Reproductive investment, gamete quality and diet in farmed salmonids

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Candidate's Declaration

I declare that the work recorded in this thesis is entirely my own work and that it is of my own composition, except were stated below. No part of this work has been submitted for any other degrees.

Chapter 3: Kristoffer Tveit aided SR with the Partial Least Square models for predicting gonad weight.

Chapter 4: Comments by two unnamed referees incorporated into this chapter.

Chapters 5 and 6: Nutreco ARC designed the experiment used in these chapters, the tagging element was added to the design by FAH and SR and all analysis was undertaken by SR.

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Thesis Summary

This thesis discusses a number of questions relating to life history strategies, gamete quality and nutrition in *salmonids*.

Chapter 1 gives a broad overview of life history strategies and nutrition in aquaculture. An organism's life history strategy determines when it will mature and it's reproductive potential. Life histories are diverse with large intra- and inter- species variation; for example in *salmonids*, some species and individuals within a population remain always in freshwater and some migrate to sea and then return to freshwater to breed. Much research has been conducted with respect to life history strategies in *salmonids* but there is still a great deal to be learned, in particular about strategies exhibited by post smolt males and females. Currently fisheries, the main source of fish for human consumption, have come under pressure and alternative production sources have been adopted; for example aquaculture, which in recent years has expanded. The future success and expansion of aquaculture is dependent upon the reproductive potential and quality of the broodstock. A better understanding of Atlantic salmon life history strategies and the ability to predict which fish will mature and produce quality gametes could be beneficial to the industry. It is also important to keep aquaculture production costs low. This can potentially be achieved by manipulating diets without compromising the quality of the final product or in the case of broodstock of the gametes.

Chapter 2 explores the possibility of predicting which individual Atlantic salmon will mature and their reproductive potential by using morphological measurements taken throughout the year proceeding maturation. Atlantic salmon that matured in a given year had higher body weights than their non-maturing counterparts in the September of the year proceeding maturation, confirming the hypothesised critical autumn maturation 'window'. They remained larger in the following May and June. Weight in May (as opposed to September and June weight) was show to be the strongest predictor of maturity in females. Unlike female maturation, no single predictor was found for male maturation. Body weight in June was shown to have a strong relationship with egg number, indicating that it could be used as a predictor of reproductive investment in

females. This chapter also highlighted a gender difference in the reproductive trade offs exhibited by males and females.

Ultrasound is used in the aquaculture industry to differentiate the gender of mature fish in a number of species. In chapter 3 it was successfully used to distinguish the sex of immature Atlantic salmon and to estimate gonad length and width of immature female salmon and maturing female rainbow trout and in the case of the latter gonad size.

Finding alternative renewable and cheaper dietary sources of lipids would be beneficial to the aquaculture industry considering the present strain fisheries are under. It has been shown that rapeseed oil is a good alternative to fish oil in on-growing Atlantic salmon diets. The study described in chapter 4 showed that a 50 % replacement of fish oil with rapeseed oil in an Atlantic salmon broodstock diet had no effect upon the reproductive investment or egg quality (measured as survival of the eggs at each of the developmental stages eyeing, hatching and first feeding).

Just as with fish oil, fish meal, the main source of dietary protein for fish food, is also a finite resource that is expensive to produce. Also if it is provided in excess in the food in conjunction with an inadequate level of lipid it can be used inefficiently by the fish producing environmental concerns. Increasing the level of lipid at the expense of protein in large rainbow trout diets was investigated in this chapter. Raising the levels of lipid improved the food conversion ratio and specific growth rate, though it did also increase the levels of fat in the flesh.

During the study performed in chapter 5, it was discovered that the majority of the fish had come into reproductive condition and as a result the effects of maturation on growth, flesh quality and energy reserves in female rainbow trout were investigated here. The data in chapter 6 showed that maturation reduces growth and energy reserves as measured by levels of fat in the flesh and size of the visceral and hepato somatic indexes, confirming previous studies.

The findings of the studies conducted are summarised in chapter 7 and the implications they have for fish biologists and aquaculture researchers and farmers are described in this final chapter. Future studies that they prompt are also considered.

Contents

Chapter 1

General Introduction

Fish in the human diet

Fish inhabit a vast range of environments, constitute 60 % of all vertebrate species and play an important role in many food webs (Bone et., al 1996). Due to the aquatic environment they live in, they have a reduced need for skeletal support and as a result have a higher muscle to bone ratio, making them a good source of protein (Bone et al., 1996). They also contain high levels of important fatty acids. Marine algae and invertebrates are very rich in the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Sargent et al., 2002). Through the actions of food webs, fish consequently also have high levels of these fatty acids. One example is the Atlantic salmon (*Salmo salar*). Torstensen et al. (2004) report that these fish generally have high levels of these important omega n-3 fatty acids. EPA and DHA are very important to cell membrane formation in the brain and central nervous system, especially during foetal development. They have also been found to be beneficial to human health, there is some evidence that they reduce the risk of heart disease (Bang and Dyerberg, 1980) may suppress tumour growth (Rose, 1997) and can potentially be beneficial to diabetic patients Malasanos and Stacpoole, 1991; Berry, 1997). Consequently fish play an important role in the human diet and they have been fished for thousands of years.

In recent years, fisheries have come under pressure from excess fishing and about 47 % of the main species fished are fully exploited with about 18 % over exploited (FAO, 2002). It is thought that fisheries are currently near their sustainable limits and have a finite capacity for greater production (Buckworth, 1998). It is very difficult to get estimates of how many *salmoinds* there are in the wild and whether stocks are in decline due to the migratory life history strategies adopted by some species. It is thought that in general wild salmon stocks in the North Atlantic and Southern Europe are in decline (ICES, 2005). This problem of excess fishing has led to the implementation of management strategies that restrict the number of fish that can be caught to protect recruitment and sustainability. The decline in and restrictions applied to fisheries have prompted the use of alternative methods for producing fish in large quantities for human consumption. One such method is the practice of aquaculture.

Aquaculture

Aquaculture is the cultivation of aquatic species under controlled conditions and it has been practised for thousands of years. Recently it has become the most rapidly developing animal food production sector with a world wide distribution and it has been recognised as alleviating poverty in rural areas (FAO report 2002). Current figures show that aquaculture has increased by more than 10 % every year since 1984 (Hempel, 1993; Bell et al., 2002) and it is predicted that it will at least double over the next year (Bell et al., 2002). The FAO (2002) report that total finfish aquaculture has increased from 26.7 million tonnes in 1996 to 35.6 million tonnes in 2000. Salmonids are important aquaculture species and their culture dates back to 1741 in Germany (Hilton and Slinger, 1981), but it was only fairly recently, in 1960, that large scale Atlantic salmon culture began in Norway (Monahan 1993). The total amount of Atlantic salmon produced by aquaculture in 1970 was 294 metric tonnes (FAO, 2005a). Bell et al. (2002) predicted that by 2005 Atlantic salmon production would have reached 1 million tonnes, this was confirmed by the FAO (2005a), who reported that 1,084,740 metric tonnes of Atlantic salmon were produced in 2002. The second most productive salmonid species in aquaculture is the rainbow trout (*Oncorhynchus mykiss*), 508,662 metric tonnes were produced in 2002 (FAO, 2005b), with total salmonid production in 2002 reaching 1,799,383 metric tonnes (FAO, 2005a). In order for the aquaculture industry to keep expanding and improving it needs fish in reproductive condition that produce good quality gametes.

Timing of maturation and reproductive investment

The sequence of events that make up an organism's life is commonly referred to as its life history. Life histories are determined by the traits that influence reproduction and death (Campbell, 1996). They are subject to natural selection and as a result are very diverse and they usually involve trade offs between aspects of survival and reproduction (Campbell, 1996). There are three fundamental issues facing all organisms with respect to reproduction; what age they will mature at, how much energy they will allocate to the process with a potential cost to future survival and the number and size of gametes they will produce (Roff, 1992; Hutchings, 1997). It is in the relationships between these issues that the trade offs and life history strategies lie. There are two very basic life

history strategies that have been identified called r-selection and K-selection. Generally r-selected species or populations mature at an early age and produces large numbers of eggs/offspring but they usually have just one reproductive effort, the eggs/offspring are small, they don't invest in parental care and have short life spans. On the other hand K-selected species or populations typically live longer, invest in parental care and have multiple reproductive efforts that produce large eggs/offspring, but they mature at a later age and only produce single or small numbers of eggs/offspring. For example the house wren (*Troglodytes aedon*) matures at one or two years of age, raises 2/3 broods of 4/5 young each season (Amadon 1964), but only reach about 5-7 years of age. Contrary to this Royal and wandering albatrosses (*Diomedea exularns* and *D. epomorpha* respectively) mature at several years age, produce one young per brood every few years provide a large amount of parental care and live in excess of 20 years (Amadon 1964). Generally, unstable, hostile environments in which offspring are likely to die result in strategies based on r-selection while stable environments with predictable resources and low predation pressures tend to lead to strategies based on K-selection being used. However there is a vast amount of inter and intra species variation in the strategies employed with some combing elements of both basic strategies.

It is not just birds that are subject to variable life-history strategies, but plants, mammals, reptiles insects and fish. Fish in general are highly fecund, producing small eggs and larvae (Houde, 1997), fecundity is typically greatest in seawater where eggs are liberated into the open environment and it decreases in freshwater where there is some form of parental care (Blaxter, 1969). For example Atlantic salmon which spawn in freshwater, into nests called redds, produce smaller numbers of eggs approximately 10^3-10^4 compared to cod (*Gadus morhus*) that release approximately 20-90 x 10^5 eggs into the pelagic environment (Blaxter, 1969). Salmonids also tend to produce the largest eggs (Wooton, 1990), approximately 5-6 mm compared to 1.1-1.6 mm for cod (Blaxter, 1969). The age at maturity of these two species are also variable, female cod generally mature around 5 years of age with males maturing slightly earlier, while Atlantic salmon can mature at 1 to several years of age with both being repeat spawners. Both cod and salmon therefore employ aspects of both r and K selected strategies. The life histories strategies of the salmonids, in particular the Atlantic salmon, are complex and very variable and can involve migrations between freshwater and seawater.

Timing of maturation in salmonids

The fish family *salmonidae*, more commonly referred to as salmonids, is made up of about 66 species that can be found in Northern Hemisphere fresh and marine water environments, with some having an anadromous existence (Nelson, 1994). They are one of the most extensively studied families of fish, especially with respect to life history theory. One of the most prominently studied species is the Atlantic salmon, due to the intricate nature of the life history strategies they demonstrate; as a result, a large amount of research has been conducted on this species. An area of Atlantic salmon life history theory that is still not completely understood is age at maturity and what triggers it. Atlantic salmon have complex life history strategies that can be confined to freshwater or involve a migratory phase to seawater. They hatch in freshwater and, after a period of initial feeding are called fry, they are then referred to as parr after one year in freshwater. Some individuals, mainly males, mature sexually as parr after one or several years (Metcalfe et al., 1988); others undergo a process known as smoltification that enables them to survive in sea water to which they migrate, becoming known as smolts. The length of time these fish spend in freshwater before becoming smolts is also variable, ranging from one year to several. What determines smoltification is believed to be dependent upon the attainment of a predetermined threshold size and energy store at a critical 'window', thought to be around the midsummer (Thorpe 1989; Thorpe and Metcalfe 1998). The migration to sea is believed to give the fish the opportunity to increase their energy reserves to a greater extent than if they remained in fresh water as, the marine environment is richer in nutrients than the freshwater one.

The length of time spent at sea is also variable; some fish return after one year only while others can remain there for several years. It is thought that maturation is repressed or inhibited, what causes the inhibitor to be removed and maturation to begin is as yet not known (Thorpe et al., 1998). Like smolting, it is believed that individuals initiate maturation upon the attainment of some predetermined threshold of size, growth and energy reserves (Rowe at al., 1991; Herbringer and Friar 1992; Thorpe 1994; Bergulund, 1995; Friedland and Hass 1996), again at a critical 'window'. It has been suggested that this 'window' occurs in the autumn of the year prior to the completion of maturation and that there is a second 'maturation window' in the intervening spring at

which they have an opportunity to arrest its development if there is not the energy reserves to complete the process (Rowe and Thorpe 1990a; Rowe and Thorpe 1990b; Prevost et al., 1992; Berglund 1995; Whalen and Parish 1999). As a result of these diverse strategies there is a large amount of variation in age at maturity of Atlantic salmon. Much of the research into maturation and the factors involved has been conducted using male parr at the population level (Rowe and Thorpe, 1990a; Rowe and Thorpe 1990b; Berglund, 1992; Prevost et al., 1992 Whalen and Parish, 1999). Even though Atlantic salmon have been well studied, there is still a lot of information to be gleaned about their life history strategies in terms of age at maturity, especially in individual post-smolt females and males.

Reproductive investment and gamete quality in salmonids

One aspect of life history strategies in fish that is more comprehensively understood than timing of maturation is that of egg number and size, which together will be termed reproductive investment in this thesis. A trade off between egg number and size is common in many organisms to try and increase reproductive success, with either large numbers of small eggs or small numbers or large eggs being produced. A negative relationship has been identified between egg number and egg size in many fresh and marine water species (Elgar 1990; Fleming 1996). Greater numbers of eggs means increased numbers of offspring are produced, but small egg size may decrease survival potential of the offspring. It has been shown that larger eggs produce larger offspring, which potentially increases their chances of survival, as they are able to compete more successfully for resources, though there is evidence that this difference in size can be overcome after a few weeks of feeding (Bromage and Springate 1992). A positive correlation has been shown to exist between female body size at the time of maturation and fecundity and egg size, larger females being more productive than their smaller counterparts (Springate and Bromage, 1985; Munro, 1990; Bromage, 1996; Bromage, 2001). When egg number and size is determined is not well understood, but there is evidence that female gamete development occurs some time in the preceding spring (Tyler and Sumpter, 1996; Stead et al., 1999). Another important aspect of reproduction is the quality of the gametes that are produced.

Gamete quality in salmonids can be measured in a number of ways, from simply egg number and weight, sperm number, frequency and percentage of motility, to the survival of offspring to the first feeding stage of development and beyond. Egg number and size are the most commonly used measures, but they do not give a clear idea of offspring survival. For a number of species in culture, including bass (*Decentrarchus labrax*), bream (*Sparus aurata*), turbot (*Scopthalmus maximus*) and halibut (*Hippoglossus hippoglossus*), there can generally be post-weaning losses of about 95 %, with those for salmonids being about 75 % (Bromage et al., 1992; Bromage, 1996). There is little information on egg quality up to the first feeding stage especially in individual females. Eggs are usually pooled as it is expensive to rear them in individual batches all the way through to first feeding (Bromage, 1996). The sustainability, quality and future development of aquaculture is dependent not only upon the reproductive investment of the broodstock, but also the quality of the gametes they produce (Bromage et al., 1992). A more comprehensive understanding of reproductive investment and gamete quality for individuals would be interesting to life history biologists and beneficial to the evolution of the aquaculture industry in terms of broodstock productivity.

Broodstock

Broodstock production is very important to the aquaculture industry. Bromage et al. (1992) estimated that more than 3 billion eggs were required per annum to maintain the 300,000t production of Rainbow trout. If broodstock productivity is poor, this will have a negative impact upon the expansion of the industry (Brooks et al., 1997). Secondary sexual characteristics that indicate which fish are maturing do not become apparent till towards the end of the maturation cycle and give no indication of reproductive potential or gamete quality. To date, the alternative methods used to detect maturation status are invasive, expensive, sometimes restricted to late in the maturation process and again do not give an indication of reproductive investment and quality. Being able to make predictions about which fish will mature and their reproductive potential as early as is possible could potentially reduce the costs of producing fish that will be of no benefit to the farmer and improving the efficiency of broodstock production. This thesis was conceived with the intention of investigating age at maturity, reproductive investment and gamete quality in salmonids. The work was part funded by an industrial sponsor

(Nutreco, a fish feed company) and as a result also included more diverse aims that involved aspects of fish nutrition in farmed salmonids.

Nutrition of farmed fish

The aim of aquaculture is to produce a high quality product as quickly and cheaply as possible and this is largely dependent upon supplying the fish with a high quality diet for the least cost (Jackson 1991). The quality of fish is measured in a number of ways, from levels of fat, astaxanthin (pigment) and moisture in the fillet to the texture (the flesh should be firm and elastic) and the amount and extent of gaping, when there is separation of the fillet segments. Criteria of quality is dependent upon the market, as different markets have different requirements. Generally the flesh is required to be well, but not excessively pigmented and not too fatty or oily. The most costly aspect of aquaculture is food production, amounting to 50-60 % of total culture costs (Sveier and Lied, 1998). Salmonids are chiefly carnivorous, the natural diet of Atlantic salmon is mainly composed of Krill, shrimp, herring, capelin, sand eels and large zooplankton, while that of Rainbow trout is mainly composed of crayfish, aquatic insects, molluscs, crustaceans and other small fish. As a result their diets are high in protein; fishmeal is the most common source of protein for fish diets. Production of fishmeal is one of the major expenses of food production and it is becoming progressively more so (Carter and Hauler 2000). Using high levels of protein in diets can lead to environmental concerns through the release of excess nitrogen in fish discharges and the break down of food that has not been consumed (Rasmussen and Osterfield 2000). In response to these concerns, a large amount of research has been conducted investigating ways of reducing protein levels in diets and making its utilisation more efficient. Research has shown that fish posses the ability to use protein more efficiently if the lipid level in the diet is increased while maintaining the level of protein at that required for maintenance and protein accretion, but no more, this effect is know as 'protein sparing'. A number of studies have shown that increasing the level of dietary lipids does not compromise growth of salmonids (Beamish and Medland, 1986; Weatherup et al., 1997; Jobling et al., 1998). As a result, the levels of lipids in salmonid diets have increased from 8-12 % to 30 or even 40 % (Stoebakken 2002). There is however a complex size and species dependent relationship between the levels of lipid presented in the diet and the levels that are deposited in the flesh (Regost et al., 2001). Some studies have show increased dietary

lipid level resulted in increased fat deposition in the body and some have shown no relationship to exist between the two (Reitz et al., 1978; Einum and Skrede 1998; Jobling at al., 1998; Regost et al., 2001). More studies are therefore required to better understand this relationship in different species of different sizes. Very few studies have been conducted on the manipulation of dietary lipid and protein levels and the effects this may have on growth and flesh quality in large (in excess of 1 kg) rainbow trout.

Fish oil is the main dietary lipid source and, like fish meal, is harvested from fisheries, is expensive to produce and is a finite resource. Therefore there is also a concerted effort into investigating the replacement of fish oils with alternatives. To date, research has focused upon using vegetable oil, as it is an easily available and renewable resource. Vegetable oils do not typically contain any of the omega n-3 fatty acids that characterise fish oils. There are however some fish species (mainly freshwater), including Atlantic salmon that can synthesis these fatty acids from their metabolic precursors. This is probably due to freshwater algae having lower levels of EPA and DHA compared to marine algae, obliging the fish to be able to synthesis the omega n-3 fatty acids. This process may have been lost in marine fish; such an ability being redundant given the abundant supply they are exposed too (Sargent et al., 2002). The EPA and DHA precursor, Linolenic acid, can be found in some vegetable oils, particularly rapeseed oil, making it a good alternative choice. It has been shown that rapeseed oil can be used as a partial or, in some cases, full replacement for fish oil without compromising growth in some Atlantic salmon (Bell et al., 2003). Although Bell et al. (2003) found that, when greater than 50 % replacements were used, there was a decrease in the levels of DHA and EPA in the flesh. Lipids are one of the main components of fish eggs (Brooks et al., 1997) and in most marine fish eggs the lipid fraction is rich in omega n-3 fatty acids (Sargent et al., 2002). As DHA and EPA are important to foetal development, using rapeseed oil in broodstock diets could have adverse effect on egg quality, especially as it is know that dietary nutrients influence fecundity and egg composition in fish (Mourente and Odriozola 1990). There is little information regarding the use of rapeseed oil in Atlantic salmon broodstock diets.

Aims of the thesis

The overall aim of the work described in this thesis was broad and involved investigating aspects of nutrition in farmed salmonids, life history strategies and the possibility of predicting which fish are likely to mature and their reproductive potential. There is still a large amount of information to be gleaned on when and what initiates maturation in post-smolt Atlantic salmon, both females and males. Being able to predict which fish will mature and their reproductive potential are important to broodstock management and the sustainability and expansion of aquaculture. The autumn of the year prior to maturation has been suggested as the point at which maturation is initiated and could therefore provide an opportunity to make an early prediction about future maturation status. With this in mind the aim of chapter 2 was to clarifying the existence of the autumn maturation window and to determine if it is possible to make predictions about which post smolt Atlantic salmon, male and female, will mature using body condition and growth measurements taken during the year preceding maturation. As well as to determine if these measurements have an effect upon reproductive investment and quality.

Recently fish farmers have been using ultrasound technology to differentiate between the genders in mature individuals in a number of species including Atlantic halibut (*Hippoglossus hippoglossus*) (Shields et al., 1993), Atlantic cod, coho salmon (*Oncorhynchus kisutch*) (Martin et al., 1983), Atlantic salmon and rainbow trout (Riemers et al., 1987; Martin-Robichaud and Rommens 2001). Ultrasound is the use of ultrasonic waves to visualise internal tissue structures in a quick and non-invasive manor. Developing this technique to measure gonadal status of individuals in a quick and non-invasive manner could be beneficial in life history studies, allowing gonad development to be followed, and broodstock management, allowing predictions to be made about productivity. The aim of chapter 3 was to determine if it was possible to measure gonad dimensions in both immature and mature Atlantic salmon and mature rainbow trout and consequently predict which fish will mature and their reproductive potential.

Reducing the costs and improving the efficiencies with which food is utilised by the fish without compromising growth and quality are important for the environment and

maximising aquaculture production. Chapter 4 describes an investigation to determine if vegetable oil can be used as a partial replacement for fish oil in broodstock diets for Atlantic salmon without compromising egg quality. Investigating the effect of replacing protein with lipid in the diet of large (> 1 kg) rainbow trout on growth, food utilisation and fillet fat levels was the aim of chapter 5. In retrospect it was discovered that the rainbow trout used in the study described in chapter 5 had come into reproductive condition. As a result, how maturation status influenced growth and flesh quality is described in chapter 6.

In chapter 7 I will summarise the findings of this thesis and discuss the wide reaching implications these findings have for life history studies and aquaculture.

Chapter 2

Body size, condition and growth rates of maturing Atlantic salmon (*Salmo salar*); implications for reproductive investment and gamete quality

Summary

Post-smolt Atlantic salmon (2-year old) with a range of body weights in September 2003 were weighed and measured repeatedly through the subsequent year to identify predictors of maturity status the following winter, reproductive investment and gamete quality. Mature males and females were heavier than their immature counter parts in September (2003), May and June (2004) and had higher specific growth rates (SGR) from September to May. Immature females had higher SGR from May to June than mature females, but immature and mature males had similar growths during this period. The best predictor of female maturity was weight in May; no single significant predictor was found for males. Female body weight in June had the strongest relationship with egg number and females that matured later in the season produced more eggs. Neither egg quality (measured as survival rates to eyeing, hatching and first feeding) nor the weight of sperm produced was influenced by any of the body weight or SGR measurements.

Introduction

Age of maturation in salmonids

Salmonids have complex and variable life history strategies. For example in a population of Atlantic salmon (*Salmo salar*) some individuals, mainly males, mature as parr while others delay maturation until after smolting and return as mature adults after varying numbers of years at sea (Metcalfe et al., 1988). This leads to intra- and inter- population variation in the number of fish that complete a reproductive cycle in any given year (Myers et al., 1986; Berglund, 1992; Herbinger and Frias, 1992; Kadri et al., 1996; Silverstein et al., 1998). The main environmental cues involved in initiating the production of gametes have been identified as photoperiod and temperature, but the initiation of the reproductive cycle is believed to occur a year in advance of its completion.

The physiological cues that determine whether an individual fish will mature in a given year are not comprehensively understood. It is believed maturation is initiated upon the attainment of some unknown combination of size, growth and energy store thresholds (Rowe et al., 1991; Thorpe, 1994; Berglund, 1995; Silverstein et al., 1997; Silverstein et al., 1998; Silverstein et al., 1999). When these thresholds must be attained have tentatively been identified as being in the autumn of the year prior to maturation and the subsequent spring and they have been entitled maturation windows. The maturation cycle is thought to be initiated at an autumn maturation window (Herbinger and Friar, 1992; Thorpe, 1994; Friedland and Hass, 1996; Silverstein et al., 1998; Thorpe and Metcalfe, 1998). Silverstein et al. (1998) reported that populations of male Chinook salmon (*Oncorhynchus tshawytscha*) parr with a greater mean size in a given September experienced a significantly higher incidence of maturation one year later. The autumn maturation 'window' has not been as well studied as the spring window. A number of studies conducted at the individual and population level, using male Atlantic and Baltic salmon (*S. salar*) parr have found that the size and growth rate experienced during the spring are important in determining the number of fish that go on to mature that year (Rowe and Thorpe, 1990a; Rowe and Thorpe,

1990b; Berglund, 1992; Prevost et al., 1992; Berglund, 1995; Whalen and Parish, 1999). Rowe and Thorpe (1990a and b) found that male *Salmo salar* parr that experience higher growth rates in the spring were more likely to mature and that maturation could be suppressed by inducing low growth during this period. They came to the conclusion that maturation is not initiated at this point, but it can be arrested if an adequate growth rate is not maintained.

Reproductive investment and gamete quality

Reproductive investment and gamete quality are also highly variable within populations even when individuals are subjected to similar light, temperature and nutritional regimes (Brooks et al., 1997; Bromage, 2001). The causative factors for such variation are as yet not fully understood. There are no universal criteria for assessing gamete quality. In females, this can be evaluated by measuring egg size and number or the percentage of eggs that survive to each of the developmental stages entitled eyeing, hatching, swim-up and first feeding (Springate et al.,1984; Craik and Harvey, 1984; Marteinsdottir and Able, 1992). Fish size at maturation has been identified as an important factor in determining egg size and fecundity, a positive correlation existing for both (Thorpe et al., 1984; Springate and Bromage, 1985; Munro, 1990; Bromage, 1996; Bromage, 2001). Fish size in the spring could also have an effect upon egg quality, as there is evidence to suggest that egg development commences around this time. Levels of the sex steroids linked to gamete development increase in salmonids around May of the year they mature (Stead et al., 1999). In rainbow trout, vitellogenesis, the principle event of oocyte growth, occurs approximately nine months prior to ovulation (Tyler and Sumpter, 1996). There is little information concerning the quality of eggs from individual fish, most results being based upon pooled samples and more information based upon individual performance is required (Bromage, 1996).

Gamete quality in males it is usually determined by the number of spermatozoas present, their percentage motility and, or the intensity and length of motility (Saad et al., 1988; Bromage, 1996). An important determinant of fitness in male salmonids is their ability to compete for access to egg-laying females. Body size

and the length of the hooked snout (kype) are important in the sexual selection of male salmonids (Quinn and Foote, 1994). Body weight and kype length therefore represent important aspects of reproductive investment in male salmonids.

Aims of the study

This study was run in conjunction with another involving dietary replacement of fish oil with rapeseed oil (chapter 4) that also required fish in reproductive condition. The aim of the present study was to track individually identified Atlantic salmon to investigate;

- Size, growth rate and body condition of maturing and non-maturing female and male individuals during the year leading up to the completion of their first reproductive cycle
- Predictors for maturity in individual adult females and males, with particular reference to the autumn 'maturation window'
- Predictors of reproductive investment (measured as egg number and size)
- Predictors of gamete quality (measured as survival rates at eyeing, hatching and first feeding)

Materials and Methods

There were a number of events that took place during this experiment and they have been summarised in a diagram (Fig. 2.1).

Experimental design and general husbandry

At the end of September 2002, 315 2-year old Atlantic salmon (*Salmo salar*) (4577 g ± 45 SEM), of the AquaGen Standard strain (supplied by AquaGen Hemme) were weighed and measured. The fish were selected to produce a large size distribution over which to test the hypothesis. Every fish had unique Visible Implant Tags (Northwest Marine Technologies, Shaw Island) inserted into the transparent tissue behind their left and right eyes, two tags being used to increase

Sep-02	START OF TRIAL	FIRST SAMPLING	AUTUMN MATURATION WINDOW
May-03		SECOND SAMPLING	SPRING MATURATION
Jun-03		THIRD SAMPLING	
Sep-03		TRANSFERRED TO FRESHWATER	
Oct-03		FIRST STRIPPING AND EGGS FIRTILISED	
Nov-03		SECOND STRIPPING	
Mar-04	END OF TRIAL	FRY WEIGHED AND COUNTED	

Figure 2.1. A summary of the main events that took place during this trial.

the chances of retaining identification. The fish were transferred to three sea cages (5 x 5 x 5 m) with 105 (4437.3 g ±SEM 82.1), 104 (4628.5 g ±SEM 76.4) and 106 (4666.0 g ± 74.3 SEM) in each. There was no significant difference in weight between the cages (one-way ANOVA $P = 0.084$, $F_{2,314} = 2.50$).

The study that was run in conjunction with this one required the use of two diets, that differed in the source of 50% of their oil inclusion, the experimental diet (rapeseed oil) was fed to two of the cages and the control diet (fish oil) was fed to the remaining cage. The diet compositions are described in chapter 4, as are the results related to this experiment. The fish were kept under ambient conditions at the Nutreco Aquaculture Research Centre, Lerang, Jørpeland (59° 01.34′N, 6° 02.50′E). The average water temperature was 10 °C (min.: 4 °C, max.: 20 °C), salinity 27 g/kg (min.: 18 g/kg, max.: 32 g/kg) and oxygen level 7.5 mg/l (min.: 6.5 mg/l, max.: 9 mg/l) at a depth of 4 m.

Sampling

During the second week in November 2002, the fish were starved for two days and anaesthetised (MS222, 0.05 g/l) to check tag retention and to allow replacement tags to be inserted if required. The fish were weighed and measured at the beginning of May 2003 and at the end of June, at which point feeding was terminated. The weights of 3 females and 4 males in May and 9 females and 14 males in June were not recorded as their tags could not be read or had been lost. Also in June 34 males were too big for the scale and no weight could be recorded.

Transfer to freshwater

At the end of September 2003 the mature fish from all cages (identified by their colour and in the case of males the kype development) were transferred to a circular 8m-land tank (84 m^3), with a salinity of 30.70 mg/l. This was reduced to 19 mg/l after two days and then to 0.5 mg/l after a further three days. The fish were identifiable by their unique tags. For stocking density reasons only 200 fish in total could be transferred. As a result, of the 89 mature fish in the fish oil cage

18 random males and one female in poor condition were not transferred. In one cages fed the rapeseed oil diet 14 random males out of a total of 94 mature fish were not transferred and in the other cage fed the rapeseed oil diet 24 random males and one female were not transferred from a total of 75 mature fish. All fish that were identified as being immature were removed at this point, killed (with a lethal dose of MS222) and dissected to confirm their maturation status and gender. Due to oversights the gender and maturity status had not been recorded for 21 individuals and the over all mortality was 23 individuals.

Fertilisation

On the 28th of October the females were tested for ripeness, indicated by the free production of eggs when they were gently squeezed in the anal area. The fish were left for 3 days, as it is believed optimal egg quality occurs between 3-6 days after the commencement of ovulation (Bromage, 1996). All females (N = 73, 28 from the fish oil cage and 21 and 24 from each rapeseed oil cage) that were ovulating were sacrificed on the 31st of October (by brain destruction), bled and hung by the tail for a few hours to allow the blood to drain away from the eggs, preventing contamination. The females were then weighed, measured and identified by their tags. An incision was made from the anus to the buccal cavity of each female and the eggs were collected in individual receptacles and kept as individual batches. Each fish was then re-weighed to establish the stripped weight. The eggs from these females will be referred to from this point as belonging to the first stripping.

The eggs from 25 of these individuals were selected for fertilisation. This number was comprised of nine individuals in one of the rapeseed oil cages, and eight individuals in each of the remaining two cages. Selection was based upon September body weight. Cumulative frequency graphs were used to approximate size categories of large, medium and small. The aim was to use the eggs from nine individuals (three large, medium and small sized females) within each cage. This was the case for one of the cages fed the rapeseed oil diet (N = 9), but two small individuals only were available from the other two cages (N = 8). Any batch of eggs found to be contaminated with blood was rinsed by gently adding a

small amount of a sterile saline solution and gently swilling the blood to the surface and then lifting out the clots until only a minimal amount was left.

All the males were anaesthetised, weighed, measured and stripped by repeatedly squeezing the posterior flanks from in front of the pectoral fin to the anal opening until no more milt was expelled. The milt was collected in individual receptacles that were placed immediately into a fridge set at 0 °C. Once all the milt was collected each sample was checked for motility under a microscope to make sure it was viable. A pool of milt for each of the three cages was created using three random males from that treatment, to ensure there was enough milt to fertilise all the respective females. 3 ml of the pooled milt was added from a sterile 5 ml plastic syringe to each individual batch of eggs from the corresponding cage. A sterile hand was used to gently mix the eggs and milt thoroughly for one minute; they were then left for a further minute and subsequently rinsed with sterile saline solution, as previously. A sub-sample of eggs were removed from each batch and rinsed in the pH buffered iodine disinfectant Buffodine (Sterner Fish Tech A/S) for 10 min, then a further sub-sample (100 ml) from each batch of the disinfected eggs were transferred to freshwater, for hardening and development.

Husbandry of eggs and fry

A common source of water supplied two parallel runways (220 x 43 x 18 cm) that contained four separate sections with sides made of perforated plastic, to allow the water to flow through. Each section was comprised of four individual compartments (22 x 20 x 15 cm) also constructed with perforated plastic. The fertilised eggs from each female were held in an individual compartment. The eggs and, after hatching, the fry were kept in continual darkness to imitate the natural conditions experienced in the redd. Water temperature and pH were maintained at 6.83 °C (± 0.06 SEM) and 6.38 (± 0.01 SEM), respectively. The eggs were bathed in the anti-fugal treatment Pyceze (Vericore Ltd) regularly over a 19 week period according to the directions from the manufacture.

Dead eggs were not picked till the day after eyeing commenced (at 294-degree days), as the eggs are very delicate prior to this point. Dead eggs were removed

and counted and defined as not reaching eyeing. The remaining eggs were checked regularly and any mortalities were removed and counted, until hatching began at 521-degree days. Eggs that did not hatch, showed no signs of a developing embryo (had not eyed) and were still translucent were defined as unfertilised and counted. After hatching dead yolk-sac fry were removed and counted. At 413-degree days post hatching (the recommended number of degree days to start feeding this strain of salmon) when there were no visual external signs of the yolk sac, all fry were sacrificed (using a lethal dose of MS222). They were counted and defined as surviving to first feeding. Samples of 20 individuals from each female were blotted dry and weighed on an individual basis. The egg batches from two of the females from one of the rapeseed oil cages were severely affected by a fungal infection and were removed from the data sets.

Reproductive investment and gamete quality

The remaining mature females from all cages (N = 43) were killed on the 11th November 2003 (by brain destruction, these individuals were not bled) weighed and measured. Due to logistical constraints only 30 were stripped of their eggs and re-weighed. The eggs collected from these females will from now on be referred to as the 2nd stripping. Any immature fish that had been mis-identified and transferred were dissected to confirm their sex.

A 200-hole egg plate counter was used to count the total number of eggs from each fertilised and second stripping female. The number of eggs that had been removed for fertilisation from each female was added to their egg count in retrospect once the exact number was known at the end of the trial. Sub-samples of 20 eggs from each female were blotted dry to remove the ovarian fluid and weighed on an individual basis and the mean weight established.

A number of quality survival parameters were calculated, for each fertilised batch of eggs, using the equations below:

Total egg no. = no. unfertilised + no. dead before eyeing + no. dead before hatchings + no. dead fry + no. fry at the end

*Fertilisation percentage = ((Total egg no. – no. unfertilised)/Total no.) *100*

*Eyed percentage = (Total egg no. – (no. unfertilised + no. dead before eyeing)/Total egg no.)*100*

*Hatched percentage = (Total egg no. – (no. unfertilised + no. dead before eyeing + no. dead before hatching)/Total egg no.)*100*

*First feeding percentage = (no. fry at the end/Total egg no.)*100*

The length of the kype in each mature male was then measured from the end to the point it merges with the head, and the width was measured across the top of the kype just in front of the head on 11th November 2003 and were stripped for milt quality assessment. The milt (sperm and seminal fluid) sample they produced was weighed (N = 55). A 1.5 ml sub-sample was frozen at –20 °C. During transportation from Norway to Glasgow university 21 samples were compromised leaving a total sample size N= 34. The sample was defrosted and spun for 30 min in a Hawksley micro-haematocrit centrifuge. The spermatocrit (amount of sperm) was established as a percentage of the total milt sample using a Hawksley reader. The weight of the sperm in the sample was then derived using the equation:

*(sample weight/100)*spermatocrit*

Data analysis

The Specific Growth Rate (SGR) was calculated from September-May (Sep.-May) and May-June for every individual using the equation:

*SGR = ((LN(Fwt)-LN(Iwt))/no.days)*100*

Fwt = Final Weight Iwt = Initial Weight

Size, growth rates and condition of mature or immature fish during the year preceding completion of the reproductive cycle

The effect of diet and cage upon female growth is described and commented upon in chapter 4. Diet did not influence male growth from September to May (Kruskal-Wallis P = 0.531, H = 0.39) or from May to June (Kruskal-Wallis P = 0.351, H = 0.87), but cage did for the fish fed the rapeseed oil diet (Kruskal-Wallis SGR September to May, P = 0.033, H = 4.56, May to June P = 0.003, H = 8.78), the reasons for this effect are discussed (chapter 4). For each gender one-way ANOVA and with data that could not be normalised, using arcsine, square root or logarithm transformations, Kruskal-Wallis analyses were performed to determine the effects of maturity status at the end of the trial on the body condition variables September, May, June weights and condition factors, and the two SGRs calculated. June weight was log10 transformed to produce a normal distribution for the male fish. For each maturation status one-way ANOVAs and, where applicable, Kruskal-Wallis tests were performed to determine if gender had any effects on the body condition and SGR variables. Paired t-tests and, for data that could not be normalised with transformations, Mann-Whitney tests, were used to determine if there were differences in the weight and SGR measurements, for both immature and mature males and females. Spearman's rank correlations, as the data could not be normalised with transformations, were then conducted on the two SGR measurements for immature and mature males and females.

Predictors of maturation

Binary logistic regressions were used to determine if September, May and June weight predicted maturation status at the end of the trial, for both genders. Only individuals that had all three of these weight measurements were used (Females, N = 130, Males, N = 81).

Predictors of reproductive investment

The effects of cage and diet upon egg number and weight for the first stripping

are discussed in chapter 4. Neither cage nor diet influenced egg number in the second stripping (nested ANOVA cage (diet), $P = 0.500$, $F_{1,29} = 0.47$, one-way ANOVA diet, $P = 0.707$, $F_{1,29} = 0.14$). Nor did they influence egg weight (nested ANOVA cage (diet), $P = 0.647$, $F_{1,29} = 0.21$, one-way ANOVA diet $P = 0.281$ $F_{1,29} = 1.12$). Linear regressions and for data that could not be normalised with transformations, Spearman's rank correlations were performed with egg number and weight and each morphological and SGR variable. Sequential bonferroni corrections were performed on the series of results produced for egg number and weight according to Rice (1989), to decrease the possibility that any of the results were positive due to chance.

Predictors of gamete quality

The effect of cage and diet upon egg quality measurements are discussed in chapter 4 with no effect detected for either, nor did they influence the weight of sperm produced (nested ANOVA cage (diet), $P = 0.788$, $F_{1,31} = 0.07$; one-way ANOVA diet, $P = 0.429$, $F_{1,31} = 0.64$). Linear regressions and for data that could not be normalised with transformations, Spearman's rank correlations, were performed between each of the egg quality variables, fry weight, kype length and width and the weight of sperm and each morphological and SGR variable. Again sequential bonferroni corrections were used on the series of results produced for each gamete quality variable. Multiple regressions were used if more than one variable was significant, to determine which had the greater influence. One-way ANOVAs were used to establish if egg number and egg weight and female September, May and June weight differed between the first and second stripped fish.

Results

Size, growth rates and condition of mature or immature fish during the year preceding completion of the reproductive cycle

Not all of the fish matured; 17.7 % of females and 6.9 % of males remained immature. Condition factor in September for both genders and SGR from May to

June in males were the only body condition variables not influenced by maturation status (Table 2.1, Fig. 2.2). In both genders, all significant variables were greater in mature fish compared to immature fish, except for the SGR from May to June in females, which was greater in the immature individuals (Fig. 2.2).

All fish significantly gained weight from September to May to June (Table 2.2, Fig 2.2). Overall the immature females had significantly higher SGRs in the period May to June (0.34 %/day ± 0.03 SEM) compared to September to May (0.17 %/day ± 0.01 SEM). Mature females had significantly higher SGRs in May to June (0.26 %/day ± 0.01 SEM) compared to September to May (0.20 %/day ± 0.01 SEM). There were no significant differences in the growth of either immature (September to May, 0.14 %/day ± 0.04 SEM, May to June 0.35 %/day ± 0.11 SEM) or mature males (September to May, 0.26 %/day ± 0.01 SEM; May to June 0.28 %/day ± 0.02 SEM) between the two periods. There was, however, substantial variation in growth rates in the mature, but not immature fish. Spearman's rank correlations on the individual data (Fig. 2.3) showed that there were significant negative relationships between the SGRs of the two periods in mature males ($P = 0.001$, $rs = -0.384$) and females ($P = 0.001$, $rs = -0.308$). No significant relationships were found in the immature fish, (males, $P = 0.57$, $rs = -0.238$; females, $P = 0.93$, $rs = -0.021$).

Mature males had significantly higher weights in September, May and June and had higher SGRs from September to May compared to the mature females. Females had higher condition factors in June. Both genders had similar SGR from May-June and condition factors in September and May (Table 2.3, Fig. 2.2). None of the body condition or SGR variables were significantly different between the genders in the immature fish, although condition factor in June was close to the <0.05 significance acceptance level (Table 2.3, Fig. 2.2).

Predictors for maturing

The binary logistic regression model for the females (mature N = 108, immature N = 22) was significant ($P = <0.001$, $G = 21.85$) and showed May weight to be the strongest and only factor in predicting maturity status ($P = 0.004$, $Z = 2.89$;

September weight P = 0.193, Z = 1.30; June weight P = 0.226, Z = -1.21). For the males (mature N = 73, immature N = 8), the model was significant (P = 0.002, G = 14.84), but no one factor was prominent in predicting maturity status (September weight P = 0.644, Z = -0.46; May weight P = 0.644; Z = 0.04; June weight P = 0.269, Z = 1.11). This could be due to the small sample size of immature fish.

Predictors of reproductive investment

The number of eggs produced by the females from the second stripping (13562 ± 316 SEM) was significantly greater than that from the first stripping (12003 ± 348 SEM) (one-way ANOVA P = 0.002, $F_{1,54}$ = 10.99). Egg number was not related to egg weight in either stripping (Pearson's correlation first stripping P = 0.404, r = -0.174, Rsq = 0.030; second stripping P = 0.265, r = 0.210, Rsq = 0.044) and the only body condition or SGR variables related to egg number were stripped weight and weight in May and June (Table 2.4). Multiple regressions showed that for females in the first stripping, weight in June was the strongest predictor of egg number and in the second stripping weight in June and stripped weight were the strongest predictors (Table 2.5). There was no significant difference in mean egg weight between the two strippings (one-way ANOVA P = 0.321, $F_{1,54}$= 1.00; 1st stripping 0.103 g ± 0.008 SEM; 2nd stripping 0.101 g ± 0.001 SEM). Mean egg weight was not related to any of the variables in the first stripping, but was positively correlated to stripped weight and weight and condition factor in June in the second (Table 2.4). Multiple regression showed these factors to be equally significant predictors of mean egg weight (Table 2.5).

There was a significant difference in the weight of the stripped females between the two strippings, the latter being heavier (one-way ANOVA P = 0.017, $F_{1,48}$ = 6.13; 1st stripping, 4617.8 g ± 127 SEM; 2nd stripping, 5057.4 g ± 118), but not in the weights from September (one-way ANOVA P = 0.780, $F_{1,54}$ = 0.08; 1st stripping, 4447.1 g ± 148 SEM; 2nd stripping, 4397.8 g ± 104 SEM), May (one-way ANOVA P = 0.543, $F_{1,54}$ = 0.37; 1st stripping, 6637.9 g ± 175 SEM; 2nd stripping, 6779.3 g ± 152 SEM) or June (one-way ANOVA P = 0.504, $F_{1,50}$ = 0.45; 1st stripping, 7675.5 g ± 182 SEM; 2nd stripping, 7851.4 g ± 180 SEM).

However, it must be remembered that the females of the first stripping had been bled, while those of the second had not. The females from the first stripping had lower SGR from September to May (0.18 %/day ± 0.02 SEM) when compared to the females from the second stripping (0.28 %/day ± 0.03 SEM) (one-way ANOVA P = 0.004, $F_{1,53}$ = 9.24). The reversed was true for the SGR from May-June (first stripping 0.27 %/day ± 0.02 SEM, second stripping 0.21 %/day ± 0.01 SEM, one-way ANOVA P = 0.016, $F_{1,50}$ = 6.24).

Neither weight of sperm produced nor kype width were related to any of the variables, the relationships with kype width that were significant becoming non-significant after a sequential Bonferroni correction (Table 2.6). Kype length was related to the May, June and stripped weight (Table 2.6).

Predictors of gamete quality

The survival rates at each post fertilisation development stage were high and remained constant from stage to stage (Table 2.7). None of these egg quality variables were correlated to the morphological or SGR variables; the significant relationships between fertilisation rate, September weight and SGR from September-June became non-significant after a Bonferroni correction (Table 2.8). Fry weight was positively related to egg weight (P = <0.001, r = 0.780, Rsq = 0.608), but to none of the other variables (Table 2.4).

Table 2.1. The results of the one-way ANOVA (F) and Kruskal-Wallis (in italics, H) tests between each listed variable and maturation status for both female and male individuals.

	Female			Male		
Variable	P	F/H	N	P	F/H	N
September Weight	0.035	4.54	145	0.025	5.13	129
May Weight	<0.001	24.97	144	*<0.001*	*15.64*	*126*
June Weight	<0.001	13.77	138	<0.001	20.66	82
September Condition Factor	*0.500*	*0.46*	*141*	*0.561*	*0.34*	*128*
May Condition Factor	*<0.001*	*14.65*	*144*	*0.003*	*9.05*	*126*
June Condition Factor	<0.001	14.96	138	<0.001	15.12	82
Sept.-May SGR	*0.027*	*4.92*	*142*	*0.001*	*11.35*	*125*
May-June SGR	*<0.001*	*13.36*	*136*	0.962	0.000	82

Table 2.2. The results of the Paired t-tests (T) and Wilcoxon tests (in italics, wilcoxon statistic) used to determine if there were any changes in weight and growth from September to May and then May to June for both genders of immature and mature fish.

	Mature				Immature			
	Female		Male		Female		Male	
Difference between	P	T/ Wilcoxon statitic	P	Wilcoxon statitic	P	T/ Wilcoxon statitic	P	T/ Wilcoxon statitic
(weight) September to May	<0.001	-27.57	*<0.001*	*6900*	<0.001	-15.18	0.003	-4.40
(weight) May to June	<0.001	-19.44	*<0.001*	*2700*	<0.001	-13.71	0.002	-4.42
(SGR) September-May to May-June	*<0.001*	*6327*	*0.676*	*1427*	*<0.001*	*246*	*0.183*	*28*

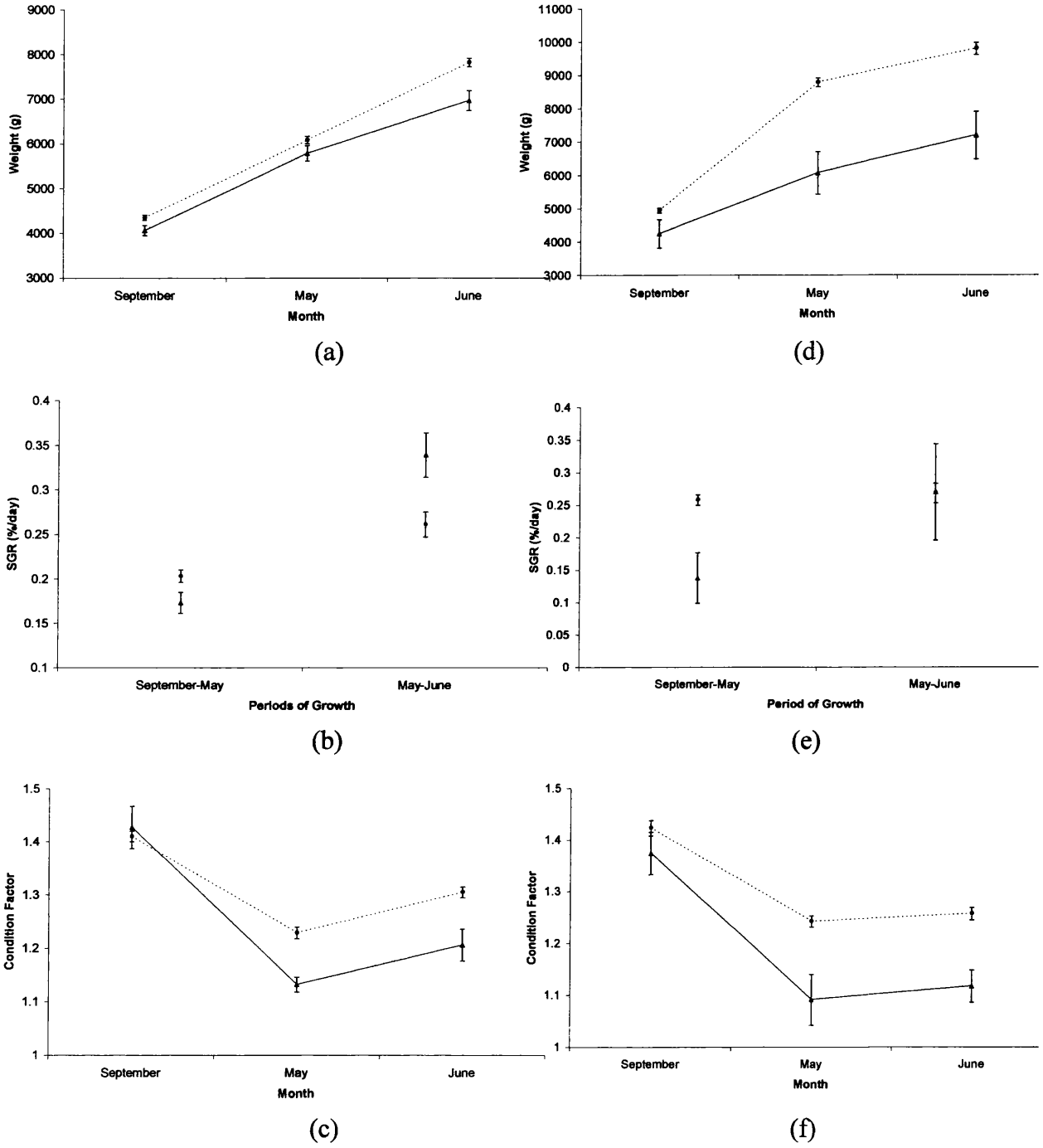


Figure 2.2. The mean (± SEM) body weights, SGRs and condition factors for the mature .....○..... and immature—△— (a-c) females □ and (d-f) males ■ .

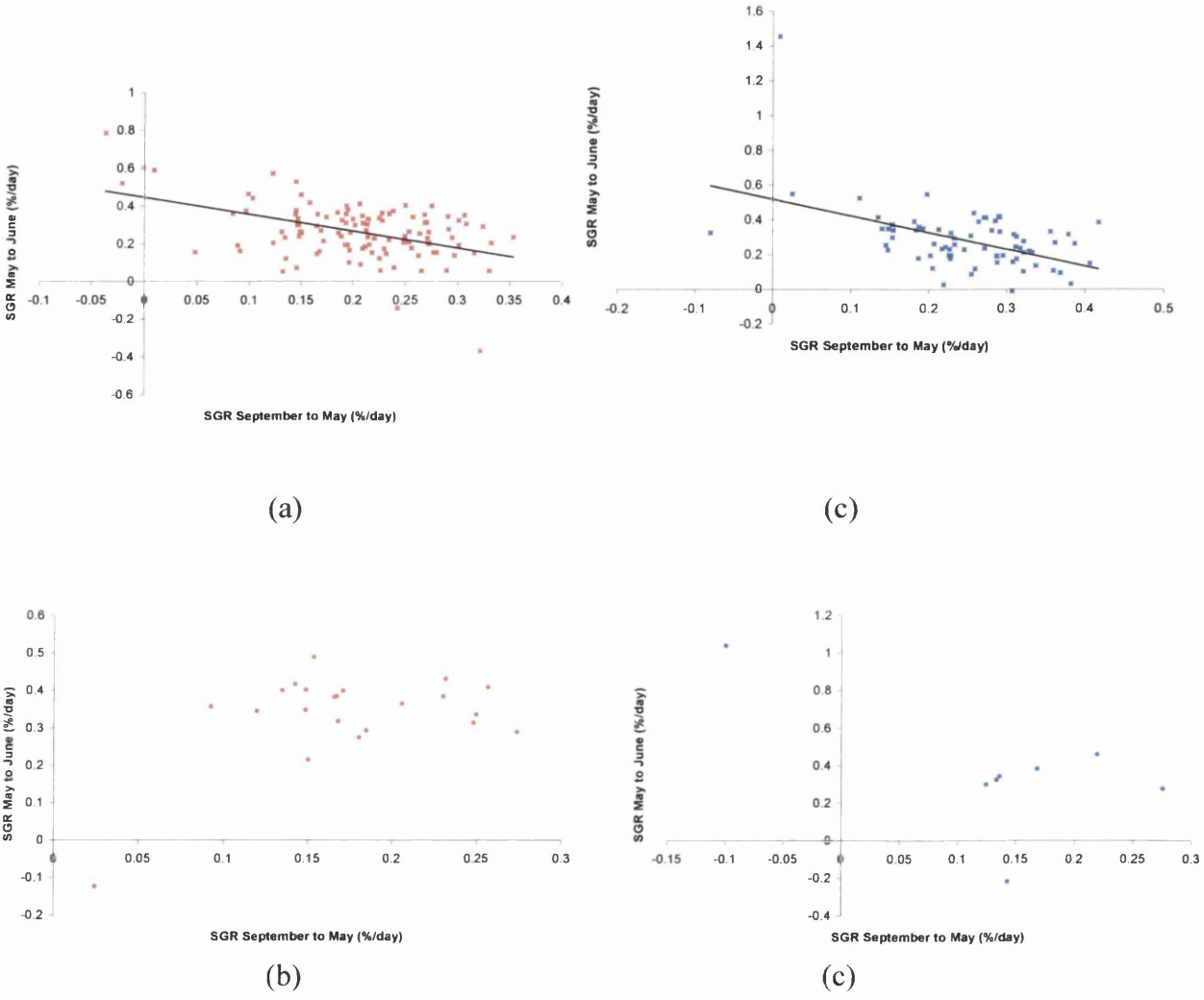


Figure 2.3. The relationships between the SGRs for both growth periods for the mature □ and immature ○ (a-b) females — and (c-d) males —. For ease of interpretation the SGR is presented as the raw data, though this variable was ranked for the purpose of statistical analysis.

Table 2.3. The results of the one-way ANOVA (F) and Kruskal-Wallis (in italics, H) tests to determine if gender influences the body condition and growth variables for both the mature and immature individuals.

Variable	Mature			Immature		
	P	F/H	N	P	F/H	N
September Weight	<0.001	40.85	241	0.556	0.35	33
May Weight	*<0.001*	*99.15*	*237*	*0.097*	*2.75*	*33*
June Weight	*<0.001*	*67.93*	*187*	0.687	0.17	33
September Condition Factor	*0.491*	*0.47*	*236*	*0.834*	*0.04*	*33*
May Condition Factor	0.409	0.68	237	0.276	1.23	33
June Condition Factor	0.002	9.53	187	*0.063*	*3.46*	*33*
SGR September-May	*<0.001*	*27.61*	*236*	*0.321*	*0.99*	*31*
SGR May-June	*0.840*	*0.04*	*186*	*0.346*	*0.89*	*32*

Table 2.4. The results from the Regressions (F) and Spearman's rank correlations (in italics, rs) for egg number, egg weight (from each stripping) and fry weight with each of the listed variable. A $^{\$}$ indicates that the result becomes non-significant after a sequential bonferroni correction.

		First Stripping			Second Stripping		
	Variable	P	F/rs	Rsq	P	F/rs	Rsq
	Stripped Weight	0.003	11.51	0.391	<0.001	21.4	0.444
	Sept. Weight	0.645	0.22	0.009	0.237	1.46	0.050
	May Weight	<0.001	22.21	0.491	<0.001	20.16	0.419
	June Weight	<0.001	37.24	0.651	<0.001	34.92	0.564
Egg	Final CF	0.343	0.94	0.039	*0.024*$^{\$}$	*0.411*	
Number	Sept. CF	0.768	0.09	0.004	0.500	0.47	0.018
	May CF	*0.112*	*0.325*		0.009$^{\$}$	7.84	0.219
	June CF	0.044$^{\$}$	4.63	0.188	0.020$^{\$}$	6.13	0.185
	Sept-May SGR	0.072	3.54	0.133	*0.067*	*0.339*	
	May-June SGR	0.647	0.22	0.011	0.352	0.90	0.032
	Stripped Weight	0.914	0.01	0.001	<0.001	15.68	0.367
	Sept. Weight	0.827	0.05	0.002	0.106	2.79	0.091
	May Weight	0.890	0.02	0.001	0.013$^{\$}$	6.99	0.200
	June Weight	0.630	0.24	0.012	<0.001	18.31	0.404
Egg	Final CF	0.112	2.74	0.106	*0.001*	*0.577*	
Weight	Sept. CF	0.960	0.00	0.000	0.541	0.38	0.015
	May CF	*0.177*	*-0.279*		0.010$^{\$}$	7.61	0.213
	June CF	0.960	0.00	0.000	0.002	12.35	0.314
	Sept-May SGR	0.981	0.00	0.000	*0.774*	*-0.055*	
	May-June SGR	0.665	0.19	0.010	0.115	2.66	0.089
	Stripped Weight	0.982	0.00	0.000			
	Sept. Weight	0.219	1.60	0.071			
	May Weight	0.730	0.12	0.006			
	June Weight	0.548	0.37	0.021			
	Final CF	0.997	0.00	0.000			
Fry	Sept. CF	0.803	0.06	0.003			
Weight	May CF	*0.211*	*-0.271*				
	June CF	0.863	0.03	0.002			
	Sept-May SGR	0.481	0.52	0.024			
	May-June SGR	0.760	0.10	0.005			

Table 2.5. The results of the multiple regression analyses used to determine the relationships between both egg number and egg weight and female stripped, May and June body weight at both strippings.

	Egg Number						Egg Weight		
		First Stripping			Second Stripping			Second Stripping	
	P	F	Rsq	P	F	Rsq	P	F	Rsq
regression	<0.001	19.95	0.81	<0.001	19.9	0.706	0.001	9.05	0.042
		T			T			T	
Stripped weight	0.084	-1.86		0.003	-3.37		0.903	-0.12	
May weight	0.168	1.45		0.176	1.39		-	-	
June weight	0.015	2.77		0.000	4.32		0.334	0.99	

Table 2.6. The results of the Regressions (F) and Spearman's Rank correaltions (in italics, rs) between the weight of milt produced (N = 32), kype length (N = 51) and width (N = 51) and the listed variables. A $^{\$}$ indicates that a result becomes non-significant after a sequential bonferroni correction.

	Weight of Sperm			Kype length			Kype width		
Variable	P	F/rs	Rsq	P	F/rs	Rsq	P	rs	Rsq
September Weight	0.284	1.19	0.038	0.100	2.81	0.054	*0.595*	*0.076*	
May Weight	0.264	1.30	0.040	*0.001*	*0.454*		*0.008*$^{\$}$	*0.369*	
June Weight	0.674	0.18	0.010	<0.001	33.14	0.486	*0.038*$^{\$}$	*0.343*	
Final Weight	0.344	0.91	0.029	<0.001	26.81	0.498	*0.007*$^{\$}$	*0.375*	
September Condition Factor	*0.700*	*0.071*		*0.468*	*-0.104*		*0.310*	*0.145*	
May Condition Factor	0.563	0.34	0.012	0.153	2.11	0.042	*0.014*$^{\$}$	*0.346*	
June Condition Factor	0.093	3.14	0.142	0.024$^{\$}$	5.59	0.138	*0.584*	*-0.093*	
Final Condition Factor	0.811	0.06	0.002	0.015$^{\$}$	6.38	0.119	*0.587*	*0.080*	
SGR September-May	*0.844*	*0.037*		*0.049*$^{\$}$	*0.279*		*0.008*$^{\$}$	*0.372*	
SGR May-June	0.421	0.68	0.034	0.861	0.03	0.010	*0.034*$^{\$}$	*-0.350*	

Table 2.7. The mean (± SEM) percentage values for the fertilised, eyed, hatched and survival to first feeding rates.

Post fertilisation variable	(%)
Fertilised	93.3 ± 0.92
Eyed	89.1 ± 1.03
Hatched	87.4 ± 1.14
Survival to first feeding	85.9 ± 1.23

Table 2.8. The results of the Pearson's (r) and Spearman's rank (in italics, rs) correlations for each of the post fertilisation quality parameters fertilised, eyed, hatched and first feeding survival percentages with each of the listed factors (N = 25). A $^{\$}$ indicates a result that becomes non-significant after a sequential bonferroni correction.

	Fertilised		Eyed			Hatched			First Feeding		
Correlation	P	r/rs	P	r/rs	Rsq	P	r/rs	Rsq	P	r/rs	Rsq
Stripped Weight	*0.199*	*0.318*	0.962	-0.021	0.000	0.667	-0.109	0.012	0.626	-0.123	0.015
Sept. Weight	*0.040*$^{\$}$	*-0.430*	0.406	-0.182	0.033	0.405	-0.182	0.033	0.371	-0.196	0.038
May Weight	*0.634*	*0.105*	0.708	-0.083	0.007	0.527	-0.139	0.019	0.390	-0.188	0.035
June Weight	*0.355*	*0.218*	0.897	-0.031	0.001	0.550	-0.142	0.020	0.527	-0.150	0.023
Egg Weight	*0.165*	*0.300*	0.715	0.081	0.007	0.767	0.065	0.004	0.981	0.005	0.000
Fry Weight	*0.321*	*0.216*	0.149	0.311	0.097	0.107	0.345	0.199	0.197	0.279	0.078
Egg No.	*0.691*	*-0.088*	0.819	-0.051	0.003	0.647	-0.101	0.010	0.598	-0.116	0.014
SGR Sept-May	*0.051*	*0.412*	0.683	0.090	0.008	0.782	0.061	0.004	0.857	0.040	0.002
SGR May-June	*0.933*	*0.020*	0.820	-0.054	0.003	0.649	-0.108	0.012	0.841	-0.048	0.002

Discussion

As predicted by the literature, not all of the fish used in this study matured, small percentages of both females and males remaining immature. Males and females that matured had greater body weights than their immature cohorts in the autumn of the previous year. This is in accordance with the results presented by Silverstein et al. (1998) for chinook salmon parr, Stead et al. (1999) for one sea winter Atlantic salmon and Berglund (1992) for Baltic salmon (*Salmo salar*) parr and would fit with the idea of an autumn maturation window (Thorpe, 1994). The maturing fish also had greater SGR from September-May and remained larger up to the following May and June. Rowe and Thorpe (1990a) showed that maturing male Atlantic salmon parr were larger than their immature counter parts in the May and June prior to the completion of maturation. The present study shows this to also be true of both male and female post-smolt Atlantic salmon.

Maturing males were significantly larger than their female counterparts in September, May and June and had higher SGRs from September to May. There was no such gender difference detected in the immature fish, all weights and growths were comparable, although it must be noted that the sample size of immature fish was small. This suggests that male and female post-smolt Atlantic salmon exhibit different reproductive trade offs. The greater energetic cost of producing eggs compared to milt (de Gaudemar 1998) means that females are required to accrue greater energy reserves, this could occur at the expense of growth, as indicated by the results of the present study. This is supported by the fact that maturing females had higher condition factors in June than the males, higher condition factors being associated with higher levels of visceral lipid content and energy reserves in salmonids (Herbinger and Friars 1991). Sexual selection in salmonids favours larger body size in males (Quinn and Foote 1994), this could also account for the preference to grow in males. Immature females had close to, but not quiet, significantly higher condition factors in June compared to their male counterparts. It could be that the females are already beginning to accumulate visceral energy reserves approaching the autumn maturation window.

Maturing females had significantly higher SGRs than immature females from September to May, but this was reversed from May to June. Both maturing and immature females had significantly higher overall SGRs from May to June compared to September to May. However the maturing females had a small but significant negative relationship between the SGRs of the two periods at the individual level, those fish that had higher growth rates from September to May had lower ones in May to June. Maturing males also had significantly higher SGRs than their immature counterparts from September to May, but in May to June they had comparable growth. There were no significant differences detected in the overall SGRs of either maturing or immature males between the two growth periods, although the sample size of the immature males was small which could have had influenced this result. As in the maturing females, there was also a small but significant negative relationship between the two SGR measurements in the maturing males at the individual level. This indicates that there is a pronounced difference in growth existing between immature and maturing female individuals from May to June that is not exhibited in males. This again could be due to the greater energetic costs associated with female maturation redirecting energy away from somatic growth into accumulating energy reserves creating a larger difference between them and the immature individuals that is not seen in males, maturing individuals achieving comparable growth to immature individuals. The reduction in individual growth during May to June seen in the present study is in accordance with the results of Stead et al. (1998). They also found that there was a decrease in the SGR of maturing Atlantic salmon in May. Kadri et al. (1995) suggested that there is a predetermined threshold for energy reserves above which accumulation ceases and utilisation begins as the fish become anorexic, which is known to occur in late spring/early summer in Atlantic salmon. This could explain the reduction in growth of the fish in this study, but food intake was not measured and so it can not be confirmed if the fish had started to reduced their food intake. Another possibility is that the initiation of gamete development had begun and as a consequence energy was directed away from somatic growth; this would fit with the hormonal evidence presented by Hunt et al. (1981), Stead et al. (1998) and Tyler and Sumpter (1996).

The best predictor of female maturation was body weight in May. This suggests that the second maturation window might be more influential in the completion of the maturation process than the autumn maturation window. There was no one specific predictor of male maturity. As before this disparity between the sexes could be due to the small sample size or again to the differential maturation cost between the genders making the difference between maturing and immature individuals less pronounced in the males compared to the females.

Egg number was influenced by stripped weight, for both the first and second strippings. It is well documented that larger fish produce greater numbers of eggs compared to smaller ones (Bromage et al. 1992). In this study, it was female body weight in June that was found to have the strongest relationship with egg number. This indicates that the number of eggs produced may be determined around the time the females become anorexic in the late spring early summer and start their migration. This would also fit with the hormonal evidence presented by Stead et al. (1999) and Tyler and Sumpter (1996) that showed an increase in the sex hormones linked to gonadal development around May. The females from the second stripping produced a larger number of eggs, suggesting that the females producing many eggs may take longer to reach full maturity. There was no relationship detected between egg weight or any of the body condition or growth variables for the eggs produced in the first stripping, but for those produced in the second stripping egg weight was positively related to female stripped weight and weight in June. In the first stripping, the fish had been bled and this could account for the disparity seen between the two strippings with reference to the effect of stripped weight. There was no significant difference in egg weight or female weight in June between the two strippings, yet for some unexplainable reason June weight was related to egg weight in the second stripping. This would indicate that egg weight, like egg number, might also be determined around the begging of the summer. The fact that no relationship was detected in the first stripping could suggest that there is more flexibility in egg weight than egg number and that egg weight could be altered later in the cycle. Contrary to the literature there was no relationship between egg number and egg weight, a negative relationship between the two has often been reported (Elgar, 1990; Fleming, 1996). The results suggest that females that reproduce later in the

season invest more into reproductive investment possibly at the cost of something else, such as a repeat spawning. They also imply that June is an important time in female post-smolt Atlantic salmon reproductive biology.

None of the body condition or growth variables were related to the fertilisation, eyeing, hatching or survival to first feeding rates of the eggs and fry. Nor were they related to fry weight only egg weight was. It could be that the fish used in this study were all in good condition and produced good quality eggs or that egg quality is preserved at the expense of another factor such as a repeat spawning. The relationship between egg and fry weight has been well documented, although it has been shown that the difference disappears after a period of feeding in some species (Springate and Bromage, 1985).

Sexual selection favours larger males and greater hooked snout length (Quinn and Foote 1994), suggesting they are important aspects of male reproductive investment and that a relationship between the two could transpire. The results presented here showed that the weight of sperm produced was unrelated to any of the morphological or growth variables including kype width and length indicating that such a relationship does not exist in the male Atlantic salmon used here.

In summary male and female Atlantic salmon that matured were larger in the September of the preceding year than those that did not. Males were larger than the females and showed different patterns of growth and reproductive investment, suggesting that they exhibit different reproductive trade offs to females. Weight in May had the strongest relationship with maturity in females. Egg number was related to stripped weight and the weight in May and June, the latter has the strongest relationship. Females that matured later in the season produced more eggs, but there was no difference in egg weight. None of the egg quality variables were related to the morphological or SGR variables, nor was the amount of sperm produced. The results also support the model for the control of maturation with body weight in the autumn of the year prior to its completion and weight and growth in June being important to the maturation process in Atlantic salmon.

Chapter 3

Using ultrasound scanning to assess the reproductive status of salmonid fish

Summary

Ultrasound scanning was used to visualise and measure the length and width of ovaries in maturing and immature rainbow trout and Atlantic salmon, to examine the possibility of using this system to sex small fish, to predict ovary weight and to measure egg size in a non-destructive manner. It was possible to sex immature Atlantic salmon (3-year old; bled weight 1500g ± 11 SEM). Significant positive correlations were found between the scanned and actual ovary length and width in maturing rainbow trout and in immature Atlantic salmon, although the relationships in the latter were weaker. It was possible to predict ovary weight from ultrasound measures of ovary length and width in salmonids with an accuracy that depends on fish size. A positive, but weak correlation was found between scanned and actual egg size in maturing Atlantic salmon. These results therefore provide further evidence that ultrasound can potentially be used successfully to assess the ovary status of salmonids, but not to measure egg size.

Introduction

Long term studies of reproductive processes in fish

Life history studies are prominent in fish biology research, especially with respect to salmonids. A number of salmonids have a complex life history pattern that involves moving between fresh water and seawater, usually associated with the maturation process. Timing of migration and maturation is variable and there is an extensive body of literature aimed at identifying the environmental and biological factors that determine when fish mature and how much energy they invest in reproductive tissue for example, Meyers et al., 1986, Berglund, 1992, Thorpe, 1994, Friedland and Hass, 1996, and Silverstien et al., 1999.

The research strategy involved in such studies frequently requires long term tracking of growth, behaviour and maturation status in individually identifiable fish. Ideally separate analyses should be conducted on males and females as gender differences may exist. For example a large number of studies have identified differences in behaviour patterns between the genders, for example, anti-preditor behaviour (Giles and Huntingford, 1984), innovation capacity in foraging (Laland and Reader, 1999) and agression (Johnsson et al., 2001). Relatively few such studies have been conducted on juveniles, yet the differences between adults could be explained by juvenile patterns (Johnsson et al., 2001). Normally gender allocation requires fish to be killed, which obviously prevents early gender assignment for life history studies. Being able to assign gender in a non-destructive way as early as possible could facilitate such studies. This would also help in studies of timing and causes of maturation, as would the early and continuous non-destructive assessment of gonad size and consequently the gonadal somatic index (GSI). Measuring GSI also generally requires the fish to be sacrificed and again this obviously prevents following fish to the conclusion of the maturation cycle. The maturation process in some salmonids seems to be initiated the year prior to its completion (Thorpe et al. 1998), but the exact timing of this process has not been fully characterised. Being able to assess gonad size in a non-destructive manner throughout this year would allow developmental

changes to be tracked from their initiation to their conclusion.

Also the early non-destructive measure of egg size could give an idea of female reproductive potential. A broad range of literature exists on egg size as a measure of egg quality through its positive relationship with initial offspring size. Ojanguren et al. (1996) found that initial offspring size in brown trout was strongly dependent on egg size, with the difference still apparent 90 days after hatching. However Springate and Bromage (1985) reported that any size differences at hatching had disappeared four weeks after first feeding in rainbow trout.

Existing methods for non-destructive assessment of gonadal status in fish

There are existing methods for early sexing of fish and assessing maturation status in a non-destructive manner. For example sex can be determined by the detection of the sex-specific steroid hormones testosterone and progesterone using radioimmunoassay techniques and the immunochemical detection of the female-specific hormone vitellogenin in the plasma and skin mucus (Gorden et al., 1984; Berlinsky and Specker, 1991). The gonadal status can also be established by these methods as the levels of the hormones increase as the gonad size increases (Gorden et al., 1984; Berlinsky and Specker, 1991). Molecular techniques have been employed in recent years to sex fish using DNA markers although this is very complex (Kovacs, et al., 2000; Devlin, et al., 2001). Ovarian biopsies and urogenital catheterization can also be performed to determine sex and gonadal status (Blythe et al., 1994; Martin-Robichaud and Rommens, 2001). Such analyses are time consuming, expensive, invasive and are often limited to certain periods of the maturation cycle (Martin et al., 1983; Blythe et al.,1994; Martin-Robichaud and Rommens, 2001). Alternative methods for assessing the maturation status in a non-invasive manner have been attempted, for example the use of morphometric measurements. Kadri et al. (1995) found a significant relationship existed between a number of morphometric variables and the gonadal somatic index ($P=<0.001$, r^2 0.717.) in maturing Atlantic salmon (body weight 1.7kg). Clearly alternative methods are needed and can be used successfully such a method is the use of ultrasound. It could provide a more

rapid, comprehensive and non-destructive method for sexing and assessing maturation status in fish.

Using ultrasound to visualise fish gonads

Ultrasound technology has been developed for the non-invasive visualisation of internal tissue structures for both humans and non-human animals. Recently it has become the preferred method of sexing maturing fish in the aquaculture industry. Notable success has been achieved in the use of ultrasound for distinguishing gender in maturing fish for a number of species including, Atlantic halibut *Hippoglossus hippoglossus* (Shields et al., 1993), Atlantic cod *Gadus morhua*, coho salmon *Oncorhynchus kisutch* (Martin et al., 1983), Atlantic salmon and rainbow trout (Riemers et al., 1987; Martin-Robichaud and Rommens, 2001). This method is non-destructive and it allows the gonads to be visualised instantly (only requiring the fish to be anaesthetised). A transducer applied to the skin produces acoustical energy that penetrates through tissue and is either reflected, scattered or absorbed. Reflected sound is converted by the transducer to an electrical signal from which a 2-dimentional image is generated (Martin et al., 1983). Mattson (1991) reported success in determining sex in immature Atlantic salmon, with a body weight of about 4.5kg, although no measure of accuracy was given. Karlson and Holm (1994) had varying success in assigning gender in small cod (body weight ~1142 g), between ~95 %-~65 % being identified correctly. On the other hand Blythe et al. (1994) were unable to assign sex in juvenile and small adult hybrid stripped bass (weight <1.8 kg) accurately with ultrasound, with less than 50 % identified correctly. Such variability in the results of predicting sex suggests that more information needs to be collected across all species. As far as assessing gonad size is concerned, Mattson (1991) found a good correlation ($r = 0.926$) between predicted female gonad diameter using ultrasound and actual measurements for the Atlantic salmon. However this did not give an accurate measure of gonad weight and as described above the study involved large fish. Although attempts have been made to make non-invasive assessments of gender and gonad dimensions using ultrasound, more information is required on sexing small immature fish and predicting gonad weight, especially with respect to salmonids.

Aims of the study

The overall aim of the present study was to explore further the possible use of ultrasound for determining gender and predicting gonad dimensions in salmonids. The data were collected during five separate experiments all with slightly different aims. Logistical constraints during these experiments restricted the measurements that could be taken at any one time. Collectively these data sets allowed us to;

- Assess the accuracy of predicting gender from ultrasound images in small, immature, seawater salmonids.
- Assess the accuracy of measuring the length and width of ovaries from ultrasound images in maturing and immature rainbow trout (*Oncorhynchus mykiss* L.) and Atlantic salmon (*Salmo salar* L.).
- Examine the feasibility of using these measurements to predict the ovary weight.
- Examine the possibility of measuring egg size in maturing, female Atlantic salmon from ultrasound images.

Materials and Methods

Measurement of rainbow trout gonads

During November 2001, 43 2-year-old female rainbow trout were provided by Hendrix SpA (Mozzecane Italy) (1573 g ± 35 SEM). Prior to being sacrificed the fish were not fed for two days, just enough time to clear the upper digestive tract and prevent food obscuring the ovaries on the image captured by the ultrasound machine. The fish were placed individually on a plastic board with their right side up. A Dynamic Imaging Concept/MC, portable, small animal ultrasound scanner (Livingston Scotland) with a 7.5 Hz transducer was used to visualise the ovaries. The transducer was repeatedly passed slowly from the vertebral column to the belly, starting at the back of the opercular cavity and continuing to the anus. Once the ovary was distinguished, it was traced to the end and its length was then recorded as the section in which this occurred. Each fish was visually

divided into four sections (1-4). The first ran from the back of the opercular cavity going to mid way between it and the dorsal fin, the second ran from this point to the front of the dorsal fin, the third ran from this point to the back of the dorsal fin and the final section ran from this point to the anus (Fig. 3.1). If the ovary was found to end at the border between two sections, half scores were assigned. It was possible to see both the left and right hand side ovaries, in 18 of the 48 individuals and the length of both of these were measured. In four fish the ovaries were recorded as non-visible.

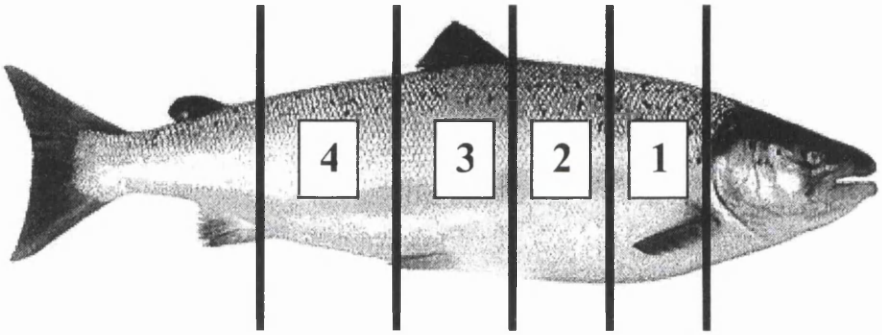


Figure 3.1. Template of a trout divided into the four sections (1-4) used for measuring ovary length.

An image of the transverse section of the ovary was saved on the scanner and the width, at its widest point (Mattson, 1991), was then measured using the scanner's image analysis software package. In six fish, the width was recorded for both the right and left ovary. At this point the fish were frozen (–20 °C) for subsequent dissection. The fish were defrosted, placed with their right side up and opened from the anus to the mouth along the length of the belly and then from under the buccal cavity following the line of the operculum to the vertebral column. The flank was then lifted to expose the ovary and the section which it ended in was recorded (1-4 see above). The ovary was carefully removed, its width was measured with callipers (to the nearest mm) at its widest point, its length was then measured with a ruler (to the nearest mm) and the ovary was weighed. Both the right and left ovaries were removed and measured from each fish. On removal three ovaries were damaged, one had not had a width measurement taken, and in six cases the width was not recorded correctly when the fish was

scanned due to an oversight. In total, sample sizes for the length and for the width were 54 and 36 respectively.

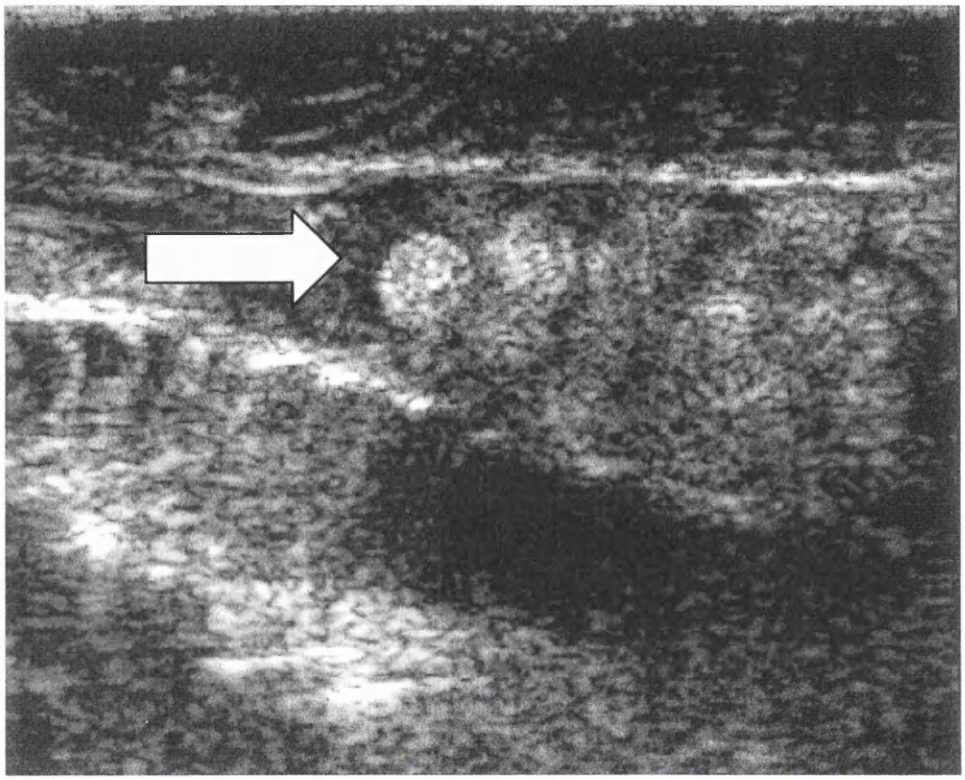


Figure 3.2. An ultrasound image showing the end of an ovary in a female rainbow trout from the January 2002 sampling.

In January 2002, 89 2-year-old female rainbow trout, 2046 g ± 47 (SEM) (provided by Hendrix SpA Mozzecane Italy), were scanned as described previously, except this time a Sonosite VET 180 plus scanner (Bothwell WA, USA) was used with a 10-5 MHz transducer and a penetration depth of 3.2 cm. When the end of the ovary was detected it was marked on the skin; the length of the ovary was then measured with a ruler to the nearest 0.5cm starting at the back of the opercular cavity to the end point. Some (N = 42) of the fish had gonads that were almost fully developed and reached all the way back to the anus; and so were not scanned. In one instance the ovary was recorded as non-visible and in two of the fish both ovaries were measured. Fig. 3.2 illustrates the end of an ovary as seen using the scanner. Upon dissection of the fish, the ovary was removed as above and the actual length measured again to the nearest 0.5 cm. Three of the actual length measurements for the trout in Jan. '02 were not

recorded due to an oversight and so these data were removed. The total sample size of length measurements for these fish was 45.

Measurement of Atlantic salmon gonads

In January 2003, 126 3-year-old Atlantic salmon (bled weight; 1500 g ± 11 SEM), provided by Marine-Harvest Loch Eil, Scotland, were scanned with the Sonosite machine as described previously. Gender was determined by the presence (female) or absence (male) of a visible gonad, a method used previously by Matteson (1991) and Blythe, et al. (1994) and recorded. In a number of randomly chosen females (time was restricted and so not all the fish could be scanned) the length of the right ovary was measured (to the nearest 0.5 cm) (N = 49). The ovary width was measured using the scanner's software (N = 48), it was then removed and frozen (-20°C). After thawing, the ovary length and width were measured with callipers, to the nearest 0.5cm and mm respectively, and the weight recorded, to the nearest 0.001g. Gender determination and ovary measuring were repeated in May 2003 (181 fish bled weight; 2603 g ± 50 SEM sexed and the ovary length measured in 66 females with the width measured in 54) and July (49 fish sexed bled weight; 3442 g ± 27 SEM and the ovary length only measured in 17 females). Fig. 3.3 shows a scanned image of an immature Atlantic salmon ovary from one of the samples with the end point highlighted and the width measurement taken.

Measurement of egg size within Atlantic salmon gonads

In November 2003, 12 3-year-old female Atlantic salmon (from a population with a mean weight of 6832 g ± 109 SEM) were individually scanned to determine their egg size. The transducer was placed directly behind the opercular cavity on the vertebral column and passed in a downward direction till an image of clear well defined eggs was achieved. The image analysis tools were used to measure the diameter of three or four clearly visible eggs at their widest point. This was then repeated in the abdominal region directly beneath the dorsal fin and again just in front of the anus. An incision was then made at the point where the image had been taken, gentle pressure was applied and three or four of the

first eggs to be expelled were collected and measured with callipers at their widest point and weighed. Fig. 3.4 shows a scanned image of the eggs and the measurements taken. The total egg sample size was 146.

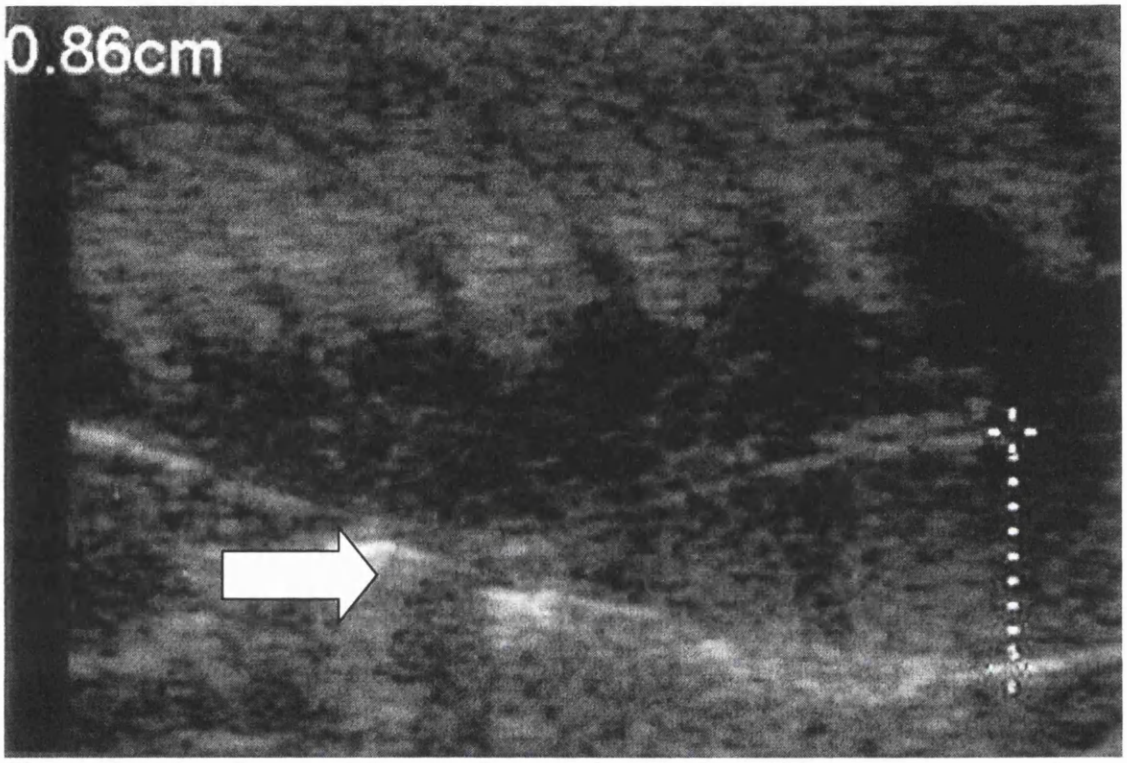


Figure 3.3. An ultrasound image of a gonad, with the width measurement and end highlighted with the arrow, of an immature Atlantic salmon from the May 2003 sample.

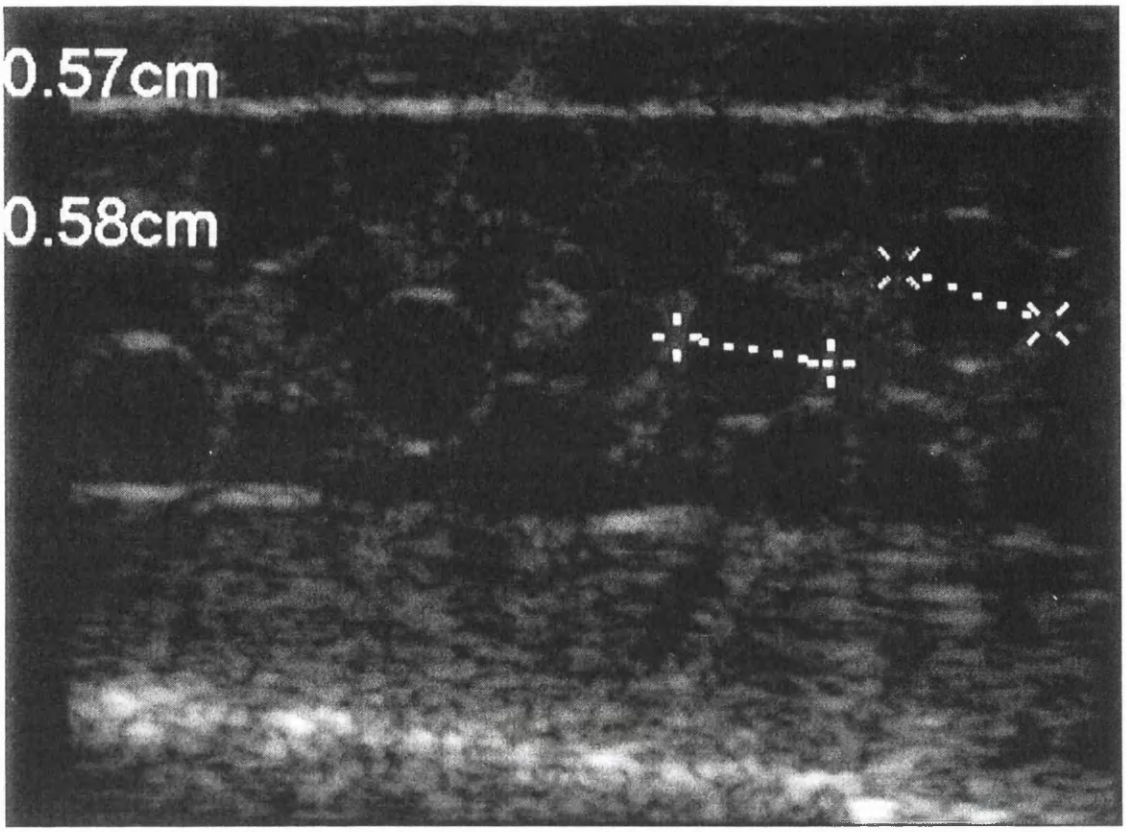


Figure 3.4. An ultrasound image of eggs within an Atlantic salmon from the November 2003 sample showing the measurements taken.

Data analysis

A Pearson's correlation was used on the scanned and actual width of the trout ovaries in Nov. '01, after testing for normality. Transformations did not normalise the scanned ovary length measurements from the rainbow trout in January '02, so a Spearman's rank correlation was performed with the actual ovary length data. Parametric and non-parametric correlations were used on the Atlantic salmon scanned and actual ovary length and width data collected in January, May and July '03.

Partial Least Square Regression analysis was employed to predict the ovary weight using the actual ovary length and width measurements taken for the rainbow trout in Nov. '01 using Design-Expert version 6 software (Stat-Ease, Minneapolis, USA). This was repeated for the Atlantic salmon data collected in

July '03. The January '03 and May '03 data could not be normalised with transformations and so was not analysed in this way.

Scanned length and width for those rainbow trout ovaries for which both measurements were taken, (N=31) in Nov.'01 as individual data points were ranked, along with the actual weight of the ovary and these ranks were used in a multiple-regression model.

Transformation of the scanned egg diameter data collected in Nov. '03 for the Atlantic salmon did not normalise the data so a Spearman's rank correlation was used to relate scanned, actual diameter and egg weight.

All analyses unless stated were conducted using MINITAB v13 and EXCEL '97.

Results

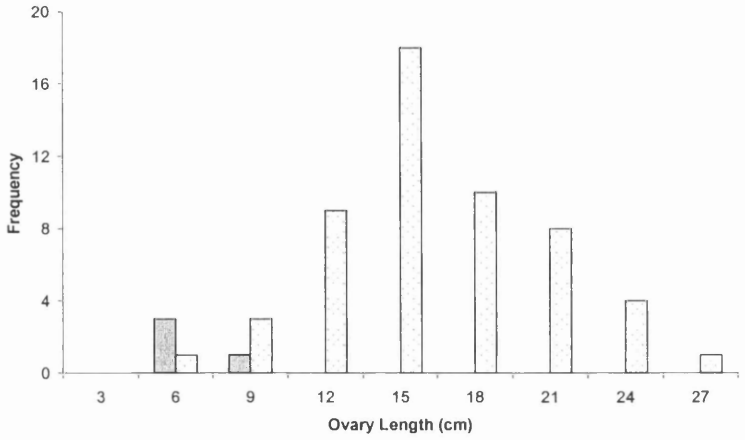
Sexing immature fish

During the Jan.'03 sampling, 96.3 % of the Atlantic salmon were correctly identified by sex, 97.24 % were correctly identified in May and 81.6 % in July.

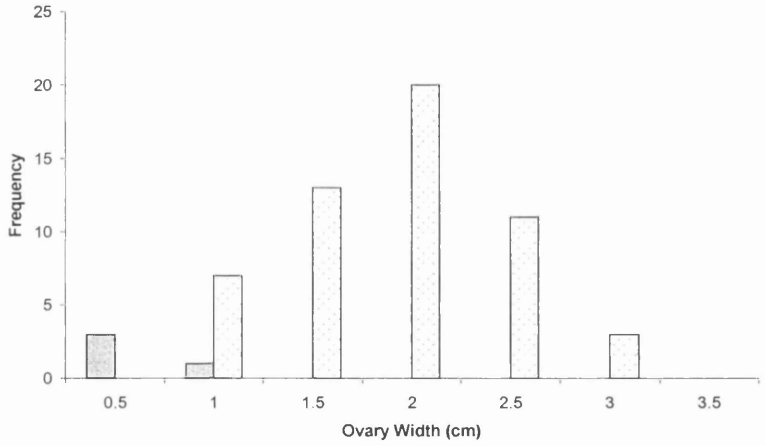
Estimation of gonad length and width

Rainbow trout (Nov.'01)

The distribution of the actual lengths and widths for these ovaries and those that were scanned can be seen in Fig. 3.5. Non-visible ovaries were recorded in four rainbow trout Nov.'01, these had actual lengths and widths of 5.1 cm ± 0.87 (SEM) and 0.51 cm ± 0.07 (SEM) respectively. The scanned length categories in Nov'01 were correct for 55.6 % of cases and wrong by only one score in 25.9 % (Table 3.1). A strongly significant correlation ($P = <0.001$, $r = 0.728$, $Rsq = 0.53$) existed between the scanned width and the actual width of the ovary (Figure 3.6a).



(a)

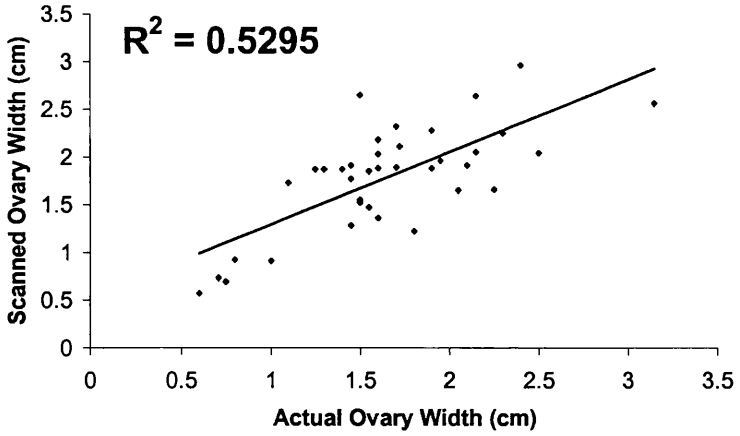


(b)

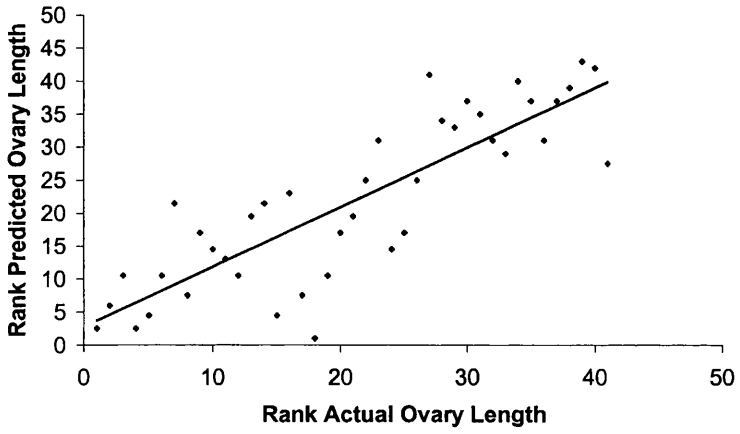
Figure 3.5. The distributions of the actual ovary (a) lengths and (b) widths for those recorded as non-visible (□) and those that were scanned (□) for the rainbow trout sampled in Nov'01.

Table 3.1. The predicted and actual ovary length scores for the rainbow trout in November 2001.

		Actual Ovary Position						
		1	**1/2**	**2**	**2/3**	**3**	**3/4**	**4**
Predicted Ovary Position	**1**	1		2				
	1/2	2						
	2			3	2	2		
	2/3			1		3		1
	3					11	4	3
	3/4				1	1	1	
	4					1	1	14



(a)



(b)

Figure 3.6. Correlations of (a) the predicted and actual ovary widths for the rainbow trout Nov '01 and (b) predicted and actual ovary length for the rainbow trout Jan. '02.

Rainbow trout (Jan.'02)

For the fish with ovaries recorded as reaching the anus 39 were successfully identified with three misidentified. The actual length of the ovary recorded as non-visible was 4.5 cm. There was one case of a measured ovary reaching right back to the anus. For the rest (N = 44) a highly significant correlation occurred between the scanned length and the actual length (P = <0.001, rs = 0.930) (Figure 3.6b). The distribution of the scanned and actual ovary lengths for these fish can be seen in Figure 3.7.

Atlantic salmon

There were no relationships between the scanned and the actual ovary length (Spearman's Rank correlation P = 0.123, rs = 0.223), or the scanned width and the actual width (Pearson's correlation P = 0.117, r = 0.229) for the Atlantic salmon sampled in Jan.'03. The mean actual ovary length and width for these fish were 3.2 cm ± 0.07 (SEM) and 0.83 cm ± 0.03 (SEM) respectively. In the salmon sampled in May'03 the correlations between the scanned and actual ovary lengths (Spearman's Rank correlation P = <0.001, rs = 0.752) and widths (Spearman's Rank correlation P = <0.001, rs = 0.750) were both significant (Figure 3.8a and b). The means of the actual ovary length and width were 4.04 cm ± 0.14 (SEM), 0.8 cm ± 0.03 (SEM) respectively. Finally the correlation between the scanned and actual ovary length, for the July'03 sampling, was significant, P = 0.012, r = 0.595, RSq = 0.35 (Figure 3.8c). The mean actual ovary length was 4.7 cm ± 0.17 (SEM).

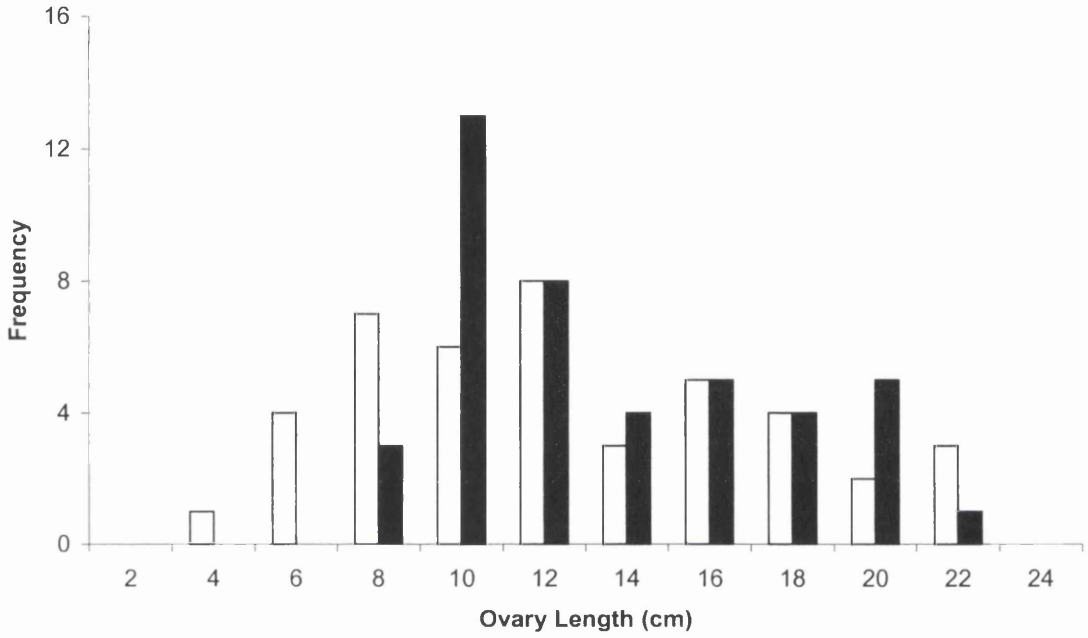
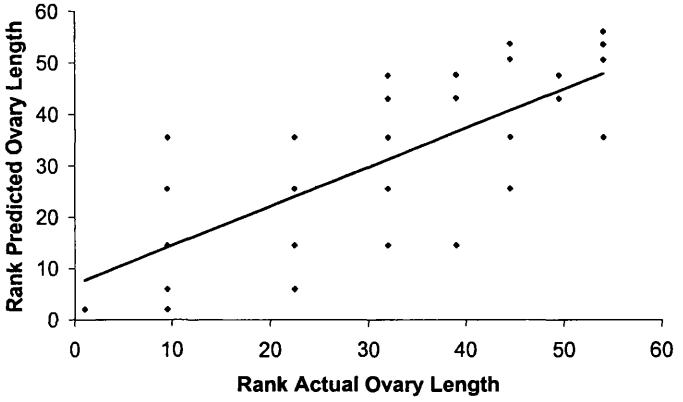
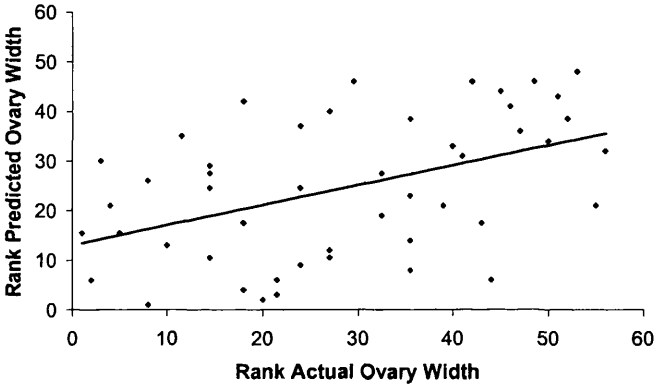


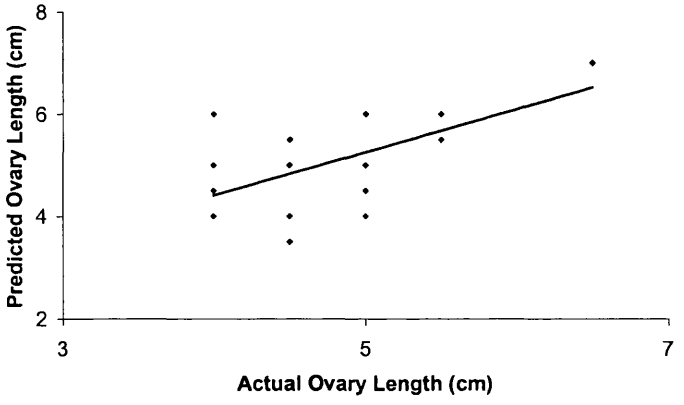
Figure 3.7. The distributions of the scanned (□) and actual (■) ovary lengths for the rainbow trout in Jan.'02 that had measured lengths.



(a)



(b)



(c)

Figure 3.8. Correlations of the Atlantic salmon (a) rank predicted and actual ovary lengths and (b) the rank predicted and actual ovary widths from the May'03 sampling and (c) the predicted and actual ovary lengths for the July '03 samples.

Predicting gonad weight using the length and width

Actual ovary weight could be predicted using the actual length and width measurements, take after dissection of the rainbow trout in Nov. '01, (Partial Least Square Regression, P = <0.001, F = 72.3, Rsq = 0.741).
The equation produced was:

*Ovary weight = -14.05 + 1.16*length + 4.33*width + 1.38*length*width*

The Rsq value for this model can be improved if the equation is raised to the power of 2 (Partial Least Square Regression, P = <0.001, F = 113.9, Rsq = 0.82).
The equation produced was:

$Ovary\ weight = (-3.33 + 0.26*length + 4.91*width + -0.78*width^2)^2$

The multiple-regression model using the rank scanned length and width with actual ovary weight was significant (P = <0.001, F = 12.59), with a Rsq of 0.473. Within the regression the length had a P value of 0.013 and the width a P value of 0.007, the equation produced was:

Ovary weight = 2.45 + 0.413 rank length + 0.434 rank width

The Partial Least Square Regression analysis to predict actual ovary weight using actual length and width for the Atlantic salmon in July'03 was not significant (P = 0.051, F = 2.89)

Measurement of eggs size

Significant correlations were found between the scanned and actual egg diameter (P = 0.002, r = 0.257, Rsq = 0.066) (Fig. 3.9) and scanned diameter and egg weight (P = <0.001 r = 0.370 Rsq = 0.137), although the RSq values for the correlations were low. The actual diameters had a mean of 0.57 cm ± 0.002 (SEM). Figure 3.10 shows that there was a tendency for the scanned diameters to be under estimated when compared to the actual diameters.

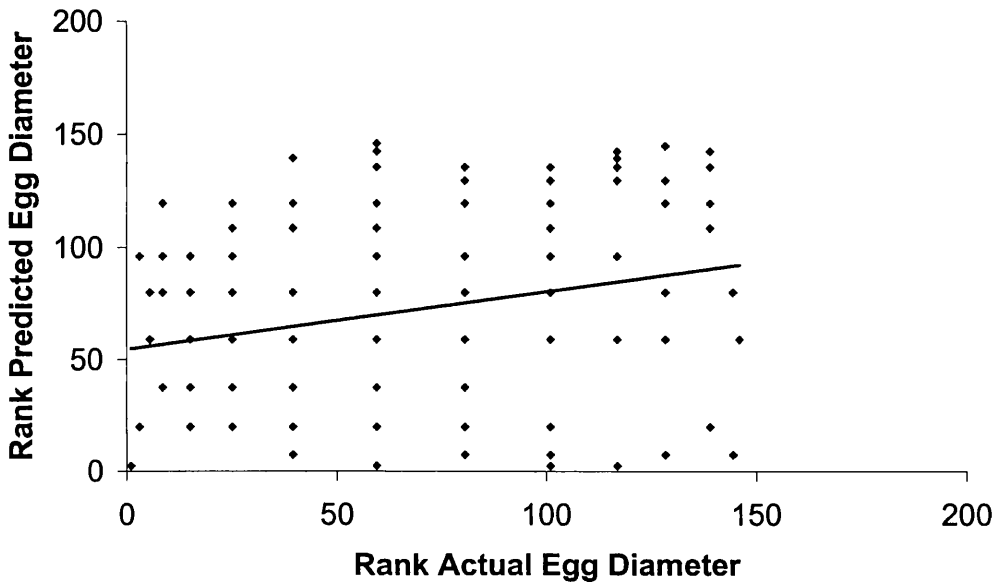


Figure 3.9. The correlation between scanned and actual egg diameter for the Atlantic salmon in November 2003.

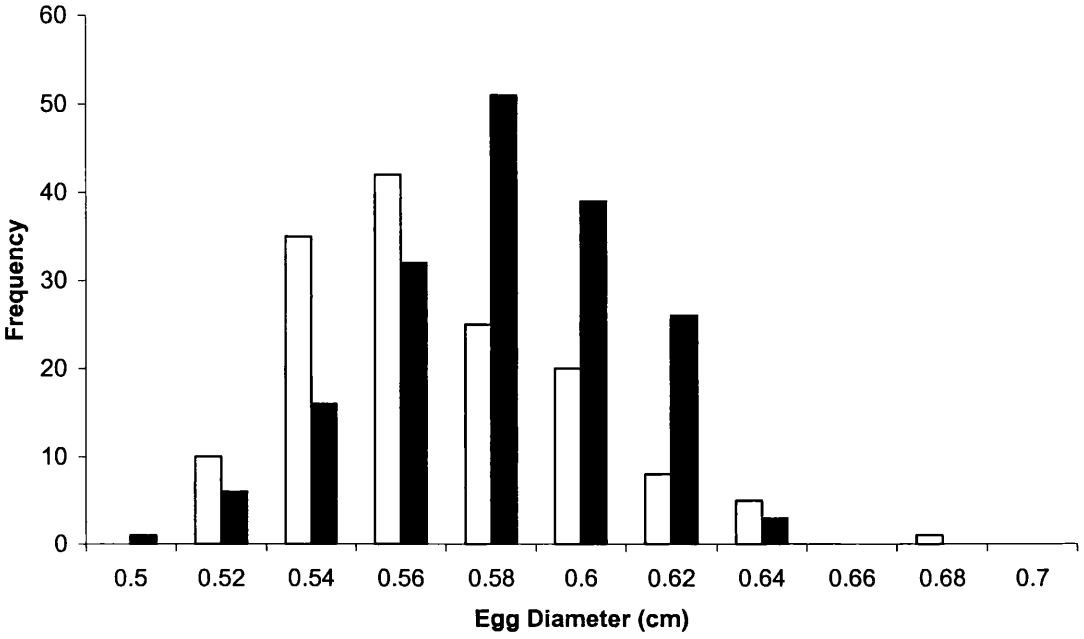


Figure 3.10. The distributions of the scanned (□) and actual (■) egg diameters.

Discussion

Ultrasound technology has been used successfully by the aquaculture industry to sex mature fish and could potentially be used by fish biologists in investigations of life history strategies in salmonids. In the current study, visualisation of the male gonads was very difficult. In the immature Atlantic salmon the female gonad was a clearly visible structure while the male gonad was not apparent at all, as reported previously for Atlantic salmon and striped bass (Matteson, 1991; Blythe et al., 1994). Gender distinction was therefore achieved on the presence or absence of a gonad. The accuracy with which this was achieved was very high, the values similar to those achieved Blythe et al. (1994) for mature stripped bass (95 %) and higher than the minimum values achieved by Karlsen and Holm (1994) (~95 %-~65 %) for immature cod. The fish used in this study were considerably smaller than the Atlantic salmon used previously by Mattson (1991, 1.5 kg compared to 4.5 kg).

The results presented here indicate that it is possible to estimate the length and width of maturing female rainbow trout ovaries using ultrasound. However estimation was less accurate for ovaries at the lower end of the size distribution; ovaries with lengths and widths of about 5.1 cm and 0.51 cm respectively were difficult to visualise consistently. In immature Atlantic salmon, significant correlations were achieved between the scanned and actual gonad length, but not for gonads below about 3.1 cm. In the rainbow trout it was possible to estimate gonad lengths of about 4.04 cm, but smaller ovaries proved difficult to visualise. Estimating width in these fish was possible, but the accuracy was variable. In Jan.'03 and May'03 actual ovary widths were on average 0.83 cm and 0.8 cm respectively, the correlation between scanned and actual was not significant for the first but was for the latter. The improvement in the accuracy of estimating ovary length from the rainbow trout to the salmon and then in estimating ovary width between salmon samplings is probably due to increased experience of the operator.

The ovary weight of a maturing female rainbow trout can be derived from an equation using the length and width. Multiple-regression using the ranked data for the rainbow trout in Nov'01 showed that a strong relationship did in fact exist between the scanned measurements and ovary weight. Indicating that it is possible to predict ovary weight from scanned measurements in maturing rainbow trout. It was not possible to predict the weight of immature Atlantic salmon ovaries from the length and the width, although it was only slightly non-significant indicating that it should be investigated further.

Although a significant correlation between the predicted and actual egg diameter was produced the accuracy was variable and that there was a tendency for the diameter to be underestimated. This could be due to the scanned image not dissecting the egg at the widest point. The relationship between scanned diameter and weight was also significant but again variable. The diameters of the eggs scanned here were similar to the widths of the gonads that were difficult to scan in previous attempts (0.57cm and 0.51cm), suggesting there is a lower size threshold that can be reduced with increased operating experience.

In summary this study indicates that it is possible to determine the sex of immature Atlantic salmon with a weight of about 1.5 kg using ultrasound. This method can also be used to measure the ovary length and width for ovaries larger than 3.2 cm in length and 0.8 cm in width, the accuracy of the latter is more variable in the case of immature fish. There is a relationship between the length, width and weight of ovaries in maturing female rainbow trout and it is possible to estimate the weight using the length and width measurements. As the length and width can be measured with the scanner this implies that weight can be too. Improved technique and more advanced transducers could lead to an improvement in the lower size threshold. It should be pointed out this research was conducted on dead fish and should be tested for repeatability with anaesthetised specimens.

Chapter 4

Long term partial replacement of dietary fish oil with rapeseed oil; effects on egg quality of Atlantic salmon *Salmo salar*

This chapter has been accepted for publication in the journal Aquaculture.

Summary

The use of vegetable oil in broodstock diets and its effect on gamete quality was investigated. During September 2002, 315 Atlantic salmon (2^{nd} sea winter) were individually tagged and divided amongst three sea cages. The fish were fed a broodstock diet with 100 % South American fish oil (at a dietary lipid level of 27.6 %) in one treatment group and with 50 % (the rest replaced with rapeseed oil) in the others. The fish were weighed and measured in November 2002, May 2003, and July 2003. Maturing fish were transferred to fresh water in October 2003 and individual fecundity and mean egg weights were measured. Dietary treatment did not affect egg number or egg weight. The eggs from a sub-set of females from each treatment were fertilised using a pool of milt from three males of the same treatment. Samples of the pre-fertilised eggs underwent fatty acid, astaxanthin and proximate composition analysis. The fatty acid profiles of the eggs and frys differed between dietary treatment, the rapeseed oil treatment producing lower levels of DHA (22:6n-3) and EPA (20:5n-3) and higher levels of 18:1n-9, 18:2n-6 and 18:3n-3, but rates of fertilisation, eyeing, hatching, survival to first feeding and the fry weight at first feeding did not. These results suggest that it is possible to replace a portion of the fish oil (South American) with rapeseed oil in a broodstock diet without compromising the early survival and initial growth of offspring. Although there could be implications for some developmental processes not tested for in this study.

Introduction

Egg quality

One of the most important constraints on the future development of aquaculture is the availability and quality of eggs and milt (Bromage, 1996; Abi-ayad et al., 1997). The continual increasing demand for cultured fish requires greater supplies of eggs (Watanabe, 1985; Cerda et al., 1994), as egg number and survival determine the number of juveniles available for the on-growing process (Watanabe, 1985). With the additional pressure of substantial losses possible during the hatchery stages, (up to two thirds in the salmonids, Bromage, 1996), the need to maximise reproductive potential is an important consideration. A predominant factor that can affect survival and juvenile quality is egg quality, which in turn is influenced by broodstock nutrition (Watanabe, 1985; Mourente and Odriozola, 1990; Abi-ayad et al., 1997; Izquierdo et al., 2001; Morehead et al., 2001). To date the majority of studies looking at nutritional influences on egg quality have been conducted with marine species and rainbow trout; the present study looks at Atlantic salmon an anadromous species that begins life in freshwater but whose oocytes develop in the seawater phase.

Alternative dietary lipid sources

Another important constraint on the aquaculture industry is the increasing pressure to find alternative primary sources of protein and oil for its diets. Strained fisheries, concern over levels of pollutants (Bruce et al., 1999; Asturiano et al., 2001) and random events such as El Nino have rendered fish oil an unpredictable raw material. As a consequence there has been a surge in research aimed at testing partial alternative constituents most notably vegetable oils. Vegetable oil is a sustainable resource and is easily available on a commercial basis, the projected production for 2002-2003 being 91.8 Mt with marine oil at 1.2 Mt (Beckman and Skrypetz, 2002).

Essential fatty acids in fish

Fish, like all animals, have a requirement for a number of fatty acids, known as

'essential fatty acids', that must be provided in their diet. Fish tissue is rich in the omega-3 essential fatty acids 22:6n-3 (DHA- docosahexaenoic acid), and 20:5n-3 (EPA-eicosapentaenoic acid) and their metabolic precursor 18:3n-3 (LNA- Linolenic acid). Especially the Southern hemisphere fish oils that are used in feed production that can contain up to twice the levels of Northern hemisphere oils. The majority of vegetable oils on the other hand are rich in omega-6 fatty acids and lack DHA and EPA, but some do contain a reasonable level of LNA. The majority of marine species have an absolute requirement for DHA and EPA as they can not synthesis them *de novo*, but Atlantic salmon can metabolise a certain level of the required DHA and EPA from LNA (Bruce et al., 1999; Sargent et al., 2002), though some should be provided in the diet. This would suggest that vegetable oil, to a certain extent, could be used in the diets of these fish, although the incorporation of vegetable oil may lead to changes in the n-3/n-6 ratio that could compromise fish health by altering their metabolism (Montero et al., 2003) as omega-6 fatty acids compete with the omega-3 fatty acids for some metabolic pathways. Rapeseed oil has moderate levels of omega n-6 fatty acids and contains LNA (Bell et al., 2003; Torstensen et al., 2004), making it a favourable candidate for partial replacement in fish diets. Bell et al. (2003) found that the growth of Atlantic salmon post-smolts was not affected when rapeseed oil was used as a partial or full replacement of fish oil, although replacement above 50% resulted in a reduction in the levels of DHA and EPA and an altered n3/n6 ratio in the flesh.

Fatty acids and lipids have an extremely important role in egg and alevin production; for example DHA is essential for neural and membrane development of the embryo (Borlongan and Benitez, 1992; Bell et al., 1997; Sargent et al., 2002). Consequently the rapidly developing embryo must be supplied with adequate levels, to ensure proper development (Bell et al., 1997). It has also been suggested that the ratio of n-3/n-6 fatty acids and DHA/EPA influence egg quality (Sargent, 1996). DHA and EPA compete for a number of metabolic pathways and a change in levels of one could have implications on the function of the other (Mazorra et al., 2003).

Broodstock

During maturation, female broodstock convey a copious supply of nutrients into the developing egg. It has been shown that changes in dietary fatty acid profiles are

reflected in those of the egg (Mourente and Odriozola, 1990; Corraze et al., 1993). Using vegetable oils in broodstock diets could consequently have an effect upon egg and subsequent fry quality (Bruce et al., 1999).

The majority of nutrient manipulation trials conducted to date have been of a short-term duration, though the development of eggs in Atlantic salmon appears to be a year long process (Thorpe et al., 1998). During this year, female Atlantic salmon accumulate a large reserve of lipids and then undergo an enforced period of anorexia as they migrate back through freshwater (Love, 1970; Kadri et al., 1996). Gonadal development during this anorexic phase is achieved using the stored nutrients (Kadri et al., 1996); it would therefore be prudent to test any dietary manipulations on a long-term basis to fully investigate any effects on egg quality in Atlantic salmon.

Aims of the study

Broodstock nutrition is a largely understudied aspect of fish nutrition yet it has a bearing upon quality of farmed fish from the very beginning of the culture process. This investigation was undertaken to determine the effects of the long time replacement of a portion of fish oil with rapeseed oil in a broodstock diet on egg and initial offspring quality, in the widely cultivated species Atlantic salmon. Specifically we studied how using 50 % rapeseed oil in place of fish oil (at a dietary lipid level of 27.6 %) would influence the pre-fertilisation variables of absolute fecundity (as total egg number), egg size and chemical composition. We also studied how the diets influenced the viability of the offspring as measured by the post-fertilisation variables fertilisation, eyeing, hatching and first feeding survival rates and the first feeding fry weight and chemical composition.

Materials and Methods

Experimental design and general husbandry

At the end of September 2002, 315 2-year old Atlantic salmon (*Salmo salar*) (4577g ± 45 SEM), of the AquaGen Standard strain (supplied by AquaGen Hemme) were weighed and measured. Every fish had a unique Visible Implant Tags (Northwest

Marine Technologies, Shaw Island) inserted into the transparent tissue behind their left and right eyes, two tags being used to increase the chances of retaining identification. The fish were transferred to three sea cages (5 x 5 x 5 m) with 105, 104 and 106 in each (an oversight during stocking led to there not being 105 in each).

Two broodstock diets (Table 4.1) were produced that differed only in the level of South American fish oil, 100 % and 50 % (50 % replaced with rapeseed oil). The latter diet was produced to be almost equal to a 100 % Northern hemisphere fish oil diet with respect to DHA and EPA levels. A high protein broodstock diet, as used here, is generally believed to produce optimal egg quality. With this in mind and given the logistical constraints of such a long term trial the 100 % fish oil, control diet, was fed to only one cage while the experimental 50 % rapeseed oil diet was fed in duplicate in the remaining two cages.

Table 4.1. The diet formulations and compositions for the experimental (rapeseed oil) and control (fish oil) diets.

Ingredients %	Rapeseed oil	Fish oil
Fish meal	45.35	45.35
Fish oil (Anchovy)	10.50	21.00
Corn gluten	12.00	12.00
Soyabean meal (semi concentrate)	10.00	10.00
Wheat	10.00	10.00
Rapeseed oil	10.50	-
Premix (minerals, vitamins and pigments)*	1.65	1.65
	100	100
Composition		
Dry matter (%)	94.3	95.0
Crude protein (%)	47.4	48.0
Crude fat (%)	28.0	27.2
Ash (%)	7.9	8.0
Gross energy (MJ/kg)	24.09	23.83
Astaxanthin (mg/kg)	50.4	49.8

* Premix (minerals, vitamins and pigments) - ground wheat as carrier:
Vitamin A 155000 IU/kg, Vitamin D 120000 IU/kg, Vitamin E 20000 mg/kg, Vitamin K3 600 mg/kg, Vitamin B1 900 mg/kg, Vitamin B2 1500 mg/kg, Vitamin B6 1200 mg/kg, Niacin 3000 mg/kg, Pantothenic Acid 9000 mg/kg, Biotin 60 mg/kg, Vitamin B12 1.25 mg/kg, Folate 600 mg/kg, Betaine 60000 mg/kg, Vitamin C 15000 mg/kg, Fe 3600 mg/kg, Cu 300 mg/kg, Zn 7200 mg/kg, Mn 800 mg/kg, Astaxanthin 3350 mg/kg

The fish were fed daily to satiation by over feeding, 60-70 % of this was distributed using automatic feed hoppers (Sterner Fish Tec, Norway) with the rest given by hand. Food waste collection was not available so food intake could not be determined. The fish were kept under ambient conditions at the Nutreco Aquaculture Research Centre, Lerang, Jørpeland (59° 01.34'N, 6° 02.50'E). The average water temperature was 10 °C (min.: 4 °C, max.: 20 °C), salinity 27 g/kg (min.: 18 g/kg, max.: 32 g/kg) and oxygen level 7.5 mg/l (min.: 6.5 mg/l, max.: 9 mg/l) at a depth of 4 m.

Feed production and chemical composition

The diets were produced as extruded, sinking 9 mm pellets from a common source of meal mixture with a constant basal composition at the Nutreco Technology Centre (Stavanger, Norway). The pellets were vacuum coated, the control diet with American fish oil (anchovy) and the experimental diet with a combination of 50 % American fish oil (anchovy) and 50 % rapeseed oil. The two diets were formulated to contain a targeted level of 47.5 % crude protein and 27.5 % crude fat.

The diets underwent a number of analyses to determine the proximate, astaxanthin and fatty acid compositions. The level of crude protein was ascertained using the Kjeldahl method (Kjeltec Auto analyser, N x 6.25). The crude fat level was analysed after pre-extraction with diethylether and hydrolysis with hydrochloric acid and extraction by diethylether in a Soxtec (Tecator) extraction apparatus. Crude moisture was measured by drying the samples at 103 °C for 16 h and gross energy by using adiabatic bomb calorimetry (Parr 1271 Bomb calorimeter, Parr instrument Company, Moline, Illinois USA) at AKVAFORSK (Sunndalsoera, Norway). The crude ash content was established by combustion at 550 °C till constant weight. The astaxanthin content in the feed was analysed according to Weber (1988). The fatty acid profiles of the two diets can be seen in Table 4.2, these were achieved using the method of Grahl-Nielsen and Barnung (1985), in which they were methylated and extracted using methanolic hydrochloric acid before water and hexane were added to create two phases. The hexane phase was then diluted with iso-octane and analysed by gas chromatography.

Table 4.2. The fatty acid profile of the two diets as the mean % area of the total fatty acid content (N=2).

Fatty acid	Rapeseed oil	Fish oil
14:0	3.4	6.1
16:0	11.8	16.4
16:1n-7	3.7	6.3
16:2n-6	0.4	0.8
18:0	2.6	3.3
18:1n-9	27.7	9.6
18:1n-7	2.8	2.6
18:2n-6	12.2	5.8
18:3n-3	4.5	1.3
18:4n-3	1.5	2.6
20:1 (sum isomers)	2.9	3.2
20:4n-6	0.4	0.7
20:4n-3	0.3	0.6
20:5n-3 (EPA)	6.8	11.7
22:1 (sum isomers)	2.7	3.3
22:5n-3	0.8	1.4
22:6n-3 (DHA)	7.7	12.8
24:1n-9	0.5	0.6
Saturated F.A. not listed	1.0	1.1
Monoenes not listed	0.3	0.4
n-6 F.A. not listed	0.7	1.3
n-3 F.A. not listed	0.3	0.6
Others	1.3	2.5
Sum saturated F.A.	18.8	26.8
Sum monoenes	40.6	26.1
Sum n-6 F.A.	13.7	8.6
Sum n-3 F.A.	21.9	31
Unknown	3.6	5.0
Unsaturated/Saturated ratio	4.0	2.5
n-3/n-6 ratio	1.6	3.61
DHA/EPA ratio	1.13	1.09

Sampling

During the second week in November 2002, the fish were starved for two days and anaesthetised (MS222, 0.05 g/l) to check tag retention and to allow replacement tags to be inserted if required. The fish were weighed and measured at the beginning of May 2003 and at the end of June, at which point feeding was terminated. A problem with the net in one of the rapeseed oil treatments meant the fish had to be moved to an adjacent cage after the May sampling.

Transfer to freshwater

After three months of self-induced starvation at the end of September 2003 the mature fish from all cages (identified by their colour and kype development) were transferred to a circular 8m-land tank (84 m^3), with a salinity of 30.70 mg/l. This was reduced to 19 mg/l after two days and then to 0.5 mg/l after a further three days. The fish were identifiable by their unique tags. For stocking density reasons only 200 fish in total could be transferred. As a result of the 89 mature fish in the fish oil cage 18 random males and one female in poor condition were not transferred. While in one of the rapeseed oil cages 14 random males out of a total of 94 mature fish were not transferred and in the other rapeseed oil cage 24 random males and one female were not transferred from a total of 75 mature fish.

Fertilisation

On the 28th of October the females were tested for ripeness, indicated by the free production of eggs when they were gently squeezed in the anal area. The fish were left for 3 days, as it is believed optimal egg quality occurs between 3-6 days after the commencement of ovulation (Bromage, 1996). All females (N = 73, 28 from the fish oil cage and 21 and 24 from each rapeseed oil cage) that were ovulating were then sacrificed on the 31st of October (by brain destruction), bled and hung by the tail for a few hours to allow the blood to drain away from the eggs, preventing contamination. The females were then weighed, measured and identified by their tags. An incision was made from the anus to the buccal cavity of each female and the eggs were collected in individual receptacles and kept as individual batches. Each fish was then re-weighed to

establish the stripped weight (body weight of the carcass without eggs).
The eggs from 25 of these individuals were selected for fertilisation. This number was comprised of nine individuals from one of the vegetable oil cages, and eight individuals from each of the remaining two cages. Any batch of eggs found to be contaminated with blood was rinsed by gently adding a small amount of a sterile saline solution and gently swilling the blood to the surface and then lifting out the clots until only a minimal amount was left.

All the males present were anaesthetised, weighed, measured and stripped by repeatedly squeezing the posterior flanks from in front of the pectoral fin to the anal opening until no more milt was expelled. The milt was collected in individual receptacles that were placed immediately into a fridge set at 0 °C. Once all the milt was collected each sample was checked for motility under a microscope to make sure it was viable. A pool of milt for each of the three treatments was created using three random males from that treatment, then 3 ml of the pooled milt was added from a sterile 5 ml plastic syringe to each individual batch of corresponding eggs. Pooled milt was used to reduce the variation due to males within each treatment, so that each female from a treatment was equally fertilised by the males from the same treatment. A sterile hand was used to gently mix the eggs and milt thoroughly for one minute; they were then left for a further minute and subsequently rinsed with sterile saline solution, as previously. A sub-sample of eggs were removed from each batch and rinsed in the pH buffered iodine disinfectant Buffodine (Sterner Fish Tech A/S) for 10 min, then 100 ml of eggs from each female were measured out and transferred to freshwater, for hardening and development.

Egg number and chemical composition

A 200-hole egg plate counter was used to count the eggs from each fertilised female. The number of eggs removed from the fertilised batches was added in retrospect once their exact number was known. Sub-samples of 20 eggs from each female were blotted dry to remove the ovarian fluid and weighed on an individual basis. Before the eggs were fertilised a small, equally sized sample of approximately 10 ml, was taken from each of the 25 females for proximate, astaxanthin, and fatty acid analysis. The methods used were those described earlier in section 2.2 for the diets, except for the astaxanthin analysis, which was achieved using an internal Nutreco method based on that of Weber

(1988). Due to the logistics of processing large numbers of samples all the analyses were conducted on the eggs from one of the rapeseed oil cages (N=8) (the cage that was not transferred) and the fish oil cage (N=8).

Husbandry of eggs and fry

A common source of water supplied two parallel runways (220 x 43 x 18 cm) that contained four separate sections with sides made of perforated plastic to allow the water to flow through. Each section was comprised of four individual compartments (22 x 20 x 15 cm) constructed with perforated plastic. The fertilised eggs from each female were held in an individual compartment. The eggs and, after hatching, the fry were kept in continually darkness. Water temperature and pH were maintained at 6.83 °C (± 0.06 sem) and 6.38 (± 0.01 sem), respectively. The eggs were bathed in the anti-fugal treatment Pyceze (Vericore Ltd) regularly over a 19 week period according to directions.

Dead eggs were not picked till the day after eyeing commenced (at 294-degree days), as the eggs are very delicate prior to this point. Dead eggs were removed and counted and defined as not reaching eyeing. The remaining eggs were checked regularly and any mortalities were removed and counted, until hatching began at 521.8-degree days. Eggs that did not hatch, showed no signs of a developing embryo (had not eyed) and were still translucent and did not turn white were defined as unfertilised and counted. After hatching dead yolk-sac fry were removed and counted. When there were no external signs of the yolk sacs remaining, at 413.5-degree days post hatching (the recommended date to start feeding this strain of salmon) all fry were sacrificed (using a lethal dose of MS222). They were counted and defined as surviving to first feeding. Samples of 20 individuals from each female were blotted dry and weighed on an individual basis. A sample of the frys were also underwent proximate and fatty acid analysis as previously in section 2.2.

Data analysis

The Specific Growth Rate (SGR) was calculated from September-May (Sep.-May), May-June and September-June (Sep.-June) using the equation:

$$SGR = ((LN(Fwt)-LN(Iwt))/no.days)*100$$

Fwt = Final Weight Iwt = Initial Weight

The SGRs from Sep.-June were analysed by diet, cage and maturity rank (Mature or Immature) using Kruskal-Wallis tests, as the data did not have a normal distribution even after appropriate transformation.

Data on egg number and mean egg weight were normally distributed; so NESTED ANOVAs were used to test for cage effects. Egg number was then analysed using a one-way ANOVA with diet and a correlation was applied with stripped weight. A General Linear Model (GLM) with an interaction term for diet and stripped weight was used on mean egg weight.

Egg moisture, protein, fat, ash and astaxanthin content (N=8) were tested for normality and arcsine transformations failed to normalise the data. Subsequently T-tests and, in the case of non-normal data, Mann-Whitney tests were used to determine if any differences occurred between the egg compositions of the two dietary treatments. This was then repeated for the individual fatty acids.

The egg batches from two of the females from one of the rapeseed oil cages were severely affected by a fungal infection and were removed from the data sets. A number of quality survival parameters were calculated, for each remaining batch of eggs, using the equations below:

Total No. = no. unfertilised + no. dead before eyeing + no. dead before hatchings + no. dead fry + no. fry at the end

*Fertilised percentage = ((Total no. – no. unfertilised)/Total no.) *100*

*Eyed percentage = (Total no. – (no. unfertilised + no. dead before eyeing)/Total no.)*100*

*Hatched percentage = (Total no. – (no. unfertilised + no. dead before eyeing + no. dead before hatching)/Total no.)*100*

*First feeding percentage = (no. fry at the end/Total no.)*100*

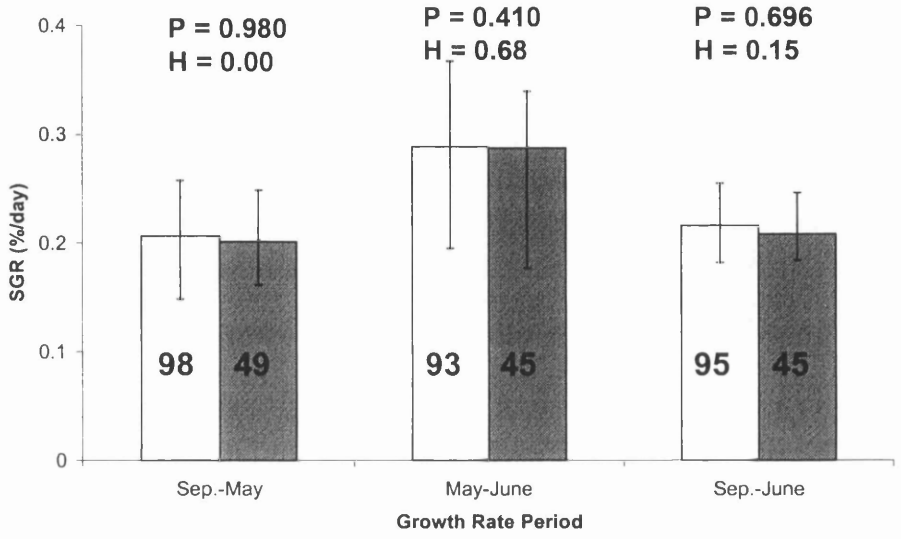
These data were then checked for normality, the eyed, hatched, and first feeding survival data were arcs transformed, but the fertilisation rate could not be normalised. NESTED ANOVAs were employed to the arcsine data to determine if there were cage effects and a Kruskal-Wallis test was used for the fertilisation data. One-way ANOVAs and a Kruskal-Wallis test were then used with dietary treatment. Finally correlations were used with each quality survival parameter, egg number and egg weight.

Fry weight was analysed as for the eggs for cage and diet effects. A correlation was performed for fry weight with egg weight. Fry composition and fatty acid profiles were analysed in the same way as the eggs. Finally T-test and Mann-Whitney (where applicable) tests were applied to the DHA-22:6n-3, EPA- 20:5n-3, 18:1n-9, 18:2n-6, 18:3n-3, n-3/n-6 ratio and DHA/EPA ratio of the eggs and fry within each dietary treatment.
All the statistical tests were carried out using MINITAB v13 (Minitab Inc. USA), the RSq values were established using EXCEL '97.

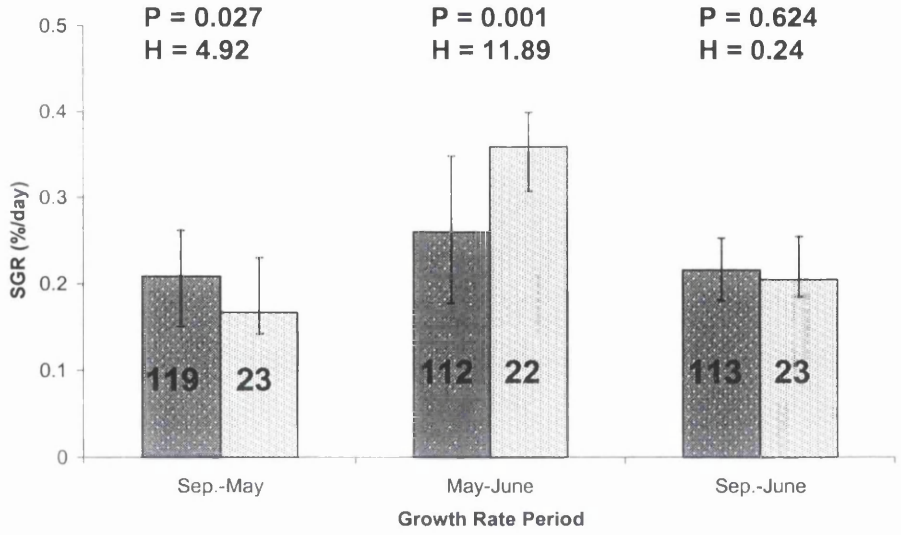
Results

Effect of diet on female growth

Diet had no significant effect on the SGRs for any of the periods measured (Sep.-May, May-June or Sep.-June; Fig. 4.1a). Cage only had an effect on the SGR from May-June ($P = 0.002$, $H = 12.31$), the post-hoc test showing this difference to be due to just one of the rapeseed oil cages. Mature fish had higher SGRs than their immature counter parts during Sep.-May; this was reversed during the May-June period. Overall though there were no differences between the two maturity categories for the period Sep.-June (Fig. 4.1b).



(a)



(b)

Figure 4.1. The median SGRs (with the inter quartile range) for each growth rate period with (a) diet□ rapeseed oil and■ fish oil and (b) maturity status ▩ mature and ▨ immature. Sample number is displayed along with the P and H values from the Kruskal-Wallis analysis.

Effect of diet on egg number, weight and chemical composition

Cage had no effect on egg number (nested ANOVA cage (diet) P = 0.736, $F_{1,24}$ = 0.12), and nor did diet (Fig. 4.2; one-way ANOVA P = 0.149, $F_{1,24}$ = 2.23). Egg number was strongly correlated with female weight in June (P = 0.000, r = 0.807, RSq = 0.651).

Mean egg weight was also not influenced by cage (nested ANOVA cage (diet) P = 0.723, $F_{1,24}$ = 0.13). When a GLM with an interaction term for diet and stripped weight was applied neither of the factors nor the interaction produced a significant effect on mean egg weight (diet P = 0.215 stripped weight P = 0.444 diet * stripped weight P = 0.336).

The proximate composition of the eggs was similar for both dietary groups (Table 4.3), although the eggs from the fish oil group had significantly higher levels of DHA and EPA, while those from the rapeseed oil group had significantly higher levels of 18:1n-9, 18:2 n-6 and 18:3 n-3 Table 4.4) in the lipid fraction. A significant difference was also found in the n-3/n-6 ratios of the two diets, yet not the DHA/EPA ratio (Table 4.4).

Effect of diet on egg and offspring viability

None of the egg quality parameters (fertilised, eyed, hatched, and first feeding survival data) were significantly influenced by cage (Kruskal-Wallis; fertilised, P = 0.76, H= 3.15, nested ANOVA cage (diet); eyed, P = 0.979, $F_{1,22}$ = 0.24; hatched P = 0.363, $F_{1,22}$ = 0.87; first feeding, P = 0.240, $F_{1,22}$ = 1.47) and no significant differences were found between the two dietary treatments for any of the quality parameters (Fig. 4.3a and b). Egg number and mean egg weights were uncorrelated with these quality parameters (Table 4.5). The mean fertilisation rates for both the rapeseed oil and fish oil treatments were very high 93.77 % ± 1.25 (SEM) and 92.38 % ± 1.23 (SEM), respectively. The rapeseed treatment survival proceeded to drop to 84.82 % ± 1.63 (SEM) at the first feeding stage, with the fish oil one dropping to 87.93 % ± 1.72 (sem) (Fig. 4.3b).

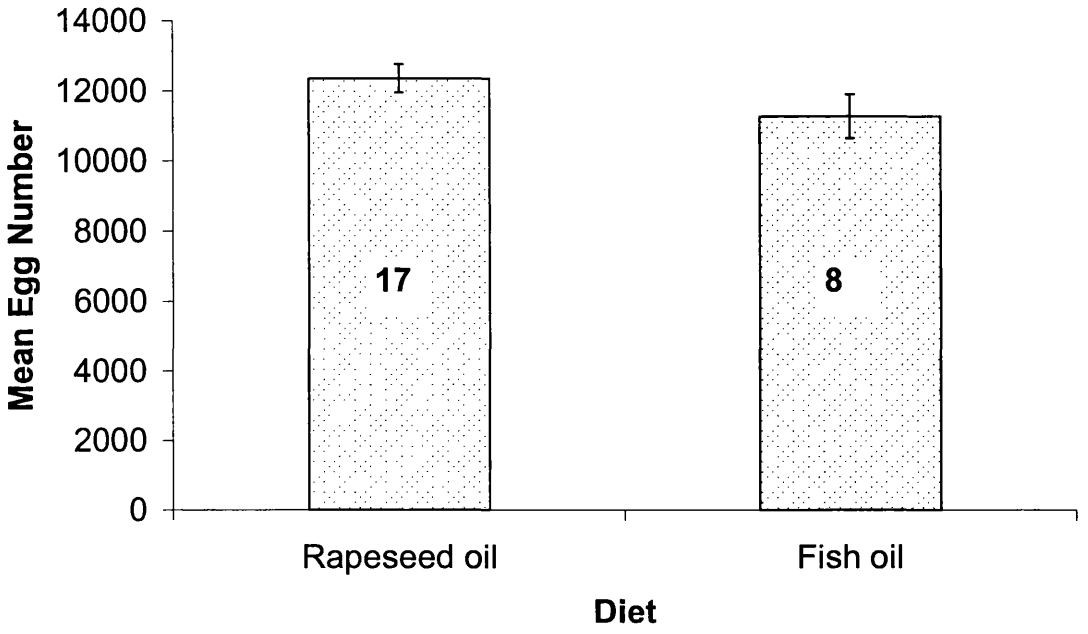


Figure 4.2. The mean egg number (± SEM) for each diet with the sample number displayed.

Table 4.3. The proximate compositions of the eggs and fry, from each dietary treatment, with the egg astaxanthin levels also included. Italics indicate a Mann-Whitney test was used instead of a t-test; all tests produced P values > 0.05.

Source	Composition (%)	Rapeseed oil	Fish oil
Egg	Moisture	*67.8 ± 0.47*	*66.6 ± 0.49*
	Protein	22.5 ± 0.37	23.0 ± 0.27
	Fat A.H.	6.3 ± 0.10	6.4 ± 0.15
	Ash	*1.7 ± 0.05*	*2.0 ± 0.20*
	Astaxanthin (mg/kg)	6. 8 ± 0.40	6.2 ± 0.36
Fry	Moisture	*83.5 ± 0.43*	*83.6 ± 0.44*
	Protein	11.7 ± 0.27	11.6 ± 0.65
	Fat A.H.	3.1 ± 0.12	2.9 ± 0.13
	Ash	1.4 ± 0.05	1.3 ± 0.26

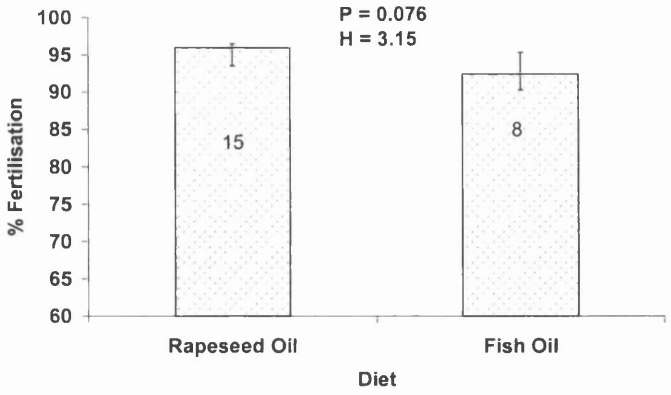
results as the mean (± SEM) (N= 8)

Table 4.4. The fatty acid profiles for the eggs produced by females from each dietary treatment as the mean (±SEM) % area of the total fatty acid content (N=8). A * indicates a P value of <0.05 produced by a t-test, or in italics a Mann-Whitney test.

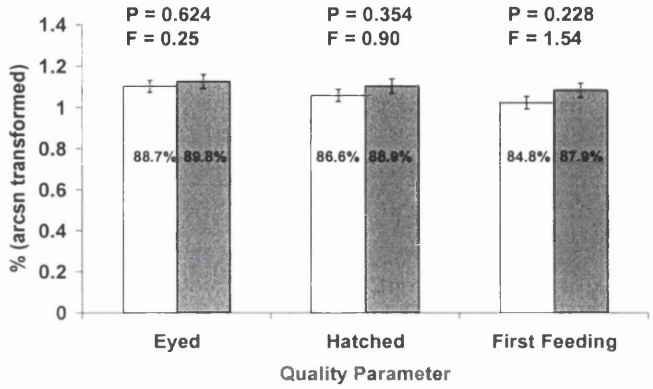
Fatty acid	Rapeseed oil	Fish oil
14:0	1.69 ± 0.02	2.13 ± 0.05 *
16:0	11.09 ± 0.06	12.28 ± 0.11 *
16: 1n-7	3.93 ± 0.09	5.15 ± 0.08 *
16: 2n-6	*0.13 ± 0.02*	*0.2 ± 0.00* *
18:0	6.13 ± 0.11	6.66 ± 0.15 *
18:1n-9	*22.73 ± 0.18*	*16.08 ± 0.18* *
18:1n-7	3.55 ± 0.03	3.75 ± 0.02 *
18:2n-6	*6.18 ± 0.14*	*4.09 ± 0.11* *
18:3n-3	*1.98 ± 0.05*	*1.04 ± 0.03* *
18:4n-3	*0.39 ± 0.01*	*0.46 ± 0.02* *
20:1(sum isomers)	2.46 ± 0.06	2.40 ± 0.07
20:4n-6	*1.38 ± 0.02*	*1.65 ± 0.02* *
20:4n-3	1.88 ± 0.06	2.41 ± 0.06 *
20:5n-3 (EPA)	7.40 ± 0.09	8.68 ± 0.08 *
22:1(sum isomers)	*0.28 ± 0.04*	*0.29 ± 0.01*
22:5n-3	5.38 ± 0.1	6.49 ± 0.07 *
22:6n-3 (DHA)	*14.80 ± 0.12*	*17.55 ± 0.21* *
24:1n-9	*0.25 ± 0.02*	*0.29 ± 0.01*
Saturated F.A. not listed	*0.24 ± 0.02*	*0.3 ± 0.00* *
Monoenes not listed	*0.2 ± 0.00*	*0.24 ± 0.02*
n-6 F.A. not listed	*1.7 ± 0.03*	*1.8 ± 0.02* *
n-3 F.A. not listed	*0.55 ± 0.02*	*0.49 ± 0.01* *
others	*0.64 ± 0.02*	*0.89 ± 0.01* *
Sum saturated F.A.	*19.14 ± 0.11*	*21.41 ± 0.21* *
Sum monoenes	33.75 ± 0.19	28.14 ± 0.26 *
Sum n-6 F.A.	*9.38 ± 0.16*	*7.13 ± 0.11* *
Sum n-3 F.A.	*32.34 ± 0.17*	*37.11 ± 0.2* *
Unknown	5.15 ± 0.07	5.36 ± 0.09
Unsaturated/Saturated ratio	*3.93 ± 0.03*	*3.39 ± 0.04* *
n-3/n6 ratio	*3.46 ± 0.07*	*5.22 ± 0.09* *
DHA/EPA ratio	2.00 ± 0.03	2.03 ± 0.04

Table 4.5. The results for the Pearson's and, in italics, Spearman's rank correlations for each of the post fertilisation quality parameters fertilised, eyed, hatched and first feeding survival with each of the listed factors N = 23.

	Fertilised		Eyed		Hatched		First Feeding	
Correlation	P	rs	P	r	P	r	P	r
Egg weight	*0.165*	*0.300*	0.715	0.081	0.767	0.065	0.981	0.005
Fry weight	*0.321*	*0.216*	0.149	0.311	0.107	0.345	0.197	0.279
Egg number	*0.691*	*-0.088*	0.819	-0.051	0.647	-0.101	0.598	-0.116



(a)



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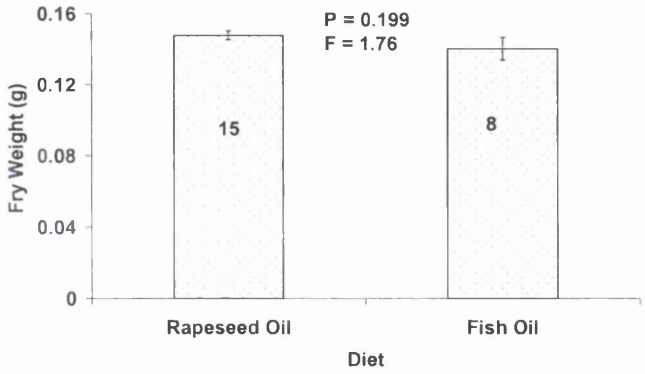


Figure 4.3. (a) Median (inter quartile range) fertilisation rates for each diet including the Kruskal-Wallis output (b) mean (± sem) arcs transformed eyed, hatched and survival to first feeding rates for each diet (□ rapeseed oil (N = 15) and ▩ fish oil (N =8)) including the one-way ANOVA output and (c) mean (± sem) fry weight for each diet including the one-way ANOVA output. The number of samples for each is also displayed.

Table 4.6. The fatty acid profiles for the fry produced by females from each dietary treatment as the mean (±SEM) % area of the total fatty acid content (N=8). A * indicates a P value of <0.05 produced by a t-test, or in italics a Mann-Whitney test.

Fatty acid	Rapeseed oil	Fish oil
14:0	1.66 ± 0.06	1.94 ± 0.06 *
16:0	13.62 ± 0.20	14.63 ± 0.26 *
16:1n-7	4.01 ± 0.14	4.85 ± 0.16 *
16:2n-6	0.08 ± 0.01	0.16 ± 0.02 *
18:0	5.91 ± 0.09	6.4 ± 0.14 *
18:1n-9	*23.64 ± 0.65*	*18.32 ± 0.72* *
18:1n-7	3.62 ± 0.05	3.84 ± 0.04 *
18:2n-6	6.30 ± 0.23	4.43 ± 0.23 *
18:3n-3	*1.86 ± 0.10*	*1.10 ± 0.09* *
18:4n-3	0.22 ± 0.02	0.32 ± 0.03 *
20:1(sum isomers)	1.53 ± 0.05	1.49 ± 0.05
20:4n-6	1.86 ± 0.05	2.12 ± 0.04 *
20:4n-3	1.8 ± 0.06	2.17 ± 0.08 *
20:5n-3 (EPA)	7.21 ± 0.14	8.12 ± 0.13 *
22:1(sum isomers)	0.05 ± 0.01	0.11 ± 0.02
22:5n-3	4.25 ± 0.13	5.09 ± 0.15 *
22:6n-3 (DHA)	15.68 ± 0.31	18.06 ± 0.34 *
24:1n-9	0.05 ± 0.02	0.12 ± 0.03 *
Saturated F.A. not listed	0.26 ± 0.02	0.4 ± 0.03 *
Monoenes not listed	0.18 ± 0.01	0.22 ± 0.01 *
n-6 F.A. not listed	1.52 ± 0.04	1.13 ± 0.03 *
n-3 F.A. not listed	0.37 ± 0.06	0.35 ± 0.04
Others	0.54 ± 0.02	0.72 ± 0.04 *
Sum saturated F.A.	21.45 ± 0.27	23.36 ± 0.37 *
Sum monoenes	33.06 ± 0.59	28.93 ± 0.63 *
Sum n-6 F.A.	9.76 ± 0.21	7.83 ± 0.22 *
Sum n-3 F.A.	31.39 ± 0.21	35.21 ± 0.22 *
Unknown	3.81 ± 0.12	3.95 ± 0.07
Unsaturated/Saturated ratio	3.47 ± 0.06	3.09 ± 0.07 *
n-3/n-6 ratio	*3.23 ± 0.11*	*4.53 ± 0.16* *
DHA/EPA ratio	2.18 ± 0.02	2.25 ± 0.04

Fry weight was not influenced by cage (nested ANOVA cage (diet) P = 0.912, $F_{1,22}$ = 0.01) or diet (Fig. 4.3c) but a strong correlation was found to exist with egg weight (P = 0.000, r = 0.780, RSq = 0.609). Like the eggs, the fry did not have significantly different proximate compositions in the two dietary treatments (Table 4.3), but the levels of the fatty acids in the lipid fraction differed significantly (Table 4.6). As in the eggs, the levels of DHA and EPA were higher in the fish oil fry, and the levels of 18:1n-9, 18:2n-6 and 18:3n-3 were higher in the rapeseed oil (Table 4.6). Again there was a significant difference in the n-3/n-6 ratio but a similar DHA/EPA ratio (Table 4.6).

Tables 4.2, 4.4 and 4.6 show that the level of DHA tended to be greater in the lipid fraction of eggs and fry than in the diet, for both of the dietary groups. In addition the level of EPA was similar in the diet, egg and fry of the rapeseed oil group and slightly decreased from diet to fry in the case of the fish oil. When comparing the levels of the main fatty acids between the egg and fry the DHA (t-test P = 0.026, T = -2.65), 18:1n-9 (Mann-Whitney P = 0.024) and the DHA/EPA ratio (P =0.001, T = -4.67) were significantly higher in the fry for the rapeseed oil group. The 18:1n-9 (Mann-Whitney P = 0.0013) and the DHA/EPA (t-test P = 0.003, T = -3.73) levels were also significantly higher in the fry for the fish oil group, as is the n-3/n-6 ratio (Mann-Whitney P =0.0014). The EPA levels are significantly lower when compared to those of the eggs (t-test P = 0.003, T = 3.69).

Discussion

A number of studies (mainly in marine fish) have shown that dietary manipulations can influence egg number, chemical composition and quality (Bromage et al., 1992; Fernandez-Palacios et al., 1995; Bruce et al., 1999; Furuita et al., 2000). The present study found no difference in the overall SGR, but there was a cage effect during the Sep.-May period, one of the rapeseed oil cages had a higher SGR than the other two. This was probably due to a change in feed intake (although it was not possible to measure this) after the problem with the net had been eliminated. The rapeseed oil eggs and fry that underwent the fatty acid analysis were not from this cage. No other factors were influenced by cage and so this cage was not removed from the other analyses. No significant differences were found in egg number, weight or proximate (moisture,

protein, lipid ash and astaxanthin) composition when 50 % of the dietary fish oil was replaced with rapeseed oil. The factor that produced the strongest influence on egg number was female weight in June, the point just prior to their onset of anorexia. Although replacing 50 % of the fish oil (South American), at a dietary lipid level of 27.6 %, with rapeseed oil did not affect the proximate composition of the eggs, which has also been shown to be the case in stripped trumpeter *Latris lineata* (Morehead et al., 2001) and in rainbow trout (Washburn et al., 1990), it did modify the egg fatty acid profiles. The use of rapeseed oil reduced the dietary levels of DHA and EPA, while elevating the levels of 18:1n-9, 18:2n-6 and 18:3n-3. This was reflected in both the eggs and the fry, as has been reported in other studies (Mourente and Odriozola, 1990; Corraze et al., 1993; Bruce et al., 1999; Morehead et al., 2001). Yet the type of diet did not have an affect upon the fertilisation, eyeing, hatching and first feeding survival; these were all equivalent for both treatments and remained high through out development (all in excess of 84.8 %). Corraze et al. (1993) also found no difference in survival of the offspring from rainbow trout fed diets with differing levels of fatty acids. However this is in contrast to the results published by Fernandez-Palacios et al. (1995) for sea bream *Sparus aurata* in which broodstock fed diets with lower lipid levels of DHA produced inferior quality eggs in terms of fertilisation rate when compared to a higher level of DHA. This could be due to a species difference, with the marine species being more sensitive to changes in fatty acids due to their inability to chain elongate fatty acids to produce DHA and EPA (Sargent, 1996). The fact that sea bream are continual spawners and readily incorporate nutrients directly from feed into egg development when compared to batch spawners with longer development phases such as Atlantic salmon (Fernandez-Palacios et al., 1995) could also explain this disparity between the two trials. Another possibility to explain the similar survival potentials of two diets in this study is the DHA/EPA ratio. Sargent (1996) reported that the ratio of DHA/EPA is important in egg biochemistry. The ratios of DHA/EPA in the present study were similar for both diets, while there was a trend for those reported in the Fernandez-Palacios et al. (1995) study to be different, increasing with the increasing DHA level.

Sargent (1996) recommends a dietary DHA/EPA ratio of is 2:1, while those supplied in the diets used in the present study were on average 1.11 these ratios increased to 2.00 and 2.03 in the rapeseed and fish oil eggs, respectively. This suggests that Atlantic

salmon are able to actively select DHA into their eggs and that the ratio suggested by Sargent (1996) is a good conservative recommendation.

Sargent (1996) also states that the ratio of n3/n-6 is important in egg biochemistry and that reductions in the levels could have implications for egg quality, recommending a ratio of 5:1 in feed. The ratios supplied in the rapeseed diet and fish oil diet used in this study were 1.6 and 3.61, respectively, yet this did not affect the egg number, weight or survival potential, with the ratios of 3.45 in the rapeseed eggs and 5.21 in the fish oil eggs. This implies that the Atlantic salmon used here were able to adjust the ratios they were provided with when producing their eggs. However the rapeseed oil fed fish did not achieve the ratio of 5:1.

Egg weight but not dietary treatment affected fry weight. The proximate (moisture, protein, lipid and ash) composition of fry, like that of the eggs, did not differ between the diets and the fatty acid profile followed that of the diet. When the levels of DHA and EPA are compared from diet to egg and fry there is an increase in DHA, while EPA appears to remain constant. This is evidence of a selective retention of DHA in the eggs and fry; such retention has been shown in Atlantic halibut (Mazorra et al., 2003) and sea bass (Bruce et al., 1999). This increase from dietary levels would be expected due to the important role DHA plays in egg and subsequent fry development. The results presented here also show that in the rapeseed oil group there is a significant increase in the amount of DHA from the egg stage to the fry stage. This is in contrast to results published by Vázquez et al. (1994) for the Senegal sole *Sola senegalensis*, in which a significant decrease in the DHA between egg and fry was detected. This would indicate that Atlantic salmon fry are able to selectively retain DHA in the lipid fraction.

In summary this study indicates that although differences in the fatty acid profiles are produced in the eggs and fry this does not influence the egg number, weight, survival potential or fry weight at first feeding. It indicates that female Atlantic salmon are able to concentrate essential fatty acids into egg production and to selectively manipulate their ratios. It also emphasises the significance of the DHA/EPA ratio. The DHA/EPA ratio has been linked to fry susceptibility to external stressors as it may influence the quality of the sensory organs (Sargent, 1996), which was not broached in this study.

Although these dietary manipulations did not affect egg and survival potential and growth there could be possible effects on later development.

Chapter 5

Effect of dietary protein and lipid levels on growth, feed efficiency and flesh quality of large rainbow trout (*Oncorhynchus mykiss*): Part I Population level analysis

Summary

The effects of feeding large (1183.1 g ± 2.7 SEM) 2-year old rainbow trout (*Oncorhynchus mykiss* L.) with varying levels of dietary protein and lipid at different feeding rates were investigated between September 2001 and January 2002. The diets used had crude protein and lipid compositions (by %) of 45.2:38.7 (HP:HL), 45.4:27.9 (HP:LL), 39.3:39.1 (LP:HL), 39.9:28.1 (LP:LL), 45.4:32.9 (HP:ML), 41.7:35.3 (MP:ML), and 38.7:32.8 (LP:ML) respectively. The first four listed were fed at three different ration levels (100 %, 80 % and 40 %). The amount of food eaten and specific growth rate fell and the food conversion ratio increased later in the study, probably due to the fact that the fish became sexually mature by the end of the study. DP/DE ratio did not influence the food conversion ratio, specific growth rate or the fat levels in the fillet. However increasing the lipid (energy) intake did improve the specific growth rate and food conversion ratio, though it also increased the level of fat in the fillet. There was no interaction between ration and diet, but ration level did influence the food conversion ratio, specific growth rate and fillet fat levels. The 40 % ration had higher food conversion ratios and lower specific growth rates and fillet fat levels than the higher rations.

Introduction

Salmonid diets

Most salmonid species, including the rainbow trout *Oncorhynchus mykiss* have a high dietary requirement for protein. However a number of concerns can arise from the use of high protein diets. Protein is expensive and providing it in large amounts can induce environmental concerns, both through the discharge of excess nitrogen and ammonia from the excretions released by the fish and waste food not consumed (Rasmussen and Osternfield 2000). Also if there is a superfluous amount of protein in conjunction with an inadequate level of energy in the diet the protein will be used less efficiently (Reinitz et al., 1978; Weatherup et al., 1997). Consequently there is an interest in finding ways to make protein utilisation more efficient.

Protein sparing

Fat contains almost twice as much energy per unit weight as protein and almost two and a half times as much as carbohydrates (Hilton and Slinger, 1981), although fish will usually obtain their energy from protein followed by fats and finally carbohydrates (Jackson, 1991). It is possible to force the fish to metabolise fat instead of protein as an energy source by manipulating the protein/energy ratio. Providing fat in abundance, maintaining the level of protein at that required for maintenance and protein accretion and no more, results in what is known as 'protein sparing' (Jackson 1991). Replacing protein in this way in fish diets increases the protein efficiency and reduces nitrogen excretions (Reinitz et al., 1978). This in turn will lead to an increase in protein utilisation, better growth, food conversion and reduced nitrogen pollution (Arzel et al., 1994). It has been known for a number of years that salmonids are capable of 'protein sparing' (Lee and Putman 1973; Beamish and Medland 1986), although it has been suggested that this ability decreases as size increases (Beamish and Medland 1986).

A number of studies on salmonids, mostly using small fish (<10 g - < 1 kg), have successfully replaced a portion of dietary protein with fat without compromising

growth, (Beamish and Medland, 1986; Hillestad and Johnsen 1994; Weatherup et al., 1997; Hillestad, et al., 1998; Jobling et al., 1998). Growth is only part of the culture process, the fish produced must be of a marketable quality. This quality is dependent upon the levels of fat found in the body. A complex size-species-dependent relationship exists between dietary lipid levels and levels of fat stored in the muscle (Regost et al., 2001). Some studies have found that increasing the level of lipid did not lead to an increase in stored fat levels. For example Jobling et al. (1998) found that in rainbow trout weighing about 90 g body and visceral fat levels were not influenced by increasing dietary fat levels from 12.6 % (gross energy, 21.2 MJ/kg) to 27.5 % (gross energy, 24.4 MJ/kg). However other studies have found that increased levels of dietary fat increase the levels of fat in whole body (2.1 g rainbow trout fed diets containing 7 – 20.7 % fat, gross energy 16.9 – 21.5 MJ/kg Reinitz et al., 1978) and or fillet fat (2.5 kg Atlantic salmon fed diets containing 25.6 – 38.9 % fat, 23.6 – 26.2 MJ/kg^{-}1 of gross energy, Einen and Skrede, 1998 and 11 – 26 % fat, 21.3 – 23.8 MJ/kg gross energy, Regost et al., 2001). Thus more research is still required.

Interaction effect between ration and diet

Another factor that is believed to influence productivity and quality of fish is the ration that they are fed (Storebakken and Austreng, 1987; Shearer, 1994). Reinitz (1983) found that rainbow trout (initial weight 2.1g) fed a high ration had higher weights when compared to a low and medium one. An interaction between diet composition and ration has been reported. For example Reinitz (1983) reported that rainbow trout fed a low fat (7.2 %):low protein (33.6 %) diet (gross energy, 17.4 MJ/kg) at a high rate produced higher weights than those fed a high fat (13.6 %):high protein (52.8 %) diet (gross energy 19.98 MJ/kg) at a low rate. Few studies manipulating dietary protein and lipid levels, at different feeding rates, have been conducted with rainbow trout, especially using fish in excess of 1 kg.

Aims of the study

This study was conducted using 2-year-old rainbow trout with an average body

weight in excess of 1kg, with the aims of: (1) examining the effect of dietary protein and lipid levels on various measures of productivity and fillet fat levels and (2) examining the possible interaction between diet composition and ration on growth, food intake and fillet fat levels. In this paper the presented results are based on the population means; a companion paper looks at their effects on body condition and flesh quality at the individual level.

Materials and Methods

Our broad strategy was to manipulate the level of protein (P) and lipid (L) in a series of diets and to feed a subset of them at different rations. The main manipulation involved two nutrient levels high (H), and low (L) used in various combinations with the extremes defined by a HP:HL and a LP:LL diet, between which were a LP:HL and LP:HL diets. These diets were then fed at three different rations, satiation (100 %), a 80 % ration and a 40 % ration. The series was completed using a medium (M) level in three further diets MP:ML, LP:ML and HP:ML fed at the 100 % rate only. The diet compositions can be seen in Table 5.1. Yttrium oxide (100 ppm) was added to each diet so that the digestibility of protein, lipid and energy could be established.

Fish husbandry

The trial was conducted at the Hendrix SpA fish trial unit (Mozzecane, Italy 45°18.34′N 10°49.02′E) from the 3rd September 2001 to the 21st January 2002. Metal grids were employed to divide 16 tanks in half to produce 32 tanks (3 m x 1 m x 1 m) of which 24 were used. The half with the water inflow (pumped directly from an underground spring) was designated upstream and the half with the outflow was designated downstream. Each tank contained 60 2-year-old rainbow trout (1183.1 g ± 2.7 SEM). The DO_2 content of the water throughout the trial was 8.75 (± 0.07 SEM) in the upstream end and 7.56 (± 0.08 SEM) in the downstream end, while the temperature was a constant 13.68 °C (± 0.03 SEM). The fish were exposed to the natural photoperiod.

Table 5.1. The formulations and compositions of all seven experimental diets.

Ingredients %	HP:HL	LP:HL	HP:LL	LP:LL	MP:ML	LP:ML	HP:ML
Low Temp. Fishmeal (Scandinavia)	57.44	47.98	55.05	45.55	51.73	43.24	56.48
Fish Oil	30.66	31.43	20.59	21.36	27.02	27.71	26.63
Wheat	11.35	19.84	23.87	32.62	20.73	28.28	16.36
Mineral and Vitamin premix*	0.62	0.81	0.55	0.54	0.59	0.83	0.59
Total	100	100	100	100	100	100	100
Composition							
Dry Matter (%)	94.70	93.20	92.00	92.40	92.40	94.30	94.00
Crude Protein (%)	45.20	39.30	45.40	39.90	41.70	38.70	45.40
Crude Lipid (%)	38.70	39.10	27.90	28.10	35.30	34.80	32.90
Ash (%)	9.40	8.30	8.40	7.60	8.70	7.90	9.60
Gross Energy (MJ/kg)	26.82	26.66	24.63	24.43	25.79	25.9	25.73
Astaxanthin (mg/kg)	47.10	46.30	46.80	46.10	46.50	46.20	47.00

*includes Yttrium oxide and astaxanthin

Food production

The diets were produced as extruded, sinking 9mm pellets from a common source of raw materials with the relevant inclusions of protein and other nutrients at the Nutreco Technology Centre (Stavanger, Norway). The pellets were then vacuum coated with the relevant level of oil and shipped (frozen) to Italy. Proximate analyses were conducted on the diets to establish the crude levels of protein, fat, and energy. Protein levels were determined using the Kjeldahl method (Kjeltec Auto analyser N x 6.25). The fat levels were ascertained after pre-extraction with diethylether and hydrolysis with hydrocholric acid and extraction by diethylether in a Soxtec (Tecator) extraction apparatus. Crude moisture was measured by drying the sample at 103 °C for 16 hours and gross energy by using adiabatic bomb calorimetry (Parr 1271 Bomb calorimeter, Parr instrument Company, Moline, Illinois USA) at AKVAFORSK (Sunndalsoera, Norway). Combustion at 550 °C till constant weight produced the ash level and the astaxanthin content was analysed according to Weber (1988).

Food distribution

The food was distributed by hand once a day from Monday to Saturday between 0900 and 0930 am until the fish finished feeding. In the 100 % tanks feeding was stopped when a few pellets became visible on the bottom, indicating the fish were no longer interested in the food. The lower rations were calculated from the previous day's 100 % intake, as the mean of the two relevant tanks. The 80 % and 40 % rations were fed gradually to make sure the whole ration was consumed. Fish in the downstream tanks were fed first (these contained the lower rations of the relevant diets found in the upstream end), to prevent the DO_2 level being reduced by the feeding action in the upstream tanks, which could have affected the intake. Although being able to detect the feeding actions in the downstream end prior to their food being introduced could have affected the upstream feeding behaviour. Once these fish had finished eating the upstream tanks were fed.

Sampling

The first sampling took place on the 5th November 2001 (the mid-point of the trial). This part of the trial was defined as the first period. A reduction in the stocking density was required to provide enough space for the fish to grow. The fish were not fed on the two days prior to the sampling, allowing enough time for the gut to be emptied. Of the 60 fish in each tank 20 were bulked-weighed and returned to the tanks, 20 were anaesthetised (120 ppm (mg/ml) of Finquel MS222 CF01), individually weighed and measured then tagged with a Visible Implant (VI) tag (Northwest Marine Technologies Ltd, Shaw Island WA) behind the left eye and returned to the tanks. To produce an equal number of fish in all tanks, allowing for previous mortalities, 15 fish from all 100 % tanks and 10 fish from all 80 % and 40 % were bulk weighed and disposed of. Finally five fish from all 100 % tanks and 10 from all 80 % and 40 % tanks were killed (by brain destruction) weighed, measured and frozen (-20 °C), for later flesh quality analyses. Each tank then contained 40 fish (20 marked) and feeding was resumed two days later.

The next sampling took place at the end of the trial on the 21st January 2002. The period up to that date was defined as the second period. All the fish were anaesthetised (as previously). Individual samples were taken as described above these were composed of the first tagged fish to be netted, all remaining fish were individually weighed and measured. The fish from the 80 % and 40 % were then disposed of while the 100 % fish were returned to their tanks and fed as usual for faecal stripping.

Faecal stripping, feed digestibility and availability

In week 21 of the trial, the fish in each 100 % tank were fed as normal in the morning, then during the afternoon they were anaesthetised (as previously) and were stripped of their faeces. The faeces were pooled by diet. Insufficient matter was collected at this first attempt, so it was repeated in week 22 and the samples combined. The frozen faecal samples were sent to Jordforsk (Norway) to determine the level of Yttrium oxide (100 ppm) present in the faeces. The digestibility of the feed was derived on an Apparent Digestible Coefficient

(ADC) for the purposes of establishing the exact amount of nutrients available to the fish:

$$ADC = 100 - 100 * \frac{\%\ maker\ feed}{\%\ marker\ faeces} * \frac{\%\ nutrient\ faeces}{\%\ nutrient\ feed}$$

The amount of digestible protein (DP), lipid (DL) and energy (DE) available to the fish on a %dry matter basis (DM) was then derived using the equation:

*(ADC faecal nutrient * crude nutrient) / 100*

Sample processing and the measurement of fat in the fillet

The fish were defrosted and filleted on both the left and right side; all remaining visible fat and bones were removed. The left fillet was then skinned and homogenised in a Moulinex food processor, bagged, frozen and sent to Nutreco's ARC laboratory in Stavanger Norway. The levels of fat in the flesh were established using the methods described in section 2.2.

Variables measured

A number of variables were established for each tank:

(a) Average food intake per fish on a dry matter basis:

Food intake / no. of fish

(b) Average intake of DP, DL and DE per fish:

*(Average food intake / no. of fish) * DP, DL or DE*

(c) Average FCR:

((Total feed eaten / no. fish)/(total weight difference/no. fish)

(d) Average SGR:

$((ln_{fw} - ln_{iw}) / no.\ days) * 100$

where fw = total final weight / no. fish and iw = total initial weight / no.fish

Statistical analysis

Data that did not have a normal distribution was transformed and if this was not possible non-parametric tests were used. Post–hoc tests for Kruskal-Wallis analyses were carried out according to Zar (1996). The experiment was designed to use regression analyses with diet effects, but the diet ration interaction was analysed using a general linear model (GLM). One of the 40% tanks produced a very high FCR of 4.45 for some unexplainable reason, but for the purposes of the interaction model it needed to be included in the analysis.

Food, nutrient and energy intakes in relation to period and diet

One-way ANOVAs were used to determine whether diet affected the overall food intake or DP, DL and DE intakes during both periods in the 100 % ration tanks. A paired t-test was used to determine if there was a difference in the amount of food eaten between the two periods.

FCR, SGR and fillet fat level in relation to period and diet

Only the 100% data was used in these analyses. Paired t-tests were used to determine if there was a difference in the FCR, SGR and fillet fat level between the two periods. Linear regressions were used, with each 100 % tank as a separate datum point to study relationships between the DP/DE ratio and FCR, SGR and fillet fat level, in both periods.

Multiple regressions were used with both protein and lipid intakes as the dependent variables and FCR, SGR and fillet fat as the response variables. Gutted weight was added as a covariant for fillet fat level in the second period, but not the first as transformations could not normalise its distribution in this period. Regressions were then used with lipid, protein and energy intakes as a single predictor of FCR, SGR and fillet fat.

FCR, SGR and fillet fat level in relation to diet ration interact*ions*

To determine if there was an interaction between diet and ration General Linear

Models (GLM) were used with SGR and fillet fat level for both periods, and FCR in the second period. Gutted weight was added to the GLM as a covariant for the fillet fat level. One-way ANOVAs were then used to look at the effect of ration. Transformation did not normalise the FCR data during the first period so a Kruskal-Wallis test was used to relate this to ration.

All the statistical tests were carried out in MINITAB v13 (Minitab Inc. USA) and EXCEL ' 97.

Results

Food, nutrient and energy intakes in relation to period and diet

There was a significant difference in the total food intake between the first and second periods (paired t-test P = <0.001, T = 9.89). On the whole there was a reduction in the food intake, but for the HP:LL diet there was an increase (Table 5.2). Total food intake was affected by diet in the second period only. This difference was largely due to a higher intake of the HP:LL diet (Table 5.2). In the first period (when food intake for the different diets was similar) intakes of protein, lipid and the DP/DE ratio mapped that of diet composition (Table 5.2). During the second period protein and lipid intakes of the HP:LL diet exceeded those of all other diets except HP:HL, the rest mapping the dietary composition again (Table 5.2). Energy intake was similar for all diets in the first period, but in the second period, fish on the HP:LL and HP:HL diets had significantly higher intakes (Table 5.2).

FCR, SGR and fillet fat level in relation to period and diet

Both the SGR (paired t-test P = 0.000, T = -6.42) and FCR (paired t-test P = 0.000, T = 10.52) were significantly different between the two periods, SGR decreasing and FCR increasing as the study proceeded.
None of the linear regressions performed between DP/DE ratio and FCR, SGR and fillet fat were significant for either period (Table 5.3).

Table 5.2. The means (±SEM) of each listed factor for all the diets, at the 100% level, with the results of the one-way analyses for both periods, different letters indicate where the differences lie within each variable and each period (DF = 6,15).

Per.	Factor	HP:HL	LP:HL	HP:LL	LP:LL	MP:ML	LP:ML	HP:ML	P
1st	Total Food (kg)	0.574 ± 0.016	0.504 ± 0.026	0.546 ± 0.004	0.48 ± 0.251	0.463 ± 0.003	0.463 ± 0.033	0.52 ± 0.07	0.198
	Protein (kg)	0.24 ± 0.01 **a**	0.18 ± 0.01 **bc**	0.23 ± 0.005 **a**	0.17 ± 0.01 **c**	0.18 ± 0.003 **c**	0.17 ± 0.012 **c**	0.22 ± 0.03 **ab**	0.013
	Lipid (kg)	0.21 ± 0.01 **a**	0.18 ± 0.01 **ab**	0.14 ± 0.00 **c**	0.13 ± 0.01 **c**	0.16 ± 0.00 **bc**	0.157 ± 0.01 **bc**	0.16 ± 0.02 **bc**	0.012
	Energy (g/MJ^{-1})	14.48 ± 0.4	12.38 ± 0.63	12.44 ± 0.09	10.53 ± 0.56	11.10 ± 0.08	11.06 ± 0.78	12.26 ± 1.74	0.071
	DP/DE (g/MJ^{-1})	16.57 ± 0.23 **b**	14.54 ± 0.07 **d**	18.09 ± 0.28 **a**	16.15 ± 0.1 **bc**	15.91 ± 0.19 **c**	15.07 ± 0.12 **d**	17.97 ± 0.1 **a**	0.000
2nd	Total Food (kg)	0.576 ± 0.01 **ab**	0.462 ± 0.05 **b**	0.698 ± 0.012 **a**	0.456 ± 0.004 **b**	0.466 ± 0.06 **b**	0.450 ± 0.04 **b**	0.475 ± 0.02 **b**	0.024
	Protein (kg)	0.24 ± 0.00 **ab**	0.17 ± 0.02 **c**	0.29 ± 0.01 **a**	0.165 ± 0.01 **c**	0.177 ± 0.02 **c**	0.157 ± 0.02 **c**	0.2 ± 0.01 **bc**	0.002
	Lipid (kg)	0.21 ± 0.00 **a**	0.17 ± 0.02 **abc**	0.18 ± 0.00 **ab**	0.12 ± 0.00 **c**	0.157 ± 0.02 **bc**	0.15 ± 0.01 **bc**	0.145 ± 0.01 **bc**	0.044
	Energy (g/MJ^{-1})	14.5 ± 0.2 **ab**	11.3 ± 1.2 **bc**	15.9 ± 0.29 **a**	10.03 ± 0.09 **c**	11.2 ± 1.43 **c**	10.76 ± 0.96 **c**	11.3 ± 0.56 **bc**	0.029
	DP/DE (g/MJ^{-1})	16.52 ± 0.22 **b**	14.57 ± 0.22 **d**	17.95 ± 0.002 **a**	16.46 ± 0.36 **b**	15.82 ± 0.06 **c**	14.56 ± 0.08 **d**	17.7 ± 0.008 **a**	0.000

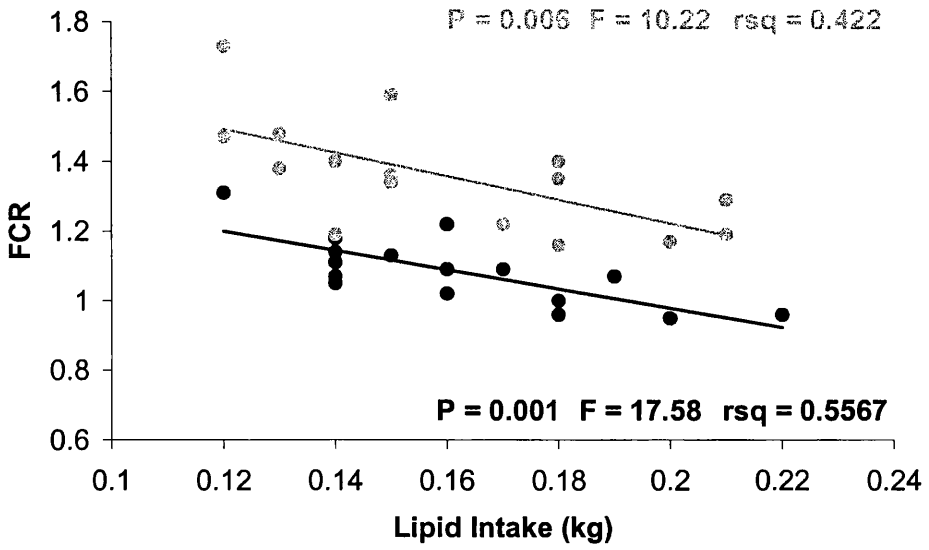
Table 5.3. The linear regression results between the DP/DE ratio and each of the listed variables for both periods (N =16).

Period	Variable	P	F	rsq
1st	FCR	0.962	0.00	0.0203
	SGR	0.166	2.13	0.132
	Fillet Fat	0.872	0.03	0.0189
2nd	FCR	0.598	0.29	0.0002
	SGR	0.172	2.07	0.129
	Fillet Fat	0.612	0.27	0.0019

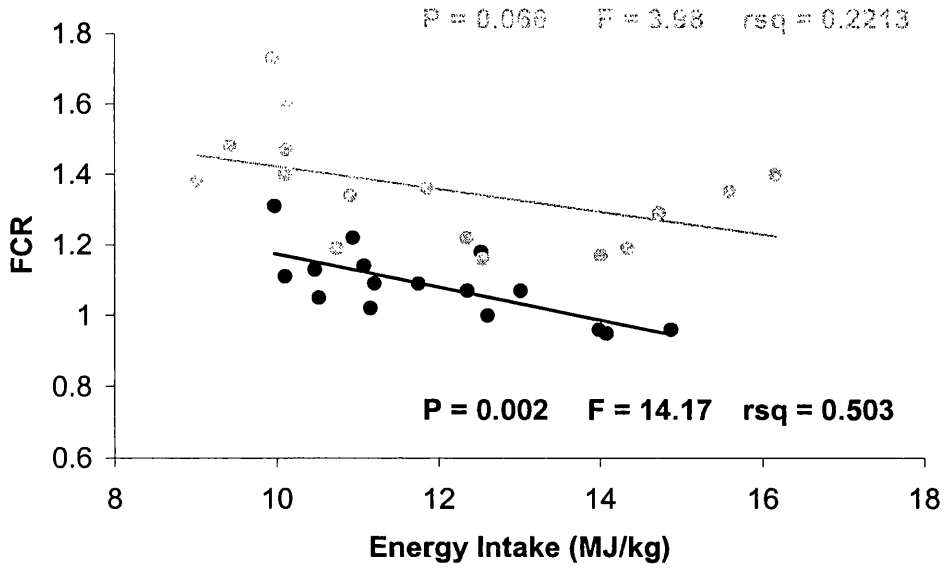
Multiple regression analyses showed that lipid but not protein intake was a significant determinant of FCR in both periods (Table 5.4). Higher lipid intakes were associated with lower FCRs (Fig. 5.1 a), and hence energy intake was also a significant predictor of FCR, a higher intake being associated with a lower FCR (Fig. 5.1 b).

SGR was related to both the protein and lipid intakes in the first period, but only to protein in the second (Table 5.4), higher SGRs being associated with higher intakes (Fig 5.2 a and b). Energy intake again had a significant influence, the greater the intake the greater the SGR (Fig. 5.2 c).

During the first period only lipid intake was significantly related to the level of fat found in the fillet (Table 5.4), an increased lipid intake produced a higher level of fillet fat (Fig. 5.3 a). In the second period neither lipid or protein intake was related to fillet fat level (Table 5.4). Energy intake was positively related to the fat level in the first period but not the second (Fig. 5.3 b).



(a)

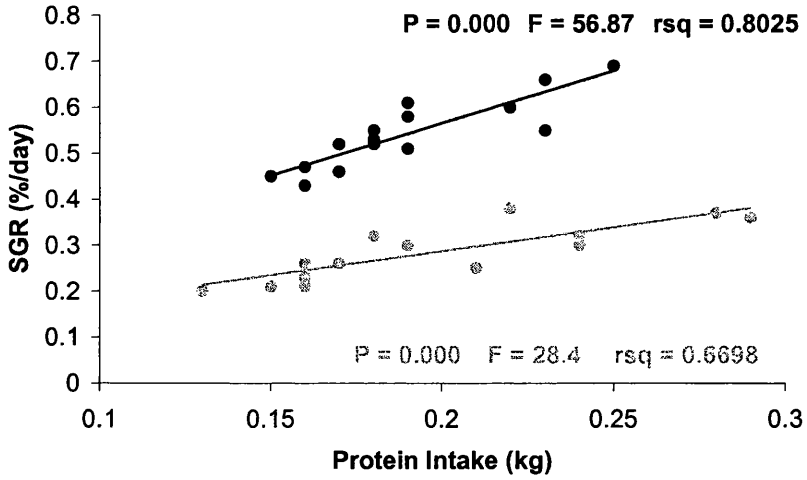


(b)

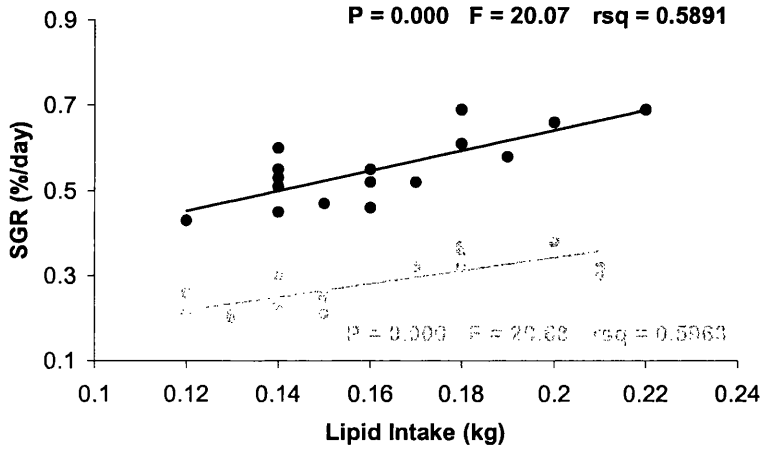
Figure 5.1. Regression analyses of FCR with each intake (a) lipid and (b) energy for the 100% ration level data in the first (●) and second (◎) periods (N = 16).

Table 5.4. The results of the multiple regression analyses used to define the relationships between both the FCR and SGR and protein and lipid intakes and between fillet fat level, lipid and protein intakes and gutted weight at the 100% ration level for both periods.

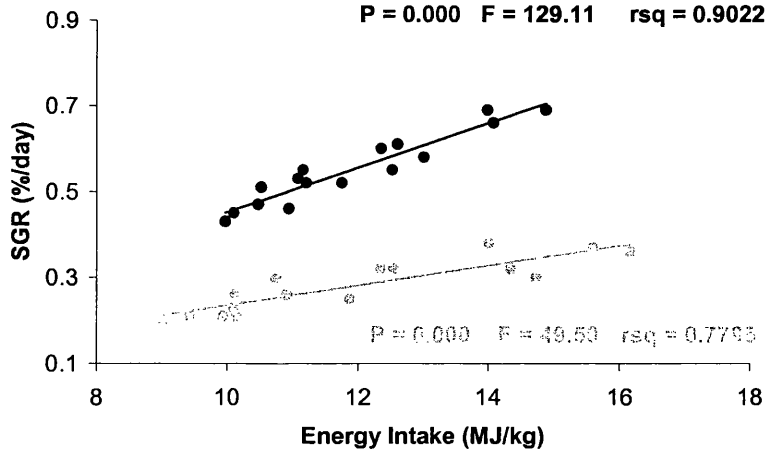
		FCR			SGR			Fillet Fat		
Period		P	F	rsq	P	F	RSq	P	F	rsq
1st	Regression	0.002	10.09	0.608	0.000	62.94	0.906	0.018	5.87	0.462
	Protein	0.214			0.000			0.703		
	Lipid	0.014			0.002			0.025		
2nd	Regression	0.02	5.33	0.450	0.000	18.35	0.738	0.128	2.31	0.366
	Protein	0.428			0.020			0.797		
	Lipid	0.016			0.088			0.902		
	Gutted weight	-			-			0.054		



(a)

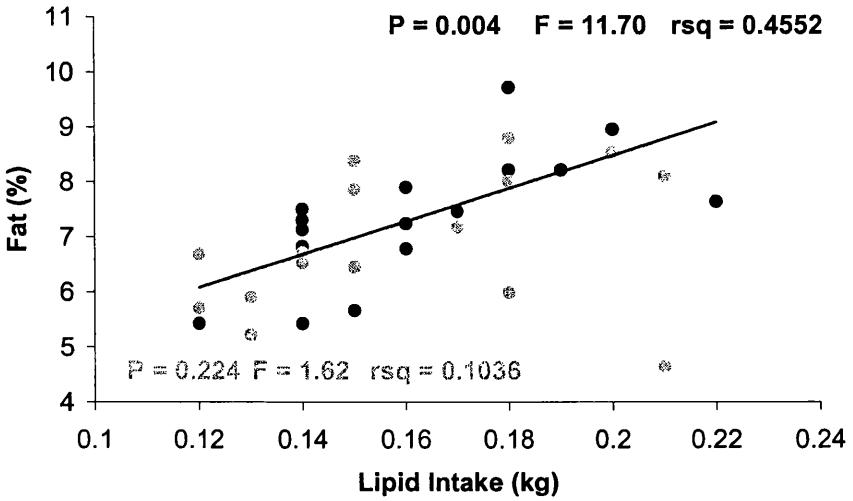


(b)

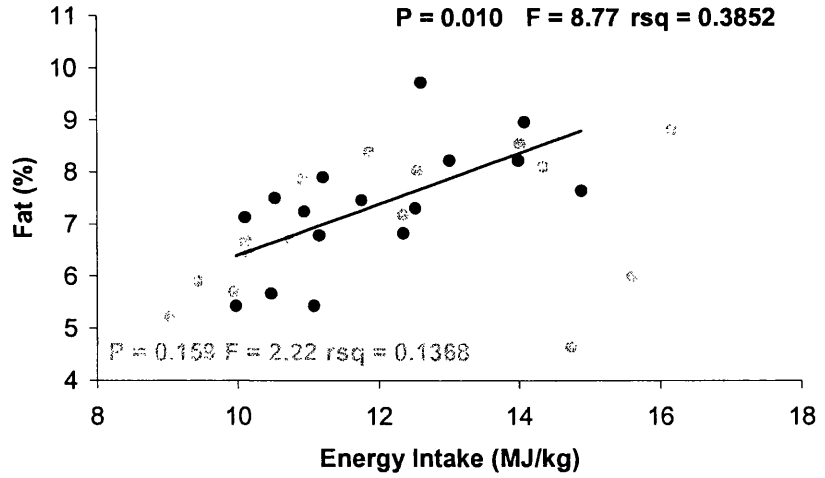


(c)

Figure 5.2. Regression analyses of SGR with each intake (a) protein, (b) lipid and (c) energy for the 100% ration level data in the first (●) and second (◎) periods (N = 16).



(a)



(b)

Figure 5.3. Regression analyses of fillet fat level with each intake (a) lipid and (b) energy for the 100% ration level data in the first (●) and second (◌) periods (N = 16).

FCR, SGR and fillet fat level in relation to diet ration interactions

No significant interactions were detected between diet and ration and FCR, SGR or fillet fat during either period, although ration did influence each factor during the first period (Table 4.5). FCR was similar for the fish fed 100 % and 80 % and highest in the 40 % ones; fillet fat was different between the 100 % and 40 % fish only, the former having the highest levels the latter the lowest (Table 4.6). SGR was highest at 100 % ration and lowest at 40 %, all rations being different (Table 4.6). In the second period, only SGR was significantly affected by ration, being lowest in the 40 % (0.195 %/day ± 0.03 SEM) fish and similar in the 100 % (0.29 %/ day ± 0.02 SEM) and 80 % (0.25 %/day ± 0.019 SEM) fish (Table 4.5).

Table 5.5. The results of the GLM and Kruskall-Wallis (in italics) analyses used to determine the relationships between each of the variables SGR, FCR and fillet fat and diet and ration and in the case of fillet fat gutted weight, during both periods (using only the diets with the three ration levels) (N = 16).

		SGR		FCR		Fillet Fat	
Period	Model	P	F/H	P	F	P	F
1st	Diet	0.059	5.96	*0.394*	*2.99*	0.037	11.63
	Ration	0.000	112.02	*0.007*	*9.99*	0.044	10.50
	Diet*Ration	0.597	0.84	-	-	0.115	4.73
	Gutted weight	-	-	-	-	0.004	61.54
2nd	Diet	0.381	1.34	0.214	2.35	0.038	11.32
	Ration	0.048	7.09	0.236	2.12	0.568	0.69
	Diet*Ration	0.265	1.98	0.336	1.61	0.107	5.03
	Gutted weight	-	-	-	-	0.068	7.81

Table 5.6. The means (±SEM) of each listed factor for each ration during the first period and the results of the one-way ANOVA analyses and in the case of the FCR the Kruskal-Wallis analysis (using only the diets with the three ration levels) (N = 16).

Factor	100	80	40	P	F/H
SGR	0.57 ± 0.029 **a**	0.43 ± 0.025 **b**	0.15 ± 0.034 **c**	0.000	45.13
FCR	1.096 ± 0.74 **a**	1.203 ± 0.04 **a**	2.36 ± 0.04 **b**	0.000	9.99
Fillet fat	7.12 ± 0.44 **a**	6.27 ± 0.57 **ab**	5.25 ± 0.1 **b**	0.037	4.31

Discussion

Fish nutrition and its consequences for productivity and quality have been studied intensively over the years, leading to continual improvements in the diets of cultured fish. It has been reported previously that fish can overcome dietary inadequacies by increasing their food intake (Lee and Putman, 1973). In the present study no difference was found in the food intakes between the diets in the first period, indicating that no such compensatory feeding was taking place or that no dietary deficiency existed. As a result in the first period the intakes of protein, lipid and energy and the DP/DE ratios followed the patterns set by the diets. In all but one diet (HP:LL) there was a reduction in the food intake in the latter part of the trial. It was discovered during the trial that the fish were maturing, the majority completing the process by the end. Anorexia or reduced food appetite in mature fish has formerly been described and it is appears this is what has occurred at the end of this trial (Kadri et al, 1997; Almansa et al., 1999). The tanks fed the HP:LL diet probably had relatively fewer mature fish compared to the rest, accounting for the constant appetite found for this diet. There was also a reduction in SGR and an increase in FCR in the latter part of the trial; again this is most likely because most of the fish were maturing and no longer investing in somatic growth. The fact that the fish matured had implications for the results in period two and it is probable that only the results from the first period are representative of non-breeding rainbow trout.

The DP/DE ratio did not influence the FCR or SGR, suggesting that protein sparing was not taking place, though it was not possible to confirm this with certainty using the variables measured in this study. This could be due to the protein and energy requirements having been met even at the lowest dietary level and that large fish were used for this study (Beamish and Medland 1986). Our results do not agree with those reported by Azevedo et al. (2004). The FCRs of the small lake trout *Salvelinus fontinalis* (body weight 47 g) and Atlantic salmon (body weight 25 g) increased when they were fed isoenergetic diets (approx. 24 MJ/kg) with increasing DP/DE ratios (18, 20, 22, 24 g/MJ) (Azevedo et al., 2004). This difference between the two studies is likely to be due to the size

difference of fish used, smaller fish having different protein requirements than large fish and the possibility that the ratios used in this study were not distinct enough to induce a difference in FCR. The DP/DE ratio did not affect the fat levels in the fillet either.

The results presented here demonstrated that increasing the level of lipid did improve the FCR irrespective of the level of protein. The FCR values achieved in the first period of this study were similar to those of Sveier and Lied (1998) (1.02 ± 0.01) for large Atlantic salmon (about 3 kg) fed a diet with 24.63 MJ/kg. A high level of lipid (energy) also improved the SGR, even when associated with increasing protein levels. The SGRs achieved here in the first period were comparable to those reported by Arzel et al., (1994) for brown trout of a similar size reared in seawater. Although increasing the level of lipid (energy) intake did increase the level of fat found in the fillet, the Rsq values were relatively low, so other factors must also be important.

There was no interaction between diet and ration in this trial, unlike that of Reintz (1983); again the fish used in that trial were much smaller than those used here, and this could account for the discrepancy. It could also be due to the fact that the combinations of ration and dietary lipid and protein levels used were too subtle. Ration itself did influence the FCR the fish fed the 40 % ration level had higher ratios than those fed the 100 % and 80 % rations. The percentage of total energy available for growth after maintenance costs have been met is lower at lower rations; this consequently leads to a higher FCR as seen here. As would be expected the 40 % ration fish had lower SGRs than both the 100 % and 80 % rations with the 100 % having the highest. The level of fat in the fillet was only reduced in the 40 % ration level

In summary the results presented here indicate that the protein requirement was met even at the lowest dietary levels. Increasing the level of lipid (energy) improved FCR and SGR. However this was also associated with an increase in fat deposition in the fillet. The use of lower rations detrimentally affect the FCR, SGR and decreased the fillet fat level independent of the dietary lipid and protein

levels used here, although similar FCRs and fillet fat levels were achieved when using an 80 % ration compared to a 100 % one.

Chapter 6

Effect of dietary protein and lipid levels on body condition and flesh quality of large rainbow trout (*Oncorhynchus mykiss*); Part II Individual level analyses

Summary

An investigation was undertaken to explore the effects of varying levels of dietary lipid and protein, fed at different rations, on body condition and flesh quality in 2-year old rainbow trout (1183.1 g ± 2.7 SEM), from September 2001 to January 2002. The diets used had crude protein and lipid compositions (by %) of 45.2:38.7 (HP:HL), 45.4:27.9 (HP:LL), 39.3:39.1 (LP:HL), 39.9:28.1 (LP:LL), 45.4:32.9 (HP:ML), 41.7:35.3 (MP:ML), and 38.7:32.8 (LP:ML) respectively and the first four listed were fed at three different ration levels (100 %, 80 % and 40 %). Diet type did not influence any of the body condition variables (visceral somatic index, hepato somatic index or condition factor) or the flesh quality variables fillet fat, moisture and astaxanthin level at a ration level of 100 %. At the end of the trial the majority of fish matured sexually. Maturity status (immature, maturating or fully mature) negatively affected the body condition and flesh quality variables, decreasing the size of the viscera, liver, level of fat and astaxanthin in the flesh while increasing moisture. Decreasing ration also negatively influenced body condition and flesh quality variables.

Introduction

Diet composition, body condition and flesh quality

The viscera is a major fat store, as to a lesser extent is the liver; the larger these stores are, the more reserves fish have to support a range of physiological needs. Changes in flesh quality can affect the market value of the fish. Flesh quality can be measured by a number of criteria, in some markets this is the level of fat, astaxanthin and moisture in the fillet. Studies have shown that replacing excess dietary protein (above the requirement level) with lipid does not compromise growth, but there is evidence that it can influence the size of the viscera and liver, the condition factor and the levels of fat and moisture stored in the flesh. Lee et al. (2002) reported that increasing dietary lipid levels from 7 to 14 % (gross energy, approx. 19.98 MJ/kg) increased the visceral somatic index of juvenile rockfish *Sebastes schlegli* (average weight 21.9 g). Increasing dietary lipid levels from 23.5 to 31.0 % (energy, 20.7 and 22.5 MJ/kg respectively) also enhanced the relative liver weight in Atlantic salmon smolts (Nordgarden et al., 2002) and Atlantic salmon of 600 g fed a higher fat diet (36.0 %) had higher condition factors than those fed a lower fat diet (26.0 %) (Solberg, 2004). Studies have also shown that dietary fat levels can influence body fat levels, depending upon species and size (Reinitz et al., 1978; Einen and Skrede, 1998; Jobling et al., 1998; Regost et al., 2001). Finally carotenoids (pigments), such as astaxanthin are fat soluble, so increasing the level of lipid in the diet could therefore increase the absorption of astaxanthin affecting the levels found in the flesh (Babosa et al., 1999). Sexual maturation is also believed to have an influence upon these body condition and flesh quality variables, as it requires a large amount of energy to complete the process.

Ration, body condition and flesh quality

Ration is another factor that influences body condition and flesh quality (Storebakken and Austreng, 1987; Shearer, 1994). Storebakken et al. (1991) found that the visceral somatic index increased with ration in rainbow trout with a body weight of about 300 g. It has also been shown that liver size is reduced

when food intake is restricted (Jezierska et al., 1982; Hemre et al., 1993), and that feeding rate influences condition factor in rainbow trout (body weight 2.1 g Reinitz, 1983). Few studies manipulating dietary protein and lipid levels, at different feeding rates, have been conducted using large rainbow trout in excess of 1 kg.

Aims of the study

In part I (chapter 5) of this study it was retrospectively discovered that the fish were sexually maturing. It was not possible in part I to look at the effects of maturation on the food conversion ratio at any point in the trial or the specific growth rate in the first half (the trial had been divided into two halves). Data on the flesh quality variables (fat, astaxanthin and moisture levels) and the body condition variables (the relative size of the viscera, liver and gonads) had been collected at the individual level from the both the first and second half of the trial and the specific growth rate in the second half. The specific aims of this study were to:

- examine the effects of maturation upon body condition, flesh quality and SGR (the latter in only the second half of the trial)
- determine if dietary levels of protein and lipid have an effect upon these variables when maturation is added to the model
- explore the interaction between diet and ration on these variables

Materials and Methods

As described in Part I (chapter 5), 2-year old rainbow trout (1183.1 g ± 2.7 SEM) were fed seven diets with differing levels of lipid and protein at three different ration levels. Half way through the trial a sub-sample of 20 fish were individually tagged, leading to the dissection of the trial into two periods the first and second. At the end of the trial flesh samples were taken for analysis of fat, moisture and astaxanthin levels. In addition to the methods described in Part I:

Sample processing and flesh quality preparation

At the beginning of this trial an initial random sample of 15 fish were killed (by brain destruction) and frozen (- 20 °C to provide baseline levels of fillet astaxanthin and fat. The viscera (stomach and associated fat), gonads and liver were removed from each sample fish and weighed individually before the fish was fillet and the left fillet homogenised.

Individual variables measured

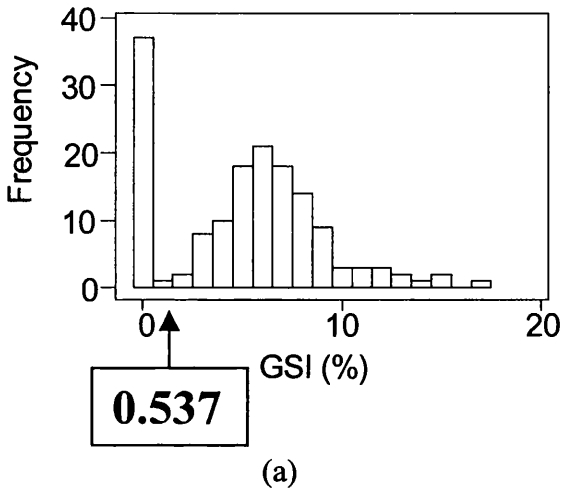
All males were removed (N = 7 for both periods) leaving 153 samples for each period. The levels of fat, astaxanthin and moisture in the flesh were established for each sample fish using the Soxtec, Weber (1988) and drying methods, respectively and are described in detail in the food production section in chapter 5. The results from one fish in both samplings and four astaxanthin measurements in the first period were not recorded due to an oversight.

A number of body condition variables were established for every individual fish taken from both the first and second periods:

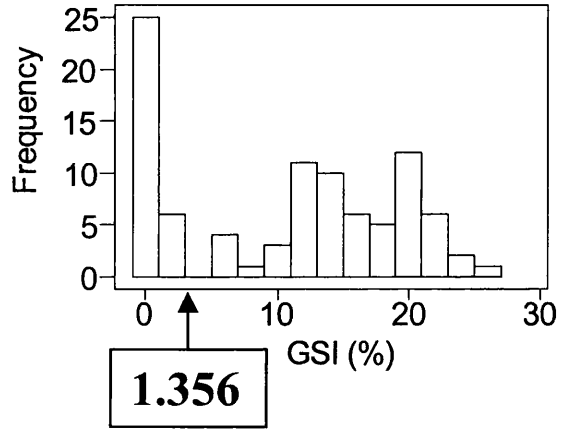
- Visceral Somatic Index (VSI): *(viscera weight/(body weight – viscera weight))*100*
- Hepato Somatic Index (HSI): *(liver weight/(body weight – liver weight))*100*
- Condition Factor : $(weight/length^3)*100$
- Specific Growth Rate (SGR): $((ln_{fw} - ln_{iw})/no.\ days)*100$ (second period only)
- Gonadal Somatic Index (GSI): *(gonad weight/(body weight – gonad weight))*100*

The GSI had a bimodal distribution in both periods and cumulative frequency graphs indicated that the distinction between the two groups occurred at a GSI of 0.537 % in the first period and 1.356 % in the second (Fig. 6.1). This lead to the formation of two maturity ranks in the first period (<0.537 % were described as immature and > 0.537 % as maturing) and three maturity ranks in the second

period (<1.356 %; immature, >1.356 %; maturing and those producing eggs defined as mature).



(a)



(b)

Figure 6.1. GSI distributions for (a) the first and (b) second periods indicating the GSI at which the bimodal distribution occurs.

Statistical analysis

Tank effects within dietary treatments

For normally distributed data tank effects were investigated using nested ANOVAs. For data that were not normally distributed a Kruskal-Wallis test was used between the tanks within each diet. All data were pooled by diet. No tank effects were detected in the first period. In the second period, tank had an effect when nested within diet for fillet fat and HSI, but when tank nested in diet was added to the GLM model with the other factors it became non-significant (Table 6.1). The Kruskal-Wallis analysis showed that there was a tank effect in two of the diets for fillet astaxanthin level and VSI and in one diet for filet moisture and condition factor in the second period.

Effects of diet on body condition and flesh quality

The 100 % data only were used for these analyses. General linear models (GLM) were used with the normally distributed fillet fat and moisture levels and HSI in the first period and fillet fat, HSI and SGR in the second to determine the effects of diet and maturity rank. Gutted weight, which was log10 transformed to normalise its distribution, was added as a covariant to the fillet fat and moisture models. The fillet astaxanthin level, VSI and condition factor in both periods and fillet moisture in the second could not be normalised with transformations so Kruskal-Wallis analyses were conducted with diet and maturity rank, with their post-hoc tests performed according to Zar (1996).

Ration and diet interactions on body condition and flesh quality

To determine if there was an interaction between diet and ration, general linear models (GLM) were used with maturity rank for the normally distributed fillet fat and moisture levels and condition factor in the first period and condition factor only in the second period. Gutted weight (log10 transformed) was added as a covariant in the models for fat and moisture levels. The rest of the data could

not be normalised by transformations and Kruskal-Wallis analyses were used with ration.

All the statistical tests were carried out in MINITAB v13 (Minitab Inc. USA).

Results

Effects of maturation and diet on body condition and flesh quality

The only variable not influenced by maturity rank in both periods was the condition factor (Kruskal-Wallis: 1st period, P = 0.733, H = 0.12, N = 75; 2nd period P = 0.600, H = 1.02, N = 74). Immature fish had lower HSI than their maturing counterparts in both periods and mature fish had the lowest in the second period (Fig 6.2.). The VSI was higher in the immature fish compared to the maturing fish and was lowest in the mature fish in the second period (Fig 6.3.). Levels of fillet fat were highest in the immature fish and lowest in mature ones in the second period, the reverse was true of the moisture levels (Fig. 6.2 and 6.3). Fillet astaxanthin was highest in immature fish and in the second period lowest in mature fish, a Pearson's correlation showed that as the GSI in the maturing fish increased, in this period, the astaxanthin level in the fillet decreased (P = 0.000, r = -0.651, rsq = 0.424 (Fig. 6.2 and 6.3). The SGR also followed this pattern (Fig. 6.2) and had a negative relationship with GSI in maturing fish (Pearson's correlation P = 0.000, r = -0.702, rsq = 0.493).
Diet did not significantly affect any of the variables in either period (Tables 6.1 and 6.2).

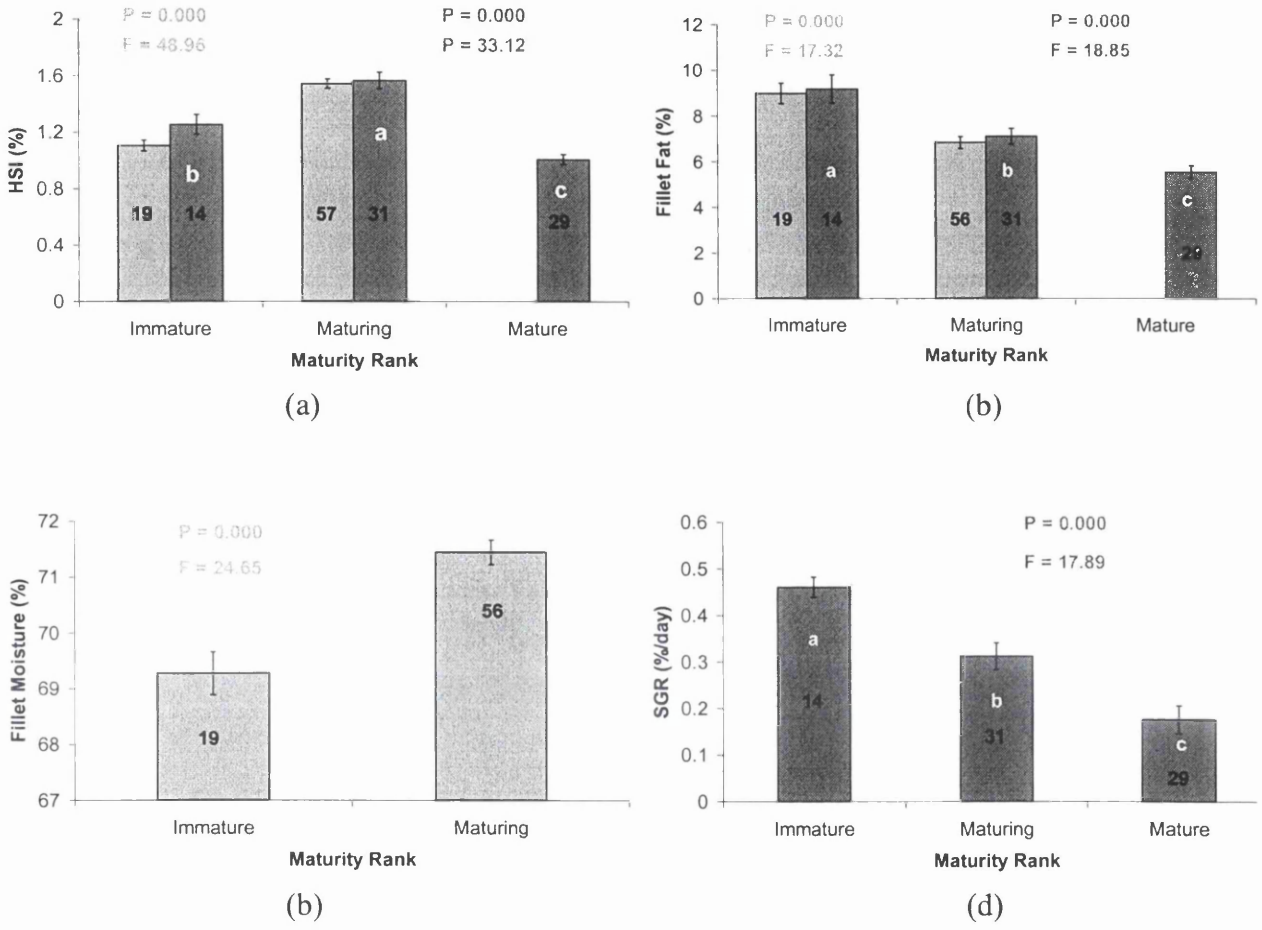
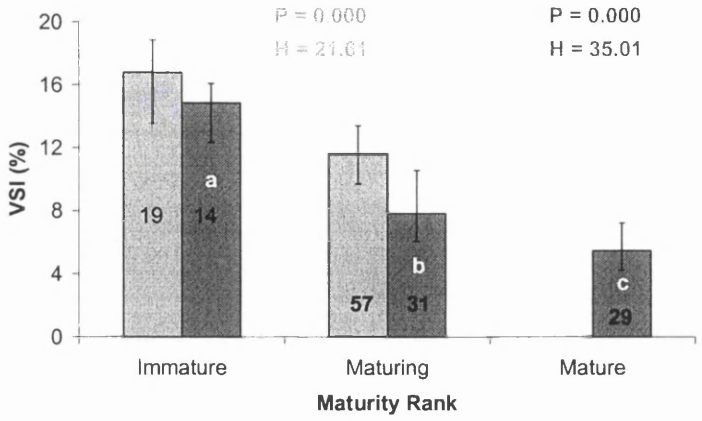
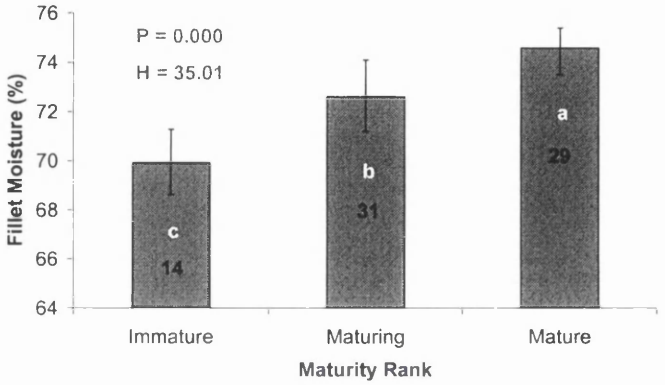


Figure 6.2. The mean (± SEM) (a) HSI, (b) fillet fat, (c) fillet moisture levels and (d) SGR by maturity rank for the first ▫ and second ▪ periods with the one-way ANOVA results and sample numbers displayed. Different letters show where the differences lie.



(a)



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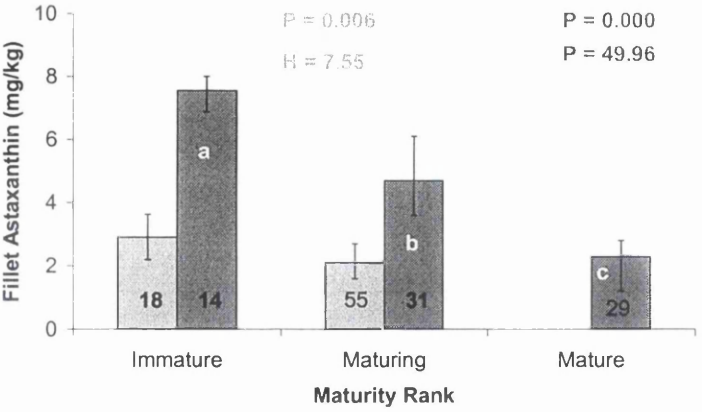

(c)

Figure 6.3. The median (inter quartile range) (a) VSI, (b) fillet astaxanthin and (c) fillet moisture levels by maturity rank for the first ▒ and second ▒ periods with the Kruskal-Wallis results and sample numbers displayed. Different letters show where the differences lie.

Table 6.1. The results of the GLMs used to determine the effects of diet, maturity rank and in some cases gutted weight on each of the variables HSI, fillet fat and moisture levels and SGR during both periods (with the exception of filet moisture in the second period and SGR in the first).

		HSI			Fillet Fat			Fillet Moisture			SGR		
Period	GLM	P	F	DF	P	F	DF	P	F	DF	P	F	DF
1st	Diet	0.291	1.3	6,75	0.217	1.43	6,74	0.426	1.01	6,74	-	-	-
	Gutted weight	-	-	-	0.002	10.5	1,74	0.001	11.6	1,74	-	-	-
	Maturity rank	0.000	47.0	1,75	0.004	8.97	1,74	0.001	13.1	1,74	-	-	-
2nd	Diet	0.520	0.9	6,73	0.463	0.96	6,73	-	-	-	0.946	0.3	6,73
	Gutted weight	-	-	-	0.004	9.23	1,73	-	-	-	-	-	-
	Maturity rank	0.000	20.3	2,73	0.000	9.12	2,73	-	-	-	0.000	15.68	2,73
	Tank (diet)	0.200	1.43	9,73	0.199	1.43	9,73	-	-	-	-	-	-

Table 6.2. The results of the Kruskal-Wallis tests used to determine the effects of diet on each of the variables VSI, fillet moisture and astaxanthin and condition factor during both periods (with the exception of filet moisture in the first period).

	VSI		Fillet Astaxanthin		Condition Factor		Fillet Moisture	
Period	P	H	P	H	P	H	P	H
1st	0.104	10.5	0.252	7.81	0.653	4.2	-	-
2nd	0.460	5.7	0.112	0.3	0.633	4.3	0.700	3.8

Table 6.3. The results of the GLMs used to determine the effects of diet, ration, maturity rank and in some cases gutted weight on each of the variables fillet fat level, fillet moisture level and condition factor during the first period (using only the diets with the four ration levels).

	Fillet Fat			Fillet Moisture			Condition Factor		
GLM	P	F	DF	P	F	DF	P	F	DF
Diet	0.000	6.61	3,114	0.004	4.94	3,114	0.573	0.67	3,114
Ration	0.019	4.12	2,114	0.194	1.66	2,114	0.000	18.05	2,114
Diet*Ration	0.052	2.17	6,114	0.077	1.97	6,114	0.550	0.83	6,114
Gutted weight	0.004	8.66	1,114	0.000	15.23	1,114	-	-	-
Maturity rank	0.006	7.91	1,114	0.000	16.05	1,114	0.322	0.99	1,114

Table 6.4. The mean (± SEM) fillet fat and moisture levels of each diet HP:HL (N = 30), LP:HL (30), HP:LL (N = 28) and LP:LL (N = 27) with the one-way ANOVA results. Different letters show where the differences within each quality variable lie.

Quality variable	HP:HL	LP:HL	HP:LL	LL:LP	P	F	DF
Fat (%)	6.84 ± 0.331 **ab**	6.93 ± 0.359 **a**	6.00 ± 0.317 **bc**	5.17 ± 0.298 **c**	0.000	6.13	3,114
Moisture (%)	71.25 ± 0.284 **a**	71.34 ± 0.301 **a**	72.02 ± 0.277 **b**	72.63 ± 0.295 **b**	0.003	4.88	3,114

Gutted weight significantly influenced fat in both periods and moisture level in the first, allowing for maturity rank (Table 6.1). As the gutted weight increased, so did the fat level (Pearson's correlation 1st period, P = 0.000, r = 0.422, rsq = 0.215; 2nd period P = 0.000, r = 0.495, rsq = 0.2454). The converse is true of moisture levels (Pearson's correlation 1st period P = 0.000, r = -0.463, rsq =0.2145; Spearman's rank correlation 2nd period P = 0.000, rs = -0.551). Gutted weight only influenced the astaxanthin level in the second period (Spearman's rank correlation 1st period P = 0.195, rs = 0.151; 2nd period P = 0.000, rs = 0.506).

There were no detectable levels of astaxanthin in the initial samples collected at the start of the study, the fish had not been fed astaxanthin or canthaxanthin additives prior to this study. The initial samples had lower fillet fat levels (4.95 ± 0.5) compared to the 100 % fed fish at the first (7.39 ± 0.3) and second (6.9 ±0.3) samplings (one-way ANOVA P = 0.001, $F_{2,14} = 7.92$).

Effect of ration and diet interactions on body condition and flesh quality

During the first period, diet and ration influenced the fat levels in the fillet and the interaction term between diet and ration was only marginally non-significant when put in a model with the other main factors gutted weight and maturity rank (Table 6.3). HP:HL and LP:HL diets tended to be different when compared to the HP:LL and LP:LL ones during the first period (Fig. 6.4). Diet and ration both influenced fat levels. The LP:HL diet had the highest level, which was statistically similar to the HP:HL diet only, the latter was also similar to the HP:LL diet which was the only diet similar to the LP:LL and the fish fed the 100 % (7.25 ± 0.268 SEM, N = 38) ration had higher fat levels than the 80 % (6.32 ± 0.291 SEM, N = 38) fish, both of which had higher levels than the 40 % (5.26 ± 0.268 SEM, N = 39) ones (Table 6.4). Neither ration nor the interactive term between diet and ration significantly influenced the moisture levels in the fillet in the first period but diet did (Table 6.3). The HP:HL and LP:HL diets had significantly lower moisture levels than the HP:LL and LP:LL diets (Table 6.4). The Kruskal-Wallis tests showed that astaxanthin levels were not influenced by ration (P = 0.159, H = 3.68, N = 111) but the HSI and VSI were (Fig 6.5.). The HSI was higher in fish fed the 100 % ration than the 40 % with all others being

similar. VSI was similar in fish fed the 100 % and 80 % rations and lowest in the 40 % ones (Fig. 6.5). The condition factor was only influenced by ration (Table 3) (one-way ANOVA P = 0.000, $F_{2,114}$ = 17.73), the 100 % (1.73 ± 0.019 SEM) ration producing higher condition factors than the 80 % ration (1.65 ± 0.021 SEM) both of which were higher then the 40% (1.55 ± 0.024 SEM).

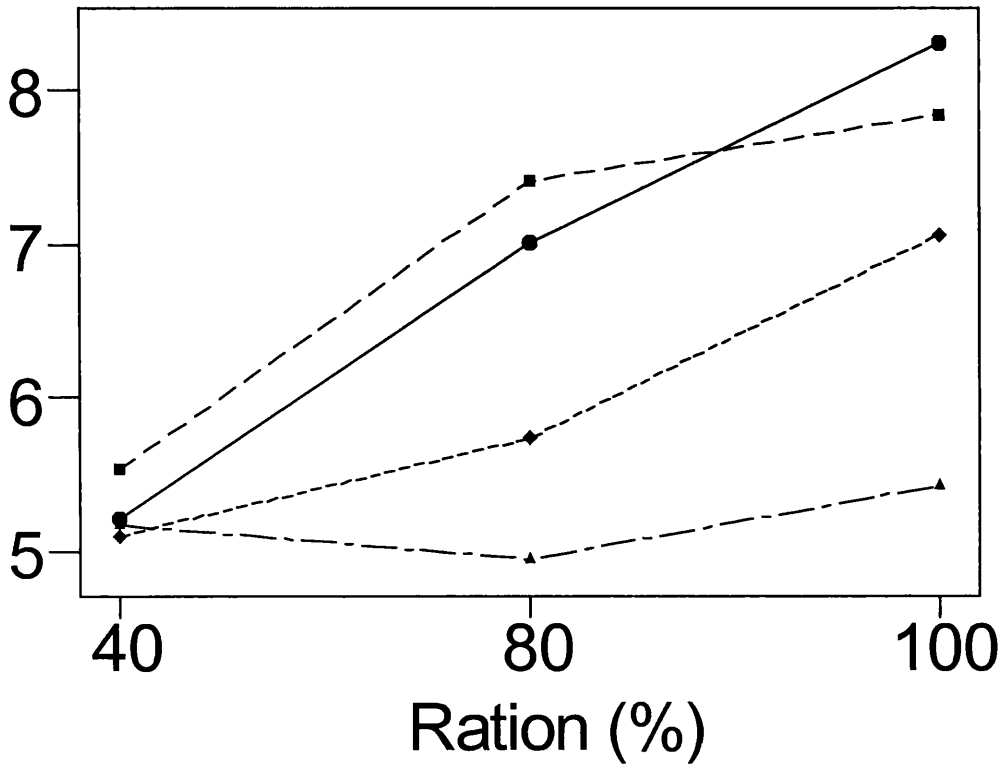

Figure 6.4. The mean fillet fat levels for each diet (HP:HL–●– LP:HL–■– HP:LL···◆··· LP:LL–·▲–·)at each ration level in the first period.

During the second period it was only possible to test for diet ration interactions with the condition factor. The interaction term did not significantly affect condition factor (GLM P = 0.401, $F_{6,113}$ = 1.04), but diet (GLM P = 0.009, $F_{6,113}$ = 4.05) and ration (GLM P = 0.000, $F_{2,113}$ = 8.80) did even when the other important factor maturity rank (GLM P – 0.012, $F_{2,113}$ = 4.58) was present in the

model. Diet became non-significant at the one-way ANOVA level ($P = 0.068$, $F_{6,113} = 2.44$) but ration remained significant (one-way ANOVA $P = 0.000$, $F_{2,113} = 9.53$). The difference occurred between the 40 % (1.57 ± 0.021 SEM) fish and 100 % (1.71 ± 0.026 SEM) and 80 % (1.66 ± 0.025 SEM) ones, the 40 % had the lowest condition factors. Ration influenced all of the variables in the second period (Fig. 5), the SGR and fillet moisture levels were different in all rations, the 100 % ration produced the highest SGR and the 40 % the lowest. The reverse was true for the moisture levels (Fig. 6.5). The levels of fillet fat and asta and the HSI and VSI were similar in the 100 % and 80 % diets, all being higher than those produced in the 40 % ration fish (Fig. 6.5).

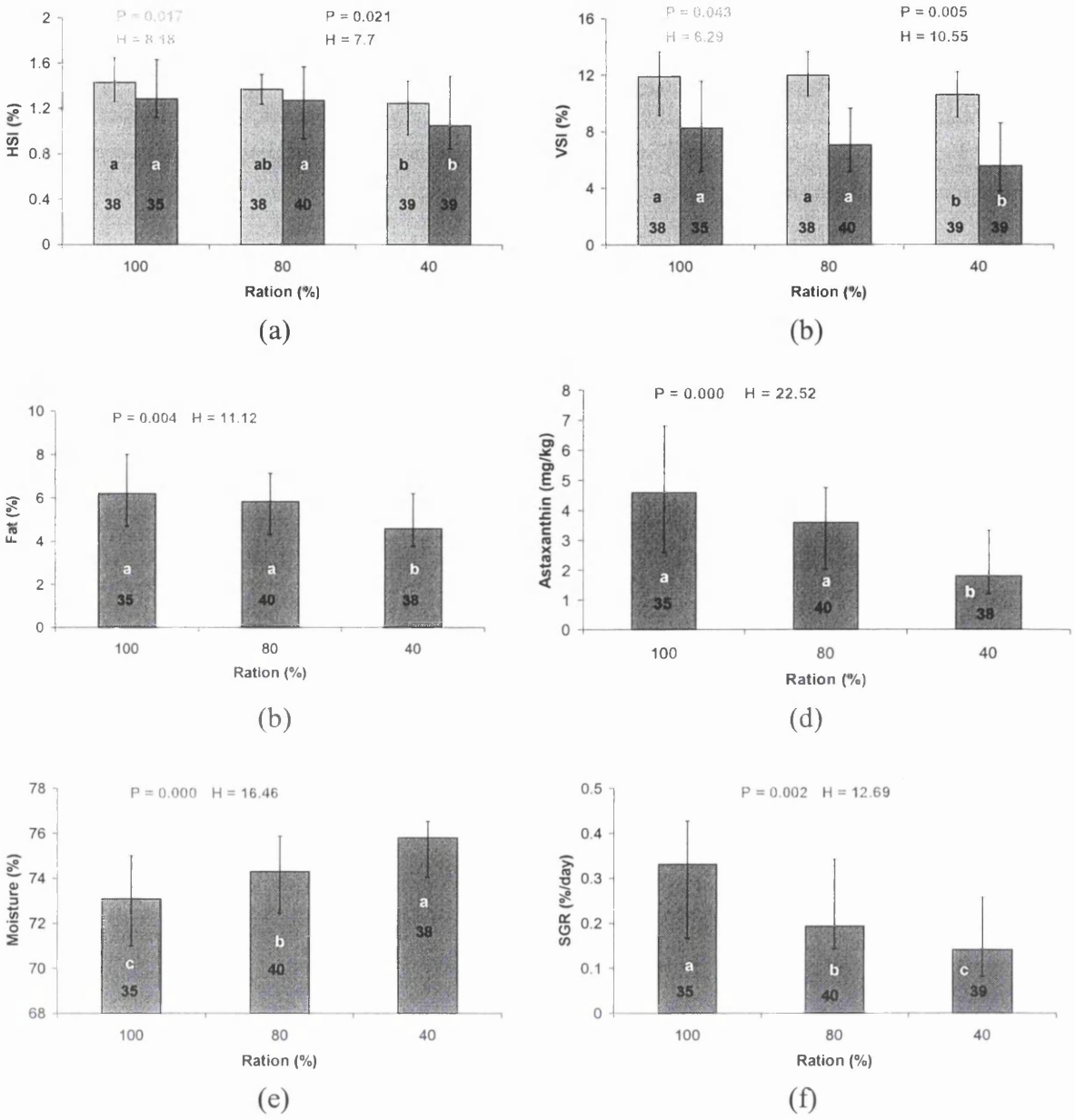

Figure 6.5. The median (inter quartile range) (a) HSI, (b) VSI, (c) fillet fat, (d) astaxanthin, (e) moisture and (f) SGR for the first and second periods with the results of the Kruskal-Wallis analyses and the sample numbers displayed. Different letters show where the differences lie.

Discussion

In part I of this study the level of dietary lipid and protein influenced the SGR and with respect to lipid the FCR, but both the SGR and FCR deteriorated in the second period of the trial, thought to be influenced by sexual maturity. The results presented here show that maturity rank did influence all of the variables measured in this study with the exception of the condition factor. It would have been expected that maturation status would be related to condition factor, fish reaching full maturation have high condition factors as they are swollen with eggs (Priede and Secombes 1991). It is unclear as to why this was not the case in the present study. As the maturity status went from immature to maturing to mature the VSI, SGR, fillet fat and astaxanthin levels declined and the fillet moisture level increased. The HSI increased from immature to maturing fish and then in the mature fish decreased to a level below that of the immature. There is a considerable expenditure of energy associated with maturation and as feeding is suspended just prior to and during maturation in salmonids the energy resources are depleted. It is believed that fish mobilise energy from the viscera, muscle and possibly the liver to fuel maturation. Henderson and Tocher (1987) state that the mesenteric fat deposited in the visceral cavity is the most obvious adipose tissue in salmon. Rowe et al. (1991) suggested that visceral fat stores are involved in the maturation process, a theory that is supported by the results presented here. The results of the present trial are in accordance with Jobling et al. (1998) who also reported that mature Arctic charr *Salvelinus alpinus* utilised liver stores. The decrease in fillet astaxanthin as maturation proceeds in the present study is what would be expected, Craik (1985) described how astaxanthin is mobilised during maturation from the muscle and liver and incorporated in the eggs. It is believed this transit occurs because pigments, such as astaxanthin, improve the quality of the eggs by serving as antioxidants, though to date this is not a particularly well studied subject (Kjorsvik et al., 1990). The reduction in the SGR is due to the suspension of somatic growth in favour of the completion of maturation and the reduction in food intake. The increase in the moisture levels in the fillet from immature fish to mature fish is almost certainly due to the very strong inverse relationship that has been show to exist between fat and moisture (Love 1970).

In the present study, diet did not influence the VSI, HSI, condition factor, levels of fillet fat, moisture or astaxanthin, in the fish fed to satiation in either period of the trial when maturation was accounted for. These results are in accordance with those of Arzel et al. (1994) who reported that dietary lipid level (21 and 29 %) did not affect the size of the viscera or liver in 1.5 kg brown trout and Jobling et al. (1998), who did not find any dietary effects (lipid, 12.6 and 27.5 %, energy, 21.2 kJ/g $^{-}$1 and 24.4 kJ/g $^{-}$1 respectively) on body fat levels in 90 g rainbow trout. Yet they contradict a number of studies showing that dietary lipid levels do influence HSI (Nordgarden et al., 2002), VSI (Lee et al., 2002), condition factor (Solberg, 2004), levels of body fat (Jobling et al., 1998) and astaxanthin (Chan et al., 2002).

Gutted weight did influence the fillet fat and moisture levels in both periods and astaxanthin in the second. Shearer (1994) recognised the need to included weight as a covariant in such analyses, as there is a possible body weight dependent effect on fat and moisture storage that needs to be taken into account. This is confirmed by the results presented here for rainbow trout, which are supported by those of Rye and Gjerde (1996) who reported that increased body size was related to an increased level of carcass fat in Atlantic salmon. The results of this trial support the results of Torrissen et al. (1995) and Torrissen (1985) who also found a positive linear relationship between body weight and astaxanthin levels in Atlantic salmon and rainbow trout, respectively. This indicates that the need to add weight as a covariant could also be extended to astaxanthin levels in salmonids.

The interaction between diet and ration was only slightly non-significant in influencing fillet fat levels and should not be dismissed entirely. The HL diets tended to perform differently than the LL ones at the different rations. The increase in fat level between the 40 % and 80 % diets was greater in the HL diets when compared to the LL ones. This indicates that at these low ration levels the energy supplied in the LL diets was perhaps enough for maintenance and limited growth but not excess storage. Diet did not affect the level of fat in the fillet with respect to the fish just fed to satiation, this is in disagreement with the all ration data. This disparity between data from just the satiated fish and that from the

three rations is most likely due to the almost significant interaction between ration and diet. During the second period it was not possible to test for diet and ration interactions due to the limited range of non-parametric tests available. Ration did influence all the variables in the second period, the HSI, VSI, condition factor, fillet fat and astaxanthin levels were similar in the 100 % and 80 % rations both being higher than in the 40 % ration.

In summary the level of dietary lipid did not influence body condition or flesh quality when maturation status was allowed for. Maturation negatively affected the VSI, HSI and flesh quality variables, as did ration. Although none of the diet-ration interactions were statistically significant, that with fillet fat level only marginally registered as non-significant and is something that should be explored in more detail. A reduction in ration to 40 % lead to a decrease in body condition and flesh quality variables but 100 % and 80 % rations produced similar results.

Chapter 7

General Discussion

Summary of aims, objectives and findings

The work presented in this thesis was diverse and involved investigating aspects of nutrition in farmed salmonids, life history strategies in post-smolt Atlantic salmon and the possibility of predicting maturation and reproductive potential.

Timing of maturation and reproductive investment (Chapters 2-3)

The objective of the study described in chapter 2 was to gain a better understanding of the life history strategies of post-smolt Atlantic salmon and to determine the possibility of predicting maturity and reproductive potential using a series of body condition measurements taken at critical and intermittent periods throughout the year. Mature individuals, both male and female, had higher body weights than their immature counterparts in September '02 a year before the completion of the maturation process in October/November '03. The mature fish also had higher SGRs from September to May and maintained this weight advantage in the intervening May and June. It was also revealed that weight in May had the strongest influence on maturation in females when placed in a model with June and September weight. There was no single influential factor for males. This could be due to the small sample size of immature males compared to mature ones or that they have similar growth patterns.

Maturing males were larger than the maturing females at all times and had higher SGRs from September to May. No such gender differences were detected in the immature fish. The greater cost of maturing faced by female fish (egg production is more energetically expensive than sperm production) may require them to accumulate energy reserves rather than somatic growth. Maturing females also had higher condition factors in June than maturing males, supporting this idea. Immature females had significantly higher overall SGRs from May to June compared to September to May, as did the maturing females, although at the individual level there was a negative relationship between the two growth periods for the mature females. The immature females had significantly higher SGRs than their maturing counterparts in the May to June period. Neither immature nor maturing males had different SGRs between the two growth periods, nor was there a difference in the SGRs between the two maturity ranks in the May to June

period. This again suggests a gender based difference in the growth patterns of post-smolt Atlantic salmon.

It was also discovered that female body weight at the time of ovulation had a significant positive relationship with egg number as has been described in previous literature, but that June weight had a stronger one. This could indicate that egg number is determined around the time they become anorexic. None of the other body condition measurements had a relationship with egg number nor did they influence egg weight, only June weight had a positive relationship with this variable in the females that matured later in the season. Contrary to the published literature there was no relationship between egg number and egg size. Nor were any of the body condition measurements on females related to egg survival at any of the developmental stages, up to and including first feeding. It is possible that the fish in this study were provided with enough food and were in a good enough condition that egg quality was not compromised by any trade offs or that they were conserved at the expense of another trade off such as a repeat spawning. Females that matured later in the season produced more eggs than those that matured earlier, there was no difference in egg weight between these early and later spawners.

Nutrition of farmed fish (Chapters 4-6)

As mentioned in the introduction, reducing dependence on fish meal and fish oil in diets would be beneficial to the aquaculture industry and the environment. Improving the protein utilisation of fish diets through protein sparing is also of interest. The aim of chapter 5 was to investigate the effect of increasing the level of dietary lipid while reducing that of protein on growth, food utilisation and flesh quality in large rainbow trout. Increasing the level of lipid improved the food conversion ratio (FCR) and specific growth rate (SGR). The results indicated that protein sparing was probably not occurring, as the DP/DE ratio (digestible protein/digestible energy) did not influence the FCR or SGR, it could be that the protein requirement was met even at the lowest level used in the study. Increased dietary lipid did increase the level of lipid in the fillet, which could have a bearing upon the quality. During this study it was discovered that the fish were maturing and, as a result, in chapter 6 the effect of maturation upon growth, fat stores and flesh quality was investigated. Maturation reduced the levels of

astaxanthin and fat in the fillet, the SGR, visceral somatic index and hepato somatic index two other measures of energy reserves.

The effects of replacing 50 % of dietary fish oil with rapeseed oil in diets for Atlantic salmon broodstock on egg quality was investigated in chapter 4. The results from this study showed that using this level of rapeseed oil did not reduce reproductive investment or egg quality. It showed that there was selective retention of DHA and EPA in the eggs, implying that the quality of the egg is conserved, as suggested by the results described in chapter 2.

Implications

Just as the aims of the work presented in this thesis were broad so are the implications. They are interesting for both fish biologists and aquaculturists.

Implications for life history theory

Many studies have been conducted that investigate the life history strategies of Atlantic salmon though little is known about those of post-smolt individuals, either male and female, and few have compared the strategies of the genders. As was mentioned in chapter 1, it is suggested that fish initiate the release of a maturation inhibitor dependent upon a threshold of size, growth and energy reserve. It is also suggested that this occurs at a theoretical critical period thought to be the autumn of the year prior to its completion (Herbinger and Friar, 1992; Thorpe, 1994, Silverstein et al., 1998). The results from chapter 2 supported the idea of an autumn maturation window for post-smolt Atlantic salmon, with respect to body weight, as larger fish in September went on to mature the following winter. However it was not possible in the present studies to investigate the influence of SGR or energy reserves with respect to this issue. The results also revealed that May body weight was the strongest predictor of maturity in females, with no one predictor prevailing for males. This suggests that May is the point at which size differences between immature and mature females are at their greatest and consequently would provide the greatest opportunity to predict which females will go on to mature.

The results from chapter 2 also indicate that male and female post-smolt Atlantic salmon exhibit different reproductive strategies. Males appear to invest more in somatic growth than energy accumulation, possibly to increase their chances of gaining access to egg-laying females, as sexual selection in salmonids favours larger body size (Quinn and Foote, 1994). Females are required to accumulate larger energy reserves than males, which appears to occur at the expense of somatic growth. The results also highlighted a difference in SGR between immature and mature females that did not occur in males, as a consequence prediction of maturation, for females may be easier than for males, the differences between immature and mature males being less pronounced than those of females.

The decrease in SGR detected at the individual level, suggests that the fish had entered the anorexic phase or perhaps that they may have started diverting energy away from growth and into gamete development.

As discussed in the introduction, female weight at the time of maturation influences egg number and weight, though little is known about when these are determined or when their development is initiated. There is hormonal evidence to suggest that gonad development in females could begin in late spring (Tyler and Sumpter, 1996; Stead et al., 1999). The fact that it was June weight that was related to egg number and, in fish that matured later in the season, egg weight supports this. This indicates that egg number could be determined around June, before the females become anorexic and migrate. It may therefore be possible to identify the females that will invest more into reproduction and as a result be more productive in terms of egg number at this juncture. While it may be possible to make predictions about egg number, egg quality appears to be unaffected by any of the measures taken in this study though it could be possible that no trade offs were required or that they were not associated with egg quality but some other factor. This suggests it would be difficult to make predictions concerning egg quality using morphological measurements alone. The quality of the milt was also unrelated to any of the measurements taken, although only one measure of male quality was used in this thesis and the strength and length of the sperm motility should be considered in any further studies.

Implications for broodstock management

The ability to identify in advance individuals that will mature would be a great benefit to broodstock farmers to help improve efficiency. Being able to make early assessments of reproductive potential would also be an advantage. From the results of the study in chapter 2, it would appear possible to predict if a fish will mature the following winter based upon its body weight in September and that in females a more defined prediction could be made in June. It also appears that females that are larger at this point will produce more eggs, but that morphological measurements can not be used to help predict egg quality which appears to be harder to predict.

In addition to these morphological predictors, ultrasound could also be used to help confirm and make a more informed prediction about potential maturation. To date ultrasound has proved useful in differentiating genders in a number of species as stated in the introduction (Martin et al., 1983; Sheilds et al., 1993; Rommens, 2001). In Chapter 3 the use of ultrasound to assess gonad size in immature and mature females and the possibility of using these measurements to make predictions about maturation was explored. It was possible to use ultrasound successfully in differentiating gender in immature Atlantic salmon and measuring the length and width of female gonads in immature Atlantic salmon and maturing rainbow trout. It was also possible using equations based on these measurements to estimate the size of the mature rainbow trout gonads. This could prove a useful accessory for both life history studies and broodstock farmers, enabling gonad development to be traced from its origins to its conclusion and perhaps making predictions of eventual reproductive investment along the way.

Implications for aquaculture nutrition

As discussed in chapter 1 food costs are a very substantial part of aquaculture production and reducing them at the same time as producing a product of a high quality is important to the development and sustainability of the culture process (Jackson 1991). In addition, improving the utilisation of dietary protein would be beneficial in relieving environmental concerns by reducing excess nitrogen production and the strain on fisheries (Reinitz et al., 1978). This could be achieved through increasing the level of dietary lipid as discussed in the introduction. As described in chapter 5, dietary protein

can be replaced with lipid without compromising growth at the same time as improving food efficiency in large rainbow trout. However it was not clear whether this was due to the action of protein sparing or whether even the lowest level used in this study were sufficient to meet their protein requirments. This implies that food companies could use higher lipid levels in diets for these fish. The study described in chapter 6 showed the importance of the energy stores and astaxanthin to the maturation process of female rainbow trout. It also indicated that the energy stored in the liver is important to the females in the final stages of maturation as ovulation neared.

Fish oil, like fish meal, is a limited and expensive resource and in recent years there has been a concerted effort to find a replacement. To date rapeseed oil has proved a worthy contender, as it has been used successfully as a partial replacement without compromising growth or flesh quality in on growing Atlantic salmon (Bell et al., 2003). Being able to replace fish oil with rapeseed oil in broodstock diets without compromising initial egg quality means that costs can be reduced and using a widely available renewable resource such as rapeseed oil can alleviate environmental concerns. The Atlantic salmon used in chapter 4 appear to be able to cope with the reduced level of DHA and EPA without compromising egg quality. Lower levels of EPA and DHA were detected in the eggs of fish fed rapeseed oil, but there was no difference in the survival percentages of each developmental stage up to that of first feeding when compared to the eggs of fish fed diets with purely fish oil. The results of this chapter imply that using a 50 % rapeseed oil replacement in Atlantic salmon diets is a feasible option in producing adequate numbers of high quality eggs at the first feeding stage. It also suggests that higher inclusions of rapeseed oil could be made. However the study undertaken as part of this thesis did not investigate the development of the offspring in their ability to feed and sense their environment. Rapeseed oil lacks the fatty acids that are important to membrane development in the brain during the embryo phase that could have implications for sensory development (Bell et al., 1997; Sargent et al., 2002). This would need to be investigated to fully understand the implications of the replacement; if the offspring are incapable of detecting and or competing for food they may have an increased mortality rate at this stage.

Future studies

Despite the large amount of research conducted upon Atlantic salmon life history strategies, there is still much to be understood. The relationship between growth rate and energy reserves approaching the autumn maturation window in post smolt fish should be investigated to better understand the 'decision' to mature. May/June has been highlighted as an important time for females in terms of maturing and egg number and weight and should also be investigated in more detail. A better understanding could be achieved by coupling physiological studies that manipulate female condition around June with the use of ultrasound to follow the physical development of the gonads to determine when development starts and the period at which most development takes place and the resultant reproductive investment and quality. At this point ultrasound has been used for female maturation predictions and its use in predicting male maturity should also be explored.

The results from chapter 6 suggest that the liver energy stores play an important role in the final stages of maturation. A more thorough study of this and the roles that the different energy reserves play in the maturation process and investigating if this energy reserve is as important to male maturation as it appears to be for females would be interesting.

Although the work presented here showed that an inclusion of 50 % rapeseed oil did not affect survival to first feeding, the effect it may have upon initial behaviour should be studied to full understand the implications of its use in broodstock diets. Also it would be advantageous to explore if the level of rapeseed oil inclusion could be raised.

Conclusions

The studies that comprise this thesis were developed by SR and Nutreco to investigate aspects of salmonid life histories and nutrition. They supported the hypothesis of the autumn maturation 'window' in post smolt Atlantic salmon, indicated that males and females exhibit different reproductive trade offs, that May/June is an important period for females in terms of maturation and reproductive investment and showed that it is possible to predict which females will mature and their reproductive investment, but not

egg quality, using morphological measurements at this point. They also showed that ultrasound can be used to gain a more comprehensive understanding of gonad status and size in female salmonids. Finally they revealed that increasing levels of dietary lipid at the expense of protein can improve food conversion and growth in large rainbow trout and that 50 % of dietary fish oil can be replaced by rapeseed oil in Atlantic salmon broodstock diets without compromising egg and fry quality to fist feeding.

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