeding treatment from the feeding ration experiment would have provided approximately the same amount of nutrients as the 100% feeding ration since the treatments were essentially the same. The 2<sup>nd</sup> day treatment would have been approximately equal to the 50% treatment in regard to the amount of nutrients the fish were receiving with a possible error in that fish fed every 2<sup>nd</sup> day would be likely to have consumed more food than those fed a measured 50% ration. The 4<sup>th</sup> day treatment would have received approximately equal nutrients to the 25% treatment, again with the probable error being that fish fed every 4<sup>th</sup> day would have increased nutrient intake through compensatory feeding. The fish in the different treatments responded differently to these allotments of nutrients. Fish fed

every day, and fed to 100% satiation had similar results in egg number (152 vs 146), similar number of spawns (14 vs 15), the same survival to eyed embryo (98.2%), and similar hatching rates (98.5% vs 99.3%). However, fish fed every 2<sup>nd</sup> day produced more eggs than fish fed on 50% ration (99 vs 72), while number of spawns, survival to eyed embryos and hatching rates remained similar. These data could be explained as a result of fish fed every 2<sup>nd</sup> day receiving slightly greater amounts of nutrients as a result of their compensatory feeding. The fish fed every 4<sup>th</sup> day and the fish fed the 25% ration, however, differed the most. Fish fed every 4<sup>th</sup> day only produced 21 eggs, half the number produced by fish on a 25% ration (50 eggs). Also, the number of spawns dropped, those fish being fed every 4<sup>th</sup> day only spawning approximately 3 times and not spawning through the entirety of the experiment while those fish fed on the 25% ration spawned for the entire experimental period. Survival to eyed embryo and hatching rate was also lower for those fish fed every 4<sup>th</sup> day as compared with those fed 25% ration. These changes in reproductive output are opposite to any *a priori* hypothesis in which it would be expected that the fish fed every 4<sup>th</sup> day had increased nutrient intake due to compensatory feeding relative to those fed a 25% ration.

This suggests that *M. splendida splendida* copes much better with lowered rations on a daily basis than infrequent feeding. From this study it may be deduced that it is not as important in this species to feed to 100% satiation every feeding as it is to feed every day. Although a 100% ration on a daily basis would still provide the best reproductive performance.

#### 4.4.3 Limitations to the study

The weight of food consumed by each fish during the feeding frequency experiment was not recorded. Studies by Gamage (2001) on *L. calcarifer* and Pirhonen

and Forsman (1998) on *S. trutta* indicated that fish fed at prolonged intervals consume more food than fish fed on a daily basis. The amount of food that a fish can consume per meal is limited, however, by their stomach capacity (De Silva and Anderson, 1995). Measurement of the amount of food consumed by each treatment group would have determined if this phenomenon was also observed in *M. splendida splendida*.

**5. Effect of energy, protein, and lipid content of broodstock diets on  
reproductive parameters**

## 5.1 General Introduction

Energy, protein and lipid contents in a diet are important. What ever is consumed in feed is needed by the fish for a variety of metabolic functions, and must first be allocated to essential needs such as respiration, digestion and excretion (De Silva and Anderson, 1995). Any excess that is available after meeting these requirements can then be allocated to other processes such as growth and reproduction (De Silva and Anderson, 1995). It is therefore necessary to ensure that diets for broodstock contain enough of these nutrients not only to meet essential metabolic functions but also to sustain egg and sperm development.

Energy content is important as energy is needed for everything that a fish does from basic metabolic functions to swimming and reproduction. The more energy provided in a diet over and above base needs may then be allocated to growth and reproduction. When limited energy is available, fewer eggs may be produced, but the more energy that is made available, the more eggs the fish will be able to yield until maximum reproduction is achieved (De Silva and Anderson, 1995).

The balance between dietary energy and protein is also vital. If the amount of dietary energy in relation to protein is low, the fish will use the protein in the diet for a source of energy (Lovell, 1984). Conversely, if too much energy is provided in the diet, it may limit food consumption by the fish and in so doing the protein requirements may not be met (Lovell, 1984). This is because fish will adjust its food intake to satisfy its need for energy (Halver, 1989, De Silva and Anderson, 1995).

Both protein and lipid are very important constituents of fish eggs. They are the primary components of vitellogenin, the lipoprotein that is the main energy source in the yolk of eggs. They are also an energy source for broodstock, therefore it is important to ensure that broodstock feeds contain enough of each so that female broodstock will have enough lipoproteins to allocate to egg production. Many studies

have previously linked increased protein level with increased reproductive ability (Smith *et al.*, 1979; Dahlgren, 1980; Gunasekera *et al.*, 1995). It has also been shown that while lipid is important to fish reproduction, too much lipid in a diet can be detrimental to egg quality (Fernandez-Palacios *et al.*, 1995; and Fernandez-Palacios *et al.*, 1997). Too little lipid or poor quality, however, can lead to a decrease in ovary size (MacFarlane *et al.*, 1993), and lower egg survival to hatching (Naves *et al.*, 1997).

In an aquaculture setting, it is necessary to determine how much energy, protein and lipid a fish needs to maximise reproduction. If too much protein or lipid is supplied in food, it is wasted and food costs increase to no useful ends. Also an excess of feed may lead to water quality problems that may be detrimental to fish health. The following three experiments were designed to determine the best feed energy, protein and lipid level to maximise reproduction so that excessive levels are not fed to fish thereby increasing food costs.

## **5.2 Methodology**

### **5.2.1 General methods**

All three experiments in this section were conducted over a period of 40 days. At the end of each experiment total number of eggs laid, total number of spawns, spawning interval, survival to eyed embryo, hatching rate and unfed larval life were determined for each treatment group.

Diets for these three experiments were prepared in the laboratory at James Cook University. Preparation of the foods followed designs by Appleford (1996), and Gamage (2001). Dry ingredients such as cornstarch, casein, fishmeal, vitamins, minerals, gelatine and cellulose were mixed for approximately 30 minutes using a Hobart mixer (Hobart Corporation, Troy, Ohio, USA) (Table 5.1). The fish oil was then added and was mixed into the dry ingredients for approximately 20 minutes. After that water was added to the mixture to allow pelleting. This mixture was extruded through a Hobart #12 chopper attachment with a 1/8-inch diameter (Hobart Corporation, Troy, Ohio). These pellets were finally broken into smaller pieces, to a suitable size for the fish used in these experiments to consume. They were then dried at 50 °C for approximately 12 hours and then stored at -18 °C in sealed plastic bags for use in the respective experiments.

### **5.2.2 Energy content experiment**

Each of 15 tanks that contained four breeding fish, ranging in size from 5.2-6.3 cm, were randomly allocated to one of three treatments. Treatment I was a 17 MJ KG<sup>-1</sup> energy diet. Treatment II was a control (14 MJ KG<sup>-1</sup>) as this diet contained similar energy, protein and lipid content as the flake food that had been fed to the fish in all earlier experiments. Treatment III was an 11 MJ KG<sup>-1</sup> energy diet (Table 5.1). All

three diets were respectively administered to five tanks of fish. Energy content in these diets was calculated by using the data of Appleford (1996) (Appendix 1). In all three diets protein was kept as similar as possible (43% protein) to keep this parameter from being a possible influence on reproduction. Lipid, however, was not able to be kept constant as fish oil, the main ingredient containing lipid also had the highest energy level. All fish were fed to satiation on a daily basis.

Statistical analyses were performed using SPSS. ANOVA (Tukey's HSD) was used for egg number, number of spawns, spawning interval, survival to eyed embryo and hatching rate and the pair wise comparison statistic was used for unfed larval life. Survival to eyed embryo and hatching rate data was arc-sine transformed prior to analysis.

**Table 5.1**

Composition of the three experimental diets. 17/43, 14/43, and 11/43 refer to the MJ KG<sup>-1</sup> energy/%protein content in that particular diet. All ingredients are given in grams.

<b>Ingredient</b>	<b>17/43</b>	<b>14/43</b>	<b>11/43</b>
Cornstarch <sup>a</sup>	145	84	0
Casein <sup>b</sup>	109	109	109
Fishmeal <sup>c</sup>	452	452	452
Fish Oil <sup>d</sup>	101	58	0
Vitamins <sup>e</sup>	30	30	30
Minerals <sup>f</sup>	5	5	5
Gelatine <sup>g</sup>	20	20	20
Cellulose <sup>h</sup>	212	312	499
Total Mass of diet	1074	1070	1115

a- Corn Starch – Anglosouce, QLD, Australia

b- Casein – Malanda, Australia

c- Fish Meal – Ridley’s Aqua Products, Brisbane, QLD, Australia

d- Fish Oil – Ridley’s Aqua Products, Brisbane, QLD, Australia

e- Vitamins – Rabar custom mix, DPI northern fisheries, Australia

f- Minerals – DPI ARI Fish mineral premix, Phone-Poulenc, Animal Nutrient Pty. Ltd., Queensland, Australia

g- Gelatine – Farmhouse Kitchen foods Pty. Ltd, QLD, Australia

h- Cellulose – Hahnflock, Hahn & Co, Germany

### 5.2.3 Protein content experiment

Each of 15 tanks that contained four breeding fish, which ranged in size from 5.4-6.2 cm, were randomly allocated to one of three treatments. All three treatments contained the 17 MJ KG<sup>-1</sup> energy content as this level yielded the best results in the energy content experiment. Treatments I, II, and III had 50%, 43%, and 35% protein content respectively (Table 5.2). Five tanks of fish were allocated to each treatment. Lipid content was kept as similar as possible in all three diets to reduce its effects on the measured parameters. All fish were fed to satiation on a daily basis.

Protein content of these diets was estimated by using data from Appleford (1996) (Appendix 1). Protein content of all three diets was further confirmed by directly measuring their protein level using the Kjeldhal method (Crooke and Simpson, 1971). Replicates of protein analysis were carried out for each diet. Crude protein of the diet was then determined using the formulae:

$$\text{Nitrogen (\%)} = \frac{\text{Titrant (ml)} \times \text{normality of titrant}}{\text{Sample weight (g)}} \times 0.014 \times 100$$

$$\text{Crude Protein (\%)} = \text{Nitrogen content (\%)} \times 6.25$$

Statistical analyses were performed using SPSS. ANOVA (Tukey's HSD) was used for egg number, number of spawns, spawning interval, survival to eyed embryo and hatching rate and the pair wise comparison statistic was used for unfed larval life. Survival to eyed embryo and hatching rate data was arc-sine transformed prior to analysis.

**Table 5.2**

Composition of the three experimental diets. 17/50,17/43, and 17/35 refer to the ( MJ KG<sup>-1</sup> energy)/% protein content in that particular diet. All ingredients are given in grams.

<b>Ingredient*</b>	<b>17/50</b>	<b>17/43</b>	<b>17/35</b>
Corn Starch	24	145	300
Casein	124	109	86
Fish Meal	517	452	357
Fish Oil	101	101	101
Vitamins	30	30	30
Minerals	5	5	5
Gelatine	30	20	20
Cellulose	229	212	191
Total Mass of Diet	1060	1074	1090

\*For the sources of each ingredient please refer to Table 5.1

#### 5.2.4 Lipid content experiment

Each of 15 tanks that contained four breeding fish, which ranged in size from 5.6-6.4 cm, were randomly allocated to one of three treatments. Treatments I, II, and III were 20%, 12%, and 9% total lipid, respectively (Table 5.3). All three treatments contained the 17 MJ KG<sup>-1</sup> energy content and 43% protein content as these amounts yielded the best results in the previous energy and protein experiments (Table 5.3). Five tanks of fish were allocated to each of the three treatments and all fish were fed to satiation on a daily basis.

Lipid content of the diets was first estimated using data from Appleford (1996) and then further confirmed by directly measuring total lipid content using the method by Folch *et al.* (1957) which had been modified by Gamage (2001). Three replicates for lipid content analysis were carried out for each diet. Fat content was calculated as:

$$\text{Fat (\%)} = \frac{\text{Weight of fat (g)}}{\text{Sample weight (g)}} \times 100$$

The percentage of abnormal larvae were also investigated in this experiment. An abnormal larva was defined as one that had a malformed spine causing the larva to swim in small circles. Many of these larvae also had problems with buoyancy.

Statistical analyses were done using SPSS. ANOVA (Tukey's HSD) was used for egg number, number of spawns, average spawning interval, survival to eyed embryo, and hatching rate and pair wise comparison statistic was used for unfed larval life. Survival to eyed embryo and hatching rate data was arc-sine transformed prior to analysis.

**Table 5.3**

Composition of the three experimental diets. 17/20, 17/12, and 17/9 refer to the MJ KG<sup>-1</sup> energy/% lipid content in that particular diet. All ingredients are given in grams

<b>Ingredient*</b>	<b>17/20</b>	<b>17/12</b>	<b>17/9</b>
Corn Starch	72	145	210
Casein	108	109	109
Fish Meal	448	452	452
Fish Oil	131	101	75
Vitamins	30	30	30
Minerals	5	5	5
Gelatine	25	20	20
Cellulose	185	212	182
Total Mass of Diet	1195	1074	1083

\*For sources of each ingredient please refer to Table 5.1

## 5.3 Results

### 5.3.1 Energy content experiment

Table 5.4 lists results from this experiment. Energy content had a significant effect on spawning success. Fish fed the 17 MJ KG<sup>-1</sup> energy diet produced on average more than twice as many eggs as those fed the control (14 MJ KG<sup>-1</sup>) ( $p < 0.05$ ), where fish in the 17 MJ KG<sup>-1</sup> energy treatment group produced  $255 \pm 15$  eggs while fish in the 14 MJ KG<sup>-1</sup> treatment group only produced  $121 \pm 11$  eggs. Fish fed the 11 MJ KG<sup>-1</sup> energy diet produced the lowest number of eggs,  $55 \pm 8$  eggs (Table 5.4). Energy content also had an effect on spawning interval; the higher the energy content of the diet the shorter the interval between egg clutches. Fish fed the 17 MJ KG<sup>-1</sup> energy diet had an average spawning interval of  $1.6 \pm 0.2$  days while those fed the 11 MJ KG<sup>-1</sup> energy content had an interval of  $3.9 \pm 0.4$  days (Table 5.4). There was no difference in survival to eyed embryo or hatching rates between fish fed the 17 MJ KG<sup>-1</sup> energy diet or the control diet (14 MJ KG<sup>-1</sup>) ( $p > 0.05$ ), but both groups had a much higher survival to eyed embryo and hatching rate than those fed the 11 MJ KG<sup>-1</sup> energy content diet ( $p < 0.05$ ). The 11 MJ KG<sup>-1</sup> energy diet treatment resulted in survival to eyed embryo of  $79.4\% \pm 4.5$  and a hatching rate of  $76.4\% \pm 5.4$  (Table 5.4).

Unfed larval life was significantly affected by energy content. Fish fed the 17 MJ KG<sup>-1</sup> energy diet had the longest unfed larval life ( $10.2 \pm 1.2$  days) of the three diets in this experiment, and also in the feeding frequency and feeding ration experiments (Tables 4.1, 4.2, 5.4). The fish on the control diet (14 MJ KG<sup>-1</sup>) had a comparable unfed larval life to that seen in the best treatment groups of the feeding frequency and feeding ration experiments, while those fed the 11 MJ KG<sup>-1</sup> energy diet had a significantly reduced life span ( $p < 0.05$ ).

**Table 5.4**

Effects of energy content on egg number, number of spawns, spawning interval, survival to eyed embryo, hatching rate and unfed larval life in *M. splendida splendida*.

<b>Treatment</b>	<b>Energy Content</b>	<b>Egg Number</b>	<b>No. of Spawns</b>	<b>Ave. Spawning Interval (Days)</b>	<b>Survival to Eyed Embryp %</b>	<b>Hatching rate %</b>	<b>Unfed Larval Life (Days)</b>
<b>1 (n = 5)</b>	<b>17 MJ KG<sup>-1</sup></b>	255 <sup>a</sup> ±	25.4 <sup>a</sup> ±	1.6 <sup>a</sup> ± 0.2	99.9 <sup>a</sup> ±	98.7 <sup>a</sup> ±	10.2 <sup>a</sup> ±
		15.0	0.5		0.9	0.6	1.2
<b>2 (n = 5)</b>	<b>14 MJ KG<sup>-1</sup></b>	121 <sup>b</sup> ±	25.4 <sup>a</sup> ±	2.1 <sup>b</sup> ± 0.1	99.8 <sup>a</sup> ±	98.6 <sup>a</sup> ±	8.9 <sup>b</sup> ±
		11.2	0.5		1.2	0.8	1.5
<b>3 (n = 5)</b>	<b>11 MJ KG<sup>-1</sup></b>	55 <sup>c</sup> ± 8.4	10.2 <sup>c</sup> ±	3.9 <sup>c</sup> ± 0.4	79.4 <sup>b</sup> ±	76.4 <sup>b</sup> ±	5.4 <sup>c</sup> ±
			0.4		4.5	5.4	0.9

The n value refers to the number of tanks of fish (two males and two females per tank) allocated to each treatment. Significance factors for egg number, number of spawns, spawning interval, survival to eyed embryo and hatching rate were determined using ANOVA (Tukey's HSD  $\alpha = 0.05$ ), while the pairwise comparison statistic was used for unfed larval life ( $\alpha = 0.05$ ). Values are mean  $\pm$  S.E; same letters indicate homogenous subsets. Survival to eyed embryo and hatching rate data was arc-sine transformed.

### 5.3.2 Protein content experiment

Table 5.5 shows the results of this experiment. The fish fed 43% and 50% protein levels did not have any significant difference in egg number, total number of spawns and spawning interval ( $p>0.05$ ). The 35% protein diet treatment did significantly lower these parameters: egg number ( $156 \pm 12$  eggs), and total number of spawns ( $19.5 \pm 0.5$  spawns), and spawning interval ( $2.1 \pm 0.4$  days) that was longer in fish fed the 35% protein diet (Table 5.5).

None of the diets had any significant effects on survival to eyed embryo or hatching rate. These parameters being similar in all diets tested (Table 5.5). The diets, however, did significantly effect unfed larval life. Fish fed the 43% protein diet had the longest unfed larval life of  $10.9 \pm 1.1$  days. This was longer than any unfed larval life period in all of the earlier experiments in this chapter and in Chapter 4 (Tables 4.1, 4.3, 5.1, 5.5). The unfed larval life of larvae from broodstock fed the 50% protein diet ( $9.8 \text{ days} \pm 1.2$ ) was slightly shorter but not significantly different than those fed 43% protein ( $p>0.05$ ). Those fed the 35% protein diet had the shortest unfed larval life of  $7.8 \pm 1.4$  days (Table 5.5).

**Table 5.5**

Effects of protein content on egg number, number of spawns, spawning interval, survival to eyed embryo, hatching rate and unfed larval life in *M. splendida splendida*.

<b>Treatment</b>	<b>Protein Content</b>	<b>Egg Number</b>	<b>No. of Spawns</b>	<b>Ave. Spawning Interval (Days)</b>	<b>Survival to Eyed Embryo %</b>	<b>Hatching Rate %</b>	<b>Unfed Larval Life (Days)</b>
<b>1 (n = 5)</b>	<b>50%</b>	241 <sup>a</sup> ±	26.4 <sup>a</sup> ±	1.5 <sup>a</sup> ± 0.1	99.8 <sup>a</sup> ±	98.7 <sup>a</sup> ±	9.8 <sup>a</sup> ±
		17.1	0.3		1.6	2.1	1.2
<b>2 (n = 5)</b>	<b>43%</b>	237 <sup>a</sup> ±	25.7 <sup>a</sup> ±	1.6 <sup>a</sup> ± 0.3	99.9 <sup>a</sup> ±	99.0 <sup>a</sup> ±	10.9 <sup>a</sup> ±
		16.9	0.6		1.2	1.3	1.1
<b>3 (n = 5)</b>	<b>35%</b>	156 <sup>b</sup> ±	19.5 <sup>b</sup> ±	2.1 <sup>a</sup> ± 0.4	99.6 <sup>a</sup> ±	98.6 <sup>a</sup> ±	7.8 <sup>c</sup> ±
		12.5	0.5		0.9	1.2	1.4

The n value refers to the number of tanks of fish (two males and two females per tank) allocated to each treatment. Significance factors for egg number, number of spawns, spawning interval, survival to eyed embryo and hatching rate were determined using ANOVA (Tukey's HSD  $\alpha = 0.05$ ), while the pairwise comparison statistic was used for unfed larval life ( $\alpha = 0.05$ ). Values are mean  $\pm$  S.E; same letters indicate homogenous subsets. Survival to eyed embryo and hatching rate were arc-sine transformed.

### 5.3.3 Lipid content experiment

Table 5.6 shows the results of this experiment where three levels of lipid content were tested and they had no significant effects on egg number, number of spawns or spawning interval ( $p>0.05$ ). Fish fed the 20% lipid diet produced a total of  $232 \pm 23$  eggs, 12% lipid diet produced  $247 \pm 17$  eggs and the 9% lipid diet produced  $239 \pm 33$  eggs (Table 5.6).

However, the treatment with the highest lipid content diet (20%) had a significantly reduced survival to eyed embryo and hatching rate as compared to that seen in the 12% and 9% lipid diet treatments ( $p<0.05$ ). The survival to eyed embryo and hatching rate of eggs produced by fish fed the 20% lipid diet being  $78.9\% \pm 3.6\%$  and  $68.7\% \pm 1.7\%$ , respectively (Table 5.6). The 9% and 12% lipid diet treatments had no significant differences between both hatching and survival to eyed embryos (Table 5.6) ( $p>0.05$ ).

Unfed larval life differed among all diet treatments. The 12% lipid diet had the longest unfed larval life ( $10.8 \pm 0.8$  days) ( $p<0.05$ ) this being similar to those seen in both the energy and protein experiment (Tables 5.4, 5.5, and 5.6). The 9% lipid diet treatment had the next higher unfed larval life ( $8.9 \pm 1.0$  days), while the 20% lipid diet treatment had the lowest unfed larval life ( $6.7 \pm 1.2$  days) ( $p<0.05$ ) (Table 5.6). There was a high percentage of abnormal larvae ( $30.6\% \pm 4.7\%$ ) that hatched from eggs produced by females fed the highest lipid diet (20% lipid) while the other two diet treatments produced no abnormal larvae (Table 5.6).

**Table 5.6**

Effects of lipid content on egg number, number of spawns, spawning interval, survival to eyed embryo, hatching rate and unfed larval life and percent of abnormal larvae in *M. splendida splendida*.

<b>Treatment</b>	<b>Lipid</b>	<b>Egg Number</b>	<b>No. of Spawns</b>	<b>Ave. Spawning Interval</b>	<b>Survival to Eyed Embryo %</b>	<b>Hatching Rate %</b>	<b>Unfed Larval Life</b>	<b>% Abnormal Larvae</b>
<b>1 (n = 5)</b>	<b>20%</b>	232 <sup>a</sup> ± 23.3	26.4 <sup>a</sup> ± 1.2	1.5 <sup>a</sup> ± 0.1	78.9 <sup>a</sup> ± 3.6	68.7 <sup>a</sup> ± 1.7	6.7 <sup>a</sup> ± 1.2	30.6 ± 4.7
<b>2 (n = 5)</b>	<b>12%</b>	247 <sup>a</sup> ± 17.8	25.7 <sup>a</sup> ± 0.9	1.6 <sup>a</sup> ± 0.1	98.7 <sup>b</sup> ± 2.5	99.8 <sup>b</sup> ± 0.9	10.8 <sup>b</sup> ± 0.8	0.0 ± 0.0
<b>3 (n = 5)</b>	<b>9%</b>	239 <sup>a</sup> ± 33.4	25.7 <sup>a</sup> ± 0.9	1.5 <sup>a</sup> ± 0.2	98.9 <sup>b</sup> ± 2.2	99.4 <sup>b</sup> ± 1.3	8.9 <sup>c</sup> ± 1.0	0.0 ± 0.0

The n value refers to the number of tanks of fish (two males and two females per tank) allocated to each treatment. Significance factors for egg number, number of spawns, spawning interval, survival to eyed embryo and hatching rate were determined using ANOVA (Tukey's HSD  $\alpha = 0.05$ ), while the pair wise comparison statistic was used for unfed larval life ( $\alpha = 0.05$ ). Values are mean  $\pm$  S.E; same letters indicate homogenous subsets. Survival to eyed embryo and hatching rate were arc-sine transformed.

## 5.4 Discussion

### 5.4.1 Energy content experiment

It was not surprising that, the highest energy level tested (17 MJ KG<sup>-1</sup> energy level diet), gave the best result in *M. splendida splendida* reproduction as it had ensured that the fish had enough energy to meet basic metabolic needs such as respiration and digestion, as well as to allocate energy to egg production. The 11 MJ KG<sup>-1</sup> energy diet may also have met these needs, but it is unclear whether these fish used protein as an energy source. If the amount of dietary energy in relation to dietary protein is low, the protein will be used as a source of energy (Lovell, 1984). While the fish on the 11 MJ KG<sup>-1</sup> energy diet did produce eggs, these eggs produced larvae with a significantly shorter unfed larval life,  $5.4 \pm 0.9$  days, only half of that from fish fed the 17 MJ KG<sup>-1</sup> diet, which indicated that the nutrients within these eggs were not as abundant or as appropriate for larval growth and development as those in the eggs of the other two treatment groups (Table 5.4). Since the larvae under starvation were not receiving any energy from external food sources, all nutrients available were those supplied within the egg yolk. Eggs with low energy content would have less energy available for use by the larvae, and as such, the larvae from a lower energy egg would die earlier than larvae hatched from an egg with a higher energy content. A broodstock diet that enabled prolonged survival of larvae during starvation, would allow more time for the larvae to adapt to external feeding and search for prey and in doing so would enhance the chances of survival during times when food sources are sub-optimal.

#### 5.4.2 Protein content experiment

The 43% and 50% protein diet treatments had similar reproductive success. The 35% protein diet treatment gave the lowest values for reproductive success in terms of egg number and number of spawns, however, the hatching rate and survival to eyed embryo were similar to those in the other treatments ( $p>0.05$ ) (Table 5.5). The 50% protein diet only differed from the 43% protein diet in the unfed larval life, which is slightly but not significantly shorter ( $9.8 \pm 1.2$ ) than that of the 43% protein diet ( $10.9 \pm 1.1$  days) ( $p>0.05$ ) (Table 5.5). This is an important parameter, despite the fact that the difference was not significant in this experiment, as it allows for a longer period of transition when larval fish must start feeding on their own. The more protein in a diet the more expensive it is to produce, and the higher probably of water quality problems it can create if overfeeding occurs as nitrogen in protein is broken down to ammonia. The 43% protein diet was deemed the more ideal diet and hence was the preferred protein level in subsequent experiments. Dahlgren (1980), showed that high feed protein levels (33%) increased reproductive success in *P. reticulata*. In *O. mykiss* fish maintained on a high protein diet (53%) produced significantly more eggs than those fed on a low protein diet (Smith *et al.*, 1979). The results of this protein content experiment on *M. splendida splendida* differed from those reported on *P. reticulata* and *O. mykiss* in that the highest protein content diet (50%) did not give an advantage over that of 45% protein in terms of reproductive success. However, the result of low reproductive success attributed to low protein in broodstock diets was in agreement with the studies of Dahlgren (1980) and Smith *et al.* (1979), in that the lowest protein content showed the worst reproductive parameters.

### 5.4.3 Lipid content experiment

Dietary lipid levels tested in this experiment did not affect number of eggs laid, number of spawns or spawning interval ( $p>0.05$ ). However, survival to eyed embryo, hatching rate and unfed larval life in the 20% lipid diet treatment were much lower than in the other two treatments ( $p<0.05$ ). This finding is in agreement with other studies that have demonstrated that too much lipid lowered larval survival (Fernandez-Palacios *et al.*, 1995; Fernandez-Palacios *et al.*, 1997). Furthermore, Springate *et al.* (1985) showed that too much lipid in a diet caused deformities in eggs and reduced hatching success in *S. aurata*. Larval deformity occurred in this experiment as well i.e. in the larvae hatched from eggs obtained from fish fed the 20% lipid content diet (Table 5.6). It is surmised that the high lipid content in this diet was the likely cause of larval deformity as it was not seen in the larvae from the 12% or 9% lipid diet treatments.

### 5.4.4 Summary

The diet containing the 17 MJ KG<sup>-1</sup> energy, 43% protein as well as the 12% lipid level maximised reproductive parameters for *M. splendida splendida*. It increased egg production from  $121 \pm 11$  (the total number of eggs produced in the control of the energy experiment Table 5.4), to  $247 \pm 17$  (in the 12% lipid diet containing 17 MJ KG<sup>-1</sup> energy and 43% protein Table 5.6). Unfed larval life was also significantly increased from  $8.9 \pm 1.5$  (Table 5.4) to  $10.8 \pm 0.9$  days (Table 5.6). This combination of nutrients was therefore used in the experiment described in Chapter 6 as the base diet.

### 5.4.5 Limitations of this experiment

The diets used in the energy experiment were noticeably different in texture, smell and palatability to the fish. The 11 MJ KG<sup>-1</sup> energy diet needed to be formulated

several times before a mixture was developed that the fish would eat. The 17 MJ KG<sup>-1</sup> energy diet was fairly oily and had a very strong smell, while in contrast the 11 MJ KG<sup>-1</sup> energy diet was fairly dry, with very little odour. These characteristics were related to the diet ingredients, therefore any effect that these characteristics had on this study could not be eliminated.

## **6. Effect of fatty acid supplementation on reproductive parameters**

## 6.1 Introduction

Essential fatty acids are fatty acids that are needed for growth and maintenance, however, the body is unable to synthesize them. In fish, fatty acids are the main component of egg membranes, maintaining structure and function (Sargent *et al.*, 1995). Fatty acids have an effect on the condition of female fish (Izquierdo *et al.*, 1992; Furuita *et al.*, 2000) as well as a significant impact on spawning performance (Fernandez-Palacios *et al.*, 1995; Bruce *et al.*, 1999; Furuita *et al.*, 2000). Fatty acids are also essential to the development of larvae, and many aspects of development may be effected by the amount and type of fatty acids in a larval diet (Dhert *et al.*, 1990; Lemm and Lemarie, 1991; Estevez *et al.*, 1999; Furuita *et al.*, 1999).

For herbivorous and omnivorous freshwater fish, many of the essential fatty acids are gained from freshwater algae. In most freshwater plants, many of the long chain n-3 and n-6 essential fatty acids are not found in abundant levels especially docosahexanoic acid (22:6n-3, DHA) (Halver, 1989). Arachidonic acid (20:4n-6, AA) and eicosapentaenoic acid (20:5n-3, EPA) are found in low levels in some freshwater invertebrates such as insect larvae, but are not in abundance, with the result that these fatty acids are still limiting (Halver, 1989). In eggs of both fresh and saltwater fish the amount of EPA and DHA are generally high. Freshwater fish eggs in addition to these two fatty acids also tend to be high in arachidonic acid (Halver, 1989). Because all three of these fatty acids are found in relatively high amounts in freshwater fish eggs, supplementing dry feed given to broodstock with these fatty acids should ensure proper egg formation and larval development.

The majority of studies done on the effects of fatty acids on reproduction or larval development have been on salt water fish species. A few studies have been carried out on temperate freshwater species such as the Murray cod, *Maccullochella peelii peelii*, the results indicating the lack of fatty acids were related to egg abnormality

(Gunasekera *et al.*, 1998). Literature on the effects of fatty acids on tropical freshwater fish reproduction and larval survival, however, is lacking especially in Australia.

The fatty acids in salt-water fish eggs differ from those found in freshwater fish eggs. Although eggs from both saltwater and freshwater fish are rich in both DHA and EPA, AA is more abundant in eggs of freshwater fish species (Halver, 1989). It is therefore reasonable to assume that freshwater fish will require all three essential fatty acids in their diet in order to maximise reproduction.

For the fatty acid supplementation experiment the, 17 MJ KG<sup>-1</sup> energy, 43% protein and 12% lipid diet was used as a base diet. This diet was then supplemented with three fatty acids, known to be found in freshwater fish eggs, to see the effects on reproductive performance of *M. splendida splendida*.

## 6.2 Methodology

Each of 25 tanks that contained four breeding fish (two males and two females), which ranged in size from 5.3-6.4 cm, were randomly allocated to one of four treatment groups or a control group. Treatment I was supplemented with arachidonic acid (20:4n-6) (AquaGrow-AA, Martek Biosciences Corporation, Columbia, Maryland, USA). Treatment II was supplemented with docosahexaenoic acid (22:6n-3) (AquaGrow-DHA, Martek Biosciences Corporation, Columbia, Maryland, USA). Treatment III supplemented with eicosapentaenoic acid (20:5n-3) (salmon oil, Ridley's Agriproducts, Brisbane, QLD, Australia). Treatment IV was supplemented with all three of the fatty acids. Treatment V was the control. Five tanks of fish were allocated to each of the five treatments. In all five diets the 17 MJ KG<sup>-1</sup> energy, 53% protein and 12% lipid levels that gave the best results in the prior experiments in Chapter 5 were used (Table 6.1). This experiment was conducted over a period of 40 days. At the end of the experiment total number of spawns and spawning interval were also determined.

Statistical analyses were done using SPSS. ANOVA (Tukey's HSD) and pairwise comparison statistic were used for various parameters. Survival to eyed embryo and hatching rate were arc-sine transformed.

**Table 6.1**

This table shows which fatty acid each treatment was supplemented with and the number of replicate tanks for each treatment group.

<b>Treatment</b>	<b>Supplementation</b>	<b>Replication</b>
<b>1</b>	AA	5
<b>2</b>	DHA	5
<b>3</b>	EPA	5
<b>4</b>	AA, DHA, EPA	5
<b>5</b>	none (control)	5

**Table 6.2**

Composition for the control diet and all four diets enriched with fatty acids expressed in grams.

<b>Ingredient*</b>	<b>Control</b>	<b>AA</b>	<b>DHA</b>	<b>EPA</b>	<b>All Fatty Acids</b>
Cornstarch	143	143	143	143	143
Fishmeal	109	109	109	109	109
Fish Oil	101	101	101	0	0
AquaGrow-AA	0	123	0	0	123
AquaGrow-DHA	0	0	123	0	97
Salmon Oil (EPA)	0	0	0	123	123
Vitamins	30	30	30	30	30
Minerals	5	5	5	5	5
Gelatine	20	20	20	20	20
Cellulose	212	212	212	212	212
Total Mass of Diet	620	743	743	642	842

\* For a list of ingredient sources see text on previous page and Table 5.1

### 6.3 Results

Table 6.2 lists the results for this experiment. There was no significant effect on reproduction for the diets that were supplemented with any one of three fatty acids ( $p>0.05$ ). No significant differences for any of the reproductive parameters such as egg number, number of spawns, average spawning interval, survival to eyed embryo, hatching rate or unfed larval life, were seen among the three diets supplemented with one of three fatty acids or the control (Table 6.2). However, the larvae from fish fed the diet containing all three fatty acids had significantly longer unfed larval life ( $11.1 \pm 1.2$  days), than any of the larvae in the other treatments in this experiment. This value was also longer than any unfed larval life from any of the previous experiments (see Chapters, 4 and 5)

Even though the differences between treatments were not statistically significant, the fish fed the diet containing all three fatty acids produced eggs with a 100% survival to eyed embryo and hatching rate. Fish in this treatment also had the highest number of eggs (254), highest number of spawns (26.4) and the shortest average spawning interval (1.5) when compared to results from the other treatments in this experiment and from the earlier experiments (Chapters 4 and 5).

**Table 6.2**

Effects of fatty acid supplementation on egg number, number of spawns, spawning interval, survival to eyed embryo, hatching rate and unfed larval life in *M. splendida splendida*.

<b>Treatment</b>	<b>Supplement</b>	<b>Egg Number</b>	<b>No. of Spawns</b>	<b>Ave. Spawning Interval (Days)</b>	<b>Survival to Eyed Embryo %</b>	<b>Hatching Rate %</b>	<b>Unfed Larval Life (Days)</b>
<b>1 (n = 5)</b>	<b>AA</b>	214 <sup>a</sup> ± 22	26.2 <sup>a</sup> ± 1.2	1.5 <sup>a</sup> ± 0.3	98.9 <sup>a</sup> ± 1.3	98.9 <sup>a</sup> ± 1.3	10.2 <sup>a</sup> ± 0.9
<b>2 (n = 5)</b>	<b>DHA</b>	232 <sup>a</sup> ± 37	25.7 <sup>a</sup> ± 0.9	1.6 <sup>a</sup> ± 0.1	99.4 <sup>a</sup> ± 2.2	99.4 <sup>a</sup> ± 2.2	10.3 <sup>a</sup> ± 1.3
<b>3 (n = 5)</b>	<b>EPA</b>	230 <sup>a</sup> ± 14	26.4 <sup>a</sup> ± 1.0	1.5 <sup>a</sup> ± 0.2	99.7 <sup>a</sup> ± 1.8	99.7 <sup>a</sup> ± 1.8	10.2 <sup>a</sup> ± 1.6
<b>4 (n = 5)</b>	<b>AA, DHA, EPA</b>	245 <sup>a</sup> ± 21	26.4 <sup>a</sup> ± 0.8	1.5 <sup>a</sup> ± 0.1	100 <sup>a</sup> ± 0.0	100 <sup>a</sup> ± 0.1	11.1 <sup>b</sup> ± 1.2
<b>5 (n = 5)</b>	<b>none (control)</b>	240 <sup>a</sup> ± 27	26.2 <sup>a</sup> ± 1.1	1.5 <sup>a</sup> ± 0.7	97.6 <sup>a</sup> ± 1.3	98.3 <sup>a</sup> ± 1.9	10.2 <sup>a</sup> ± 0.8

The n value refers to the number of tanks of fish (two males and two females per tank) allocated to each treatment. Significance factors for egg number, number of spawns, spawning interval, survival to eyed embryo and hatching rate were determined using ANOVA (Tukey's HSD  $\alpha = 0.05$ ), while the pairwise comparison statistic was used for unfed larval life ( $\alpha = 0.05$ ). Values are mean  $\pm$  S.E; same letters indicate homogenous subsets. Survival to eyed embryo and hatching rate were arc-sine transformed.

## 6.4 Discussion

### 6.4.1 Discussion of general findings

None of the treatment groups fed a diet supplemented with a singular fatty acid showed any significant differences in the measured reproductive parameters. This could be because all three of the fatty acids were limiting to reproduction and the inclusion of one but not all acids was not sufficient to cause a difference. Alternatively, it may be that the broodstock were receiving enough of each fatty acid in their diet even without supplementation for it to not be a limiting factor to egg production. It was also seen that fatty acid supplementation had no significant effect on survival to eyed embryo or hatching rate. Fish fed the diet containing all three of the fatty acids did, however, produce larvae with a longer unfed larval life indicating that these fatty acids were limited for larval growth. Dhert *et al.*, (1990) showed that newly hatched *L. calcarifer* larvae had higher physiological condition resulting in lower mortality rates when fed *Artemia* enriched with EPA and 22:3n-6. Navarro *et al.*, (1988) showed that a deficiency of dietary n-3 HUFA resulted in low survival of *D. labrax* larvae, while increased growth and survival of *P. olivaceus* fed diets enriched by n-3 HUFA was seen by Furuita *et al.*, (1999). Most studies done, however, look at the effect of fatty acids on larvae already feeding. By including higher percentages of fatty acids in the diets of broodstock it may allow the females to allocate more to the eggs. This in turn may result in the larvae having a higher percentage in their body on hatching so they can live for longer without needing to find a new source of fatty acids.

Fatty acids are an important aspect of cell membrane structure and function (Sargent 1995). In this study no significant effect was seen on survival to eyed embryo or hatching rate of eggs in any of the groups. It could be assumed that there was already enough of these fatty acids in the diet to ensure proper membrane structure. The addition of more fatty acids did not affect egg structure, but allowed for more fatty

acids to be used and stored by the larvae. Arachidonic acid, EPA and DHA are all found in abundant levels in freshwater fish eggs (Halver, 1989). It is possible that it is the embryo that needed additional supplementation of fatty acids in order to develop properly and later after hatching for growth.

#### 6.4.2 Limitations of this study

The major limitation to this experiment was that although fatty acids were able to be included in the diet, in order to meet the protein and lipid content it was not possible to look at the effects of a fatty acid deficient diet. Using a purified diet, it is possible to investigate nutrient requirements by using deficient diets and noting growth and reproductive performance. However, purified diets vary significantly from practical diets used in aquaculture and interpreting the data obtained using purified diets is difficult (De Silva and Anderson, 1995). Also it is notable that freshwater fish are able to chain elongate and desaturate short chain saturated or unsaturated fatty acids to long chain PUFA. This allows freshwater fish to use other fatty acids which may be in abundance to make less available fatty acids. As such, it is not possible to know if the diet already contained an appropriate amount of the given fatty acids to maximise reproduction. Even with this limitation, however, the final diet trialed, containing 17 MJ KG<sup>-1</sup> of energy, 43% protein 12% lipid and supplemented with the three fatty acids significantly improved the unfed larval life period relative to that in the other diets tested (Table 6.2).

A further difficulty was finding a source of EPA that was not also a source of DHA. Salmon oil was chosen for the source of EPA because the ratio of EPA to DHA was much higher in this oil than in any other source for EPA. The EPA diet would also have had a slightly elevated level of DHA, although the amount in this diet was less than in the DHA diet.

## **7. General discussion and conclusions**

## 7.1 Summary

### 7.1.1 General discussion

These experiments conducted on *M. splendida splendida* are important for two reasons. Firstly, few studies have been done on the nutritional needs for reproduction on tropical freshwater species. Secondly this species is of commercial value in the ornamental fish trade as it is being sold in many aquarium stores.

The first experiment on reproductive parameters and the effects of starvation was conducted to establish effective reproductive parameters for use in the later experiments. In *M. splendida splendida*, the size of broodstock did not have significant effects on the size of eggs produced ( $p>0.05$ ), but it did significantly impact on the number of eggs ( $p<0.05$ ) (Table 3.1). Similarly, during periods of starvation the impact on egg diameter was not significant. Starvation did however impact on egg number and caused broodstock to cease egg production very quickly. There are two strategies that a fish can adopt for egg production either produce lots of similar sized eggs or produce fewer but larger eggs. The results suggested that in *M. splendida splendida*, the strategy for producing many eggs of similar size had been adopted.

For broodstock fish of the same size range, differences in reproduction could be seen with both feeding frequency and feeding ration. The fish fed daily and to 100% satiation produced the most eggs, had the shortest spawning interval, the highest survival to eyed embryo, hatching rate and the longest unfed larval life (Table 4.1 and 4.2) (Chapter 4)

Under conditions where broodstock were fed to 100% satiation on a daily basis, the energy content was observed to significantly affect reproductive performance ( $p<0.05$ ). Fish fed the  $17 \text{ MJ KG}^{-1}$  energy diet had the highest reproductive success ( $p<0.05$ ) (Table 5.4). The results of the protein content experiment again enhanced reproductive success with the 43% and 50% protein diets increasing the unfed larval life

(Table 5.5). While there was no significant difference in the length of unfed larval life between these two treatments ( $p>0.05$ ) fish fed the 43% protein diet had a longer unfed larval life. The 43% protein level from this experiment was chosen for further trials because of this advantage (even though it wasn't significant) and also because a lower protein content would reduce the food cost and decrease the chances of nitrogen and sulphur related water quality problems. In the lipid content experiment, the 12% lipid treatment gave the best results with the period of unfed larval life being significantly longer than in the 20% and 9% lipid content treatments ( $p<0.05$ ). Further the 20% lipid diet also resulted in a high number of abnormal larvae and a significantly shortened unfed larval life ( $p<0.05$ ).

A very dramatic increase in reproductive success could be seen when comparing fish fed the 14 MJ KG<sup>-1</sup> energy control diet modeled on a commercially available flake (Nutrafin®) and fish fed the 17 MJ KG<sup>-1</sup> energy, 43% protein, 12% lipid diet supplemented with arachidonic acid, DHA and EPA, hereafter to be called the “final diet”. Egg number doubled increasing from  $121 \pm 11$  eggs to  $245 \pm 21$  eggs (Table 7.1). The number of spawns significantly increased while average spawning interval decreased (Table 7.1). There was no significant difference in survival to eyed embryo and hatching rate, however, fish fed the final diet achieved a 100% survival to eyed embryo and hatching rate which is quite notable. The unfed larval life increased significantly as well, increasing by over two days from  $8.9 \pm 1.5$  to  $11.1 \pm 1.2$  days (Table 7.1). The increase in egg number, unfed larval life and decrease in spawning interval achieved by replacing the control diet with the final diet showed that a very marked increase in reproductive success had been demonstrated between these two diets for *M. splendida splendida*.

**Table 7.1**

Differences in reproductive parameters of fish fed a control diet (similar to the flake food) and the end diet (17 MJ KG<sup>-1</sup> energy, 43% protein, 12% lipid diet supplemented with AA, DHA and EPA.

<b>Treatment</b>	<b>Control</b>	<b>Final Diet</b>
<b>Egg number</b>	121 ± 11	245 ± 21
<b>Total spawns</b>	19.3 ± 0.3	26.4 ± 0.8
<b>Ave. Spawning Interval (Days)</b>	2.1 ± 0.1	1.5 ± 0.1
<b>Survival to eyed embryo %</b>	99.8 ± 1.2	100 ± 0.0
<b>Hatching rate %</b>	98.6 ± 0.8	100 ± 0.1
<b>Unfed Larval Life (Days)</b>	8.9 ± 1.5	11.1 ± 1.2

### 7.1.2 Conclusion

These experiments in this research project have covered a variety of topics on freshwater fish nutrition in *M. splendida splendida* ranging from identifying what reproductive factors are affected by nutrition to how diet composition affected these factors. This study determined that egg number as opposed to egg diameter was affected by nutritional status, and that it is best to feed *M. splendida splendida* daily to satiation in order to maximise reproduction. The best diet trailed in this study for maximising reproduction was one with a 17 MJ KG<sup>-1</sup> energy content, 43% protein and 12% lipid content, that was supplemented with AA, DHA, and EPA. The energy content in the diet effected egg number the most, while the protein content, lipid content, and fatty acid supplementation maximised unfed larval life.

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## Appendix 1

### Proximal Composition of Feed Components

Proximal composition as determined by analysis of feed ingredients used in the preparation of experimental diets (Appleford, 1996).

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a – values are percentage dry weight

b – values are kJ/g dry weight

## Appendix 2

### Glossary of Common and Scientific Names

<u>Common name</u>	<u>Scientific name</u>
African catfish	<i>Clarias gariepinus</i>
Anchoveta	<i>Engraulis ringens</i>
Atlantic cod	<i>Gadus morhua</i>
Atlantic salmon	<i>Salmo salar</i>
Banded damsel fish	<i>Abudefduf abdominalis</i>
Barbel	<i>Barbus sclateri</i>
Barramundi	<i>Lates calcarifer</i>
Black carp	<i>Mylopharyngodon piceus</i>
Black porgy	<i>Acanthopagrus schlegeli</i>
Brown Trout	<i>Salmo trutta</i>
Clownfish	<i>Amphiprion percula</i>
Coho salmon	<i>Oncorhynchus kisutch</i>
Common carp	<i>Cyprinus carpio</i>
Common sole	<i>Solea solea</i>
Desert goby	<i>Chlamydogobius eremus</i>
Eastern rainbowfish	<i>Melanotaenia splendida splendida</i>
Fathead minnow	<i>Pimephales promelas</i>
Gilthead seabream	<i>Sparus aurata</i>
Goldfish	<i>Carassius auratus</i>
Guppy	<i>Poecilia reticulata</i>
Haddock	<i>Melanogrammus aeglefinus</i>
Herring	<i>Clupea harengus</i>
Japanese flounder	<i>Paralichthys olivaceus</i>
Largemouth bass	<i>Micropterus salmoides</i>
Milkfish	<i>Chanos chanos</i>
Murray cod	<i>Maccullochella peelii peelii</i>
Nile tilapia	<i>Oreochromis niloticus</i>
Tilapia	<i>Preochromis niloticus</i>
Pink Salmon	<i>Oncorhynchus gorcusha</i>
Rainbow trout	<i>Oncorhynchus mykiss</i>
Rainbow trout	<i>Salmo gairdneri</i>
Red seabream	<i>Pagrus major</i>
Ruffe	<i>Gymnocephala cornus</i>
Sable fish	<i>Anoplopoma fimbria</i>
Sakhalin taimen	<i>Hucho perryi</i>
Sand Goby	<i>Pomatoschistus minutus</i>
Saratoga	<i>Scleropages leichardti</i>
Sea bass	<i>Dicentrarchus labrax</i>
Sockeye salmon	<i>Oncorhynchus nerka</i>
Striped bass	<i>Morone saxatilis</i>
Three-spined stickleback	<i>Gasterosteus aculeatus</i>
Tilapia	<i>Tilapia zillii</i>
Turbot	<i>Scophthalmus maximus</i>
Upland bully	<i>Gobiomorphus breviceps</i>
Yellowtail flounder	<i>Limenda ferruginea</i>
Yellowtail rock fish	<i>Sebastes flavidus</i>