EXAMINING FISH QUALITY - THE EVALUATION OF THE USE OF LIPIDS AS A MEASURE OF CONDITION IN WILD ATLANTIC SALMON

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Examining fish quality – The evaluation of the use of lipids as a measure of condition in wild Atlantic salmon

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Abstract

Considering the response of organisms to their environment is difficult; it is made more so if population numbers cannot be closely monitored. In such cases different methods of population assessment are required. This thesis uses lipids as a measure of Atlantic salmon (*Salmo salar* L.) quality and investigates its usefulness in indicating fish condition.

The first study examines the relationship between fish total lipid content and W_R condition factor; this study clearly demonstrates that there is a significant positive relationship between the condition factor of a fish and its total lipid content. In the following study the lipid storage between the different tissues of the Atlantic salmon is considered. This indicates that the red muscle and the adipose tissues hold higher concentrations of lipid than the white muscle. However, the white muscle makes up the majority of lipid tissue mass in the Atlantic salmon so contains the bulk of stored lipid in a fish, at low concentration.

The next study investigates the effect of spawning on Atlantic salmon condition. Salmon can be seen preferentially conserving lipid in their musculature and drawing down the lipid stored in their adipose tissues. The following study looked at one key lipid group, triacylglycerides, in salmon. Triacylglycerides are energetically important in fish and this study found that the spawning process depleted triacylglyceride reserves, but that the red muscle conserves triacylglycerides even after spawning.

The final study considers the relationship between maternal quality and egg quality, identifying that longer Atlantic salmon produce eggs with more lipid after spawning migration. Egg lipid concentrations were comparably maintained between fish. Monitoring quality in this way is a useful tool to determine population wellbeing and help indicate where populations are compromised.

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Contents

1. General introduction

Fisheries scientists use a wide range of measures to evaluate the quality of fish. A variety of morphological and physiological metrics are available for use, with different attributes. These metrics, originally developed for use in humans to describe health, have been translated to help us better understand fish health (Lloret *et al.* 2014a). The usefulness of such metrics is dependent on the question asked and the availability of resources.

These metrics are often described as measuring fish condition, a fish in 'good' condition is assumed to be healthy. A healthy fish is assumed to have large energy reserves and be in optimum condition. However there are many definitions of what determines health, even in humans, it is not just considered to be the absence of disease, it also includes a measure of wellbeing (WHO 1946). In fish we term this condition.

The condition of fish has been assessed in a number of ways, from using simple morphometric measures such as; length, weight or condition factors, to complex physiological measures such as; lipid content, triacylglyceride concentration or liver function (Lloret *et al.* 2014a). Whilst all the metrics have their own advantages and disadvantages there are no clear rules that assess which are most effective and efficient to apply.

This thesis aims to delve deeper into this problem by focusing on the use of such metrics in one particular troubled species, the Atlantic salmon (*Salmo salar*). By considering the ease of use and reliability of a few of these metrics the hope is to draw a clear conclusion on which metrics are best to use and which circumstances these can easily be applied for Atlantic salmon.

The Atlantic salmon problem

Living in a climate-altered environment is already the case for many organisms. Management of aquatic life has always been complex, as it is challenging to study aquatic organisms in their natural environment, and the difficulty of this has increased due to the complex nature of climate change. Good examples of this are recent reports of expanding sea ice in Antarctica, where increasing temperatures have lead to an increase in sea ice. Suggestions to explain why sea ice is increasing range from an influx of cold water from melting shelf-ice (Bintanja *et al.* 2013), natural variability between years (Stammerjohn *et al.* 2008) and satellite error (Eisenman *et al.* 2014).

A major indicator of climate change in the ocean is the melting of this sea ice, in both the Antarctic and Arctic (IPCC 2013); but there are also variations in sea surface temperatures (SSTs) (Rayner *et al.* 2006; IPCC 2013). Ocean warming is a key threat of climate change, and the IPCC (Intergovernmental Panel on Climate Change) reported that the upper 75m of the ocean warmed by 0.09-0.13^{o}C between 1971 and 2010 (IPCC 2013). Sea surface temperature variations may influence marine animal productivity and survival (Beaugrand & Reid 2003; Friedland *et al.* 2005), and can cause important economical and biological changes and shifts (Klyashtorin 1998).

Considering the effect of climate change on a single species can help illuminate this threat. A large number of fisheries have been shown to be affected by temperature variations: these include reports for sand eels (*Ammodytes marinus* Raitt) (Frederiksen *et al.* 2006), blue whiting (*Micromesistius poutassou*) (Hátún *et al.* 2009) and also herring (*Clupea harengus* L.) (Klyashtorin 1998). These studies have led to increasing levels of worldwide concern regarding climate change and its influence on marine ecosystems (Cury *et al.* 2008).

The Atlantic salmon (*Salmo salar* L.) has been shown to be influenced by changes, climatic and otherwise, in both the freshwater (Russell *et al.* 2012) and marine environment (Beaugrand & Reid 2003; Todd *et al.* 2008). However, it has been repeatedly suggested that these influences are not direct and occur as a result of variation in the availability and/or quality of the salmon's prey (Beaugrand & Reid 2003; Richardson & Schoeman 2004; Stokstad 2004; Todd *et al.* 2008). This implies that this system is subject to a form of bottom-up control, whereby a species is regulated by the species at a lower trophic levels (Cury *et al.* 2008).

Recent declines in salmon populations have been attributed to the period of time spent at sea rather than time in freshwater (Mills 2000). Currently disproportionately less is known about the life of salmon at sea (Mills 2000; Beaugrand & Reid 2003). However, it is more complex than this; salmon are influenced by their freshwater environment and have been found to be leaving freshwater earlier over recent years (Russell *et al.* 2012). If Atlantic salmon leave their rivers at younger ages they tend to be smaller, thus even smaller fish have to survive the compromised marine environment (Todd *et al.* 2010).

Todd *et al.* (2008) found that ocean climate impacted upon wild Atlantic salmon populations. They were able to demonstrate that not only have stock abundances decline over the past three decades, but that salmon had also declined in terms of condition. Todd *et al.* use two independent populations to demonstrate an overall decline of between 11-14% in the past 10 years. This work also demonstrate the Atlantic salmon of poor condition factor also had compromised their lipid stores by up to 80%. They suggested that measures of condition should be incorporated into analysis of compromised populations.

The aim of this thesis is to help inform these investigations of compromised populations by considering a variety of metrics to inform better collection of

Atlantic salmon condition data. The final aim of this work is to update the discussion on developing a meaningful and accurate method to assess fish condition.

Atlantic salmon life history

Atlantic salmon are thought to have historically occurred in every river which flowed into the North Atlantic Ocean and Baltic Sea (Mills 1991). Although much of this population has been lost Atlantic salmon still occur in many rivers of Northern Europe, Iceland and North America. The Atlantic salmon goes through a distinctive lifecycle, so much so that for a long period of time the different phases were considered as separate species; only in the 1830s and 40s was it finally realised that they were the same species (Mills 1991). This confusion was largely due to the salmon's lifecycle depending upon two environments, fresh and marine water (Thorstad *et al.* 2010), because salmon are anadromous.

Atlantic salmon follow a variety of lifecycles, but most conform to a general pattern: salmon hatch from eggs in river and spend their juvenile phase in freshwater; after this they migrate to sea for feeding and growth and then return to freshwater to spawn (Thorstad *et al.* 2010). Juvenile fish (smolts) emigrate to sea in the springtime (Todd *et al.* 2010).

Adult Atlantic salmon can migrate back to their natal river with a high level of accuracy (Youngson *et al.* 2003). The time spent at sea before the salmon's return to the rivers as adults varies, giving rise to two different names for salmon (one-sea and multi-sea winter salmon) dependent on their time at sea (Thorstad *et al.* 2010). Fish which spend one winter at sea, one-sea-winter (1SW) fish, commence their return to freshwater after 12-18 months, whereas fish which spend more than one winter at sea, multi-sea-winter (MSW) fish, can return at any time of year. But this seasonality varies with latitude. Most salmon will return between the months of May and October, although there is a high

level of seasonal variation with latitude, particularly in Scotland where salmon return to rivers all year round (Klemetsen *et al.* 2003).

This spawning migration is thought to occur when Atlantic salmon become sexually mature (Thorstad *et al.* 2010); however it is not yet apparent which environmental, genetic or other intrinsic factors might initiate maturation. The spawning migration can be split into two phases; the journey from sea to river mouth and then the journey from river mouth to home river (Hansen *et al.* 1993). Migration is very energetically costly and Atlantic salmon can swim at speeds of up to 100 km day^{-1} (Hansen & Quinn 1998).

When salmon begin their return to the rivers they stop feeding and are reliant upon stored energy reserves (Mills 1991). After their return the salmon will remain in the river until late autumn when they begin laying their eggs, spawning only occurs between November and December. As a consequence some salmon will spend almost a year in the river until they spawn (if they return early in the season) (Klemetsen *et al.* 2003). In Scotland, for example, some salmon re-enter the river in November but choose not to spawn until November of the following year.

The Atlantic salmon is an iteroparous species, a small proportion of spawners will survive reproduction, emigrate to sea once again, and perhaps return to spawn on future occasions (Klemetsen *et al.* 2003). Unlike the semelparous Pacific salmon (*Oncorhynchus spp.*) which die after spawning, individual Atlantic salmon may survive and return in subsequent years (Thorstad *et al.* 2010). Some individuals may spawn seven or more times but the majority Atlantic salmon will only spawn once or twice (Fleming 1996).

The recent declines of Atlantic salmon

Salmon have an iconic status in Scotland; they are a key source of food, income and have considerable cultural and socio-economic significance (Shelton 2009). It is estimated that the farmed salmon industry in Scotland was worth £584m in 2011 and wild salmon tourist angling is valued at £95-100m (SSPO 2011), with many people reliant upon the salmon fishing industry for income.

Examinations of the historical records have found that wild Atlantic salmon numbers have been decreasing since the 1980s and have reached the lowest phase since recording began (ICES 2012), declining from ~12000 tonnes in the 1960s to less than 2000 tonnes in 2011. Atlantic salmon are not only important economically but they are also the dominant anadromous fish in many North Atlantic river systems (Paterson *et al.* 2004). Due to expanding commercial interests in salmon this species is increasingly being put under exploitation pressure. Fisheries of Atlantic salmon in Scotland are in decline (Youngson *et al.* 2003) and are under increasing pressure to close, due to these widespread declines.

The abundance of wild Atlantic salmon has been shown to be declining across their whole geographical range and survival has been correlated with a nutrient-rich environment (ICES 2012). Catch numbers of returning wild Atlantic salmon in both North America (Condron *et al.* 2005) and the North East Atlantic (Europe) (Beaugrand & Reid 2003) have been declining since the late 1980s and have increasingly become a cause for concern (Friedland *et al.* 2003b; ICES 2012). Estimates suggest a decrease of approximately 3 million individuals between 1980 and 2008 (ICES 2009; Hindar *et al.* 2010). These declines have led to the introduction of fishing quotas upon fisheries and the restriction of the Greenland fisheries for local use (ICES 2012).

However, it should be noted that both oceanic and freshwater conditions may

influence the survival of salmon (Otero *et al.* 2011), and the possibility of interaction between these distinct environments in this context cannot be precluded. Nonetheless, the recent pattern of a marked decline in mean size and condition factor of 1SW grilse returning to Scottish rivers between 1993 and 2007 has been specifically correlated with anomalous warming of the surface waters in the oceanic feeding grounds of salmon exploiting the Norwegian Sea (Todd *et al.* 2008).

Recent studies highlight increasing sea surface temperatures (SSTs) as an indirect effect of this decline in quality. Increases in temperature, as high as 2^{o}C, have been recorded in the Atlantic salmon's feeding grounds in the eastern North Atlantic (Todd *et al.* 2008). These increases can be correlated with the declining condition of salmon. These sea surface temperature variations have also been linked with the shift of zooplankton populations (Beaugrand *et al.* 2002; Beaugrand & Reid 2003; Richardson & Schoeman 2004), with northerly movements of warm water species and declines in abundances of cold-water species.

Zooplankton have been identified as having a 'bottom-up' controlling effect on salmonid success (Richardson & Schoeman 2004), in a process which is dependent on larval production. Shifts in the fauna of the North Atlantic (the phytoplankton – zooplankton - blue whiting - pilot whale food chain) have been documented from the 1920s (Hátún *et al.* 2009), and have been shown to have influence bottom-up through the food chain, plankton numbers control the number of whiting and pilot whales. Increases in sea surface temperature correlate with declining Atlantic salmon quality (Friedland *et al.* 2003a), however it should be noted that both oceanic and freshwater conditions have been documented as influencing the survival of salmon (Otero *et al.* 2011).

Salmon feeding patterns

Atlantic salmon are generalist, opportunist, visual predators of zooplankton and nekton at the ocean surface (Haugland *et al.* 2006). Their growth response to prey availability and quality are therefore likely to be complex, and moreover there now is considerable evidence of the importance of size-related mortality impacting post-smolt salmon abundances (Friedland *et al.* 2003a). More specifically, increases in the growth rate of individuals have been linked with a switch from preying upon smaller zooplankton to piscivory, or the targeting of larger crustaceans, and this probably occurs only following the attainment by the fish of a size threshold (Rikardsen & Dempson 2010).

But the extent to which salmon are specifically selective of prey items - or largely responding to prey availability - remains a research challenge and prey choice appears to vary according to location (Friedland *et al.* 2009; Rikardsen & Dempson 2010). It now is well established that large-scale changes in sea surface temperature can influence the distribution and abundance of North Atlantic zooplankton populations, resulting in regime shifts in community structure (Beaugrand *et al.* 2002; Beaugrand & Reid 2003). Such large-scale ecological change may well exert 'bottom-up' control on salmonid success (Richardson & Schoeman 2004), and increases in sea surface temperature have been correlated with declining Atlantic salmon quality (Friedland *et al.* 2003a).

Research considering the effect of changing sea temperature upon Atlantic salmon has focused primarily upon abundance (Beaugrand & Reid 2003; Friedland *et al.* 2009; Vøllestad *et al.* 2009; ICES 2012). The introduction of quotas on salmon fisheries makes it uncertain whether these measure offer a truly accurate measure of fish numbers. Thus other measures must be used to measure salmon quality (Blackwell *et al.* 2000).

Morphometric measures of fish quality

Morphometric measures of fish quality are generally considered to be simple measures that assess the length or weight data of a fish (Lloret *et al.* 2014a). They are constructed from simple weight and length data that can be easily gathered by non-destructive methods (Murphy *et al.* 1990). This makes them affordable to implement and requires the minimum amount of equipment.

These metrics work on the underlying principle that a heavier fish of a given length is in better condition than a lighter fish of the same length (Jones *et al.* 1999). This implies that a standard fish of a species should be of a given weight and length, and those fish that do not meet these criteria indicate signs of relative condition. These metrics when applied to a fish they are called its condition factor.

Condition factors

The use of condition factors to measure fish quality is not a new phenomenon. Since the 1900s they have been used to identify the condition of fish (Murphy *et al.* 1990). They have remained popular due to their ease of application and the fact that they can readily be applied to historical datasets that commonly recorded length and weight data of fish (Froese 2006). There have been a number of reviews assessing the reliability and controversies surrounding the use of condition factors, but the review performance by Blackwell *et al.* in 2000 is particularly informative on the successes and failures on the two of the commonly used measures of condition factor.

Fulton's condition factor was the first widely used morphometric condition factor applied in fisheries science (Ricker 1973). Fulton's condition factor or K is calculated using this formula; $K = (W/L^3) \times 100{,}000$, where W is the weight of the

fish and L its length. A fish of K=1 is considered to be a standard fish, with fish of K>1 considered to be of good condition.

Fulton's K makes one major assumption; that fish grow in an isometric pattern, meaning that fish must grow in a uniform pattern putting on weight to length in relationship in the order of 3 (Lloret & Ratz 2000; Froese 2006). This is true for some species, such as cod, but needs to carefully applied to other fish species to ensure this is also true.

An alternative to using Fulton's K is using Relative Weight condition factor, known as W_R. This condition factor is based upon the principle that fish from different populations may show difference sizes and shapes but still be of the same quality (Lloret *et al.* 2014a). It is calculated using the formula; $W_R = W/W_S$, where W is the weight of a fish and W_S is a length-specific standard weight predicted from a weight-length regression of the whole population (Blackwell *et al.* 2000).

In doing this is removes some of the bias that Fulton's K has on growth patterns; W_R does not assume isometric growth. The draw back with using W_R is that to get a meaningful result the population studied needs to have standard weight measures available from prior analysis (Blackwell *et al.* 2000). This can limit its application to many species, however, commercially important species, like the Atlantic salmon, have extensive historical records detailing this information to which W_R condition factor can be applied (Todd *et al.* 2008).

A comparison of using W_R condition factor and Fulton's K

Todd et al. (2008) and Bacon et al. (2009) used these two different morphological metrics to measure the quality of the same Atlantic salmon populations; by analysing trends in historical datasets from a number of Scottish

rivers. Todd *et al.* and Bacon *et al.* both found declines in the length and weight of one-sea winter (1SW) salmon over time (Bacon *et al.* 2009) and these studies confirmed that there was a marked decline in body condition (calculated from the length-weight relationship)(Blackwell *et al.* 2000; Weber *et al.* 2003).

However the effect of using these different condition factors to measure fish condition became apparent when both groups examined the relationship between increasing sea-surface temperature (SST) and condition. Bacon et al. (2009) use Fulton's K and found no influence of increasing SST on these returning fish, whereas Todd et al. (2008) use river specific W_R condition factors and were able to find a correlative effect. This led to Bacon *et al.* claiming that the correlation found by Todd *et al.* could not be substantiated for any of the six fisheries studied over wider timescales. This is not the case but instead clearly demonstrates the issues involved in the misuse of morphological metrics of condition factor, in particular Fulton's K.

The basic assumption of morphological metrics is that they are able to offer an indication of the energy reserves of a fish. However there have been a number of reviews disputing this idea (Froese 2006; Stevenson & Woods 2006; Davidson & Marshall 2010), with Stevenson and Woods suggesting the morphometric measures only really compare the shapes of fish.

Physiological measures of fish quality

Other proxies for fish quality have been variously used and applied (Lloret *et al.* 2014b). Several authors have suggested that other phenotypic and biochemical measures - such as individual fecundity (Berg *et al.* 2001), protein content (Hillestad & Johnsen 1994), stable isotope analysis (Hanson *et al.* 2013), and lipid reserves (Davidson & Marshall 2010) may offer more tractable information to assist in the interpretation of the possible proximate effects of large-scale environment change on marine populations (Litzow *et al.* 2006).

Lipids as a measure of fish quality

Variations in lipids and other dietary substances can be influenced by climatic fluctuations (Litzow *et al.* 2006), so this relationship needs to be considered. It is problematic that the shifts seen are very rarely direct, are part of complex ecosystems and often influenced by either bottom-up or top-down control (Litzow *et al.* 2006). It appears that salmonids are not just directly affected by fluctuations in sea surface temperature, but they are also limited by their prey (Beaugrand & Reid 2003). The change in salmon condition can be traced to ocean climatic variation (Todd *et al.* 2008), but the question of whether this variation impacts upon lipid content of fish then arises.

Lipids are the major energy reserves of all fin fish (Leaver *et al.* 2008). In Atlantic salmon lipids are stored in the body tissue, providing a constant source of energy and energy reserves for the fish (Zhou *et al.* 1996; Nanton *et al.* 2007). Atlantic salmon acquire their lipid supply from their diet, so they are reliant upon their prey supply. To consider this link between salmon and their prey lipid levels should be investigated.

Todd et al. (2008) demonstrated that total lipid content is related to fish condition factor in a non-linear pattern. This is potentially problematic if one were to infer lipid reserves from historical data of weight at length that show declining somatic condition. The implication is that lipid content also is declining, but this needs to be directly observed and confirmed. It is, nonetheless, to be expected that these declines will have an impact upon the survival and fecundity of individual fish (Thorpe *et al.* 1998; Todd *et al.* 2008) because salmon make a spawning migration they expend energy (Thorstad *et al.* 2010). Increasing mortality rates during the sea phase of the Atlantic salmon lifestyle and declines in salmon fecundity will therefore cause considerable management problems.

Aims and structure

The original aim of this research was to examine and investigate the measures of quality for Atlantic salmon populations. This thesis does this by examining the physiological responses of salmon through various metrics as indicators for environmental change. To achieve these aim new methodologies for examining lipid and TAG concentrations of salmon tissues were developed and then investigated with respect to changing body condition.

Chapter 2 focuses solely upon the relationship between the use of a morphometric measure of quality, condition factor, and a physiological metric, lipid content. The hypothesis that lipid content of salmon increases with condition factor was upheld; however, this thesis indicates that the relationship is not simple. The non-linearity of this relationship makes it impossible to hind-cast lipid contents based on somatic condition factors that can be calculated from archive datasets.

The following chapter sought to study the informativeness of sampling lipids from the different body tissues of Atlantic salmon. Chapter 3 focuses upon the lipid contents of the white muscle, red muscle, adipose and liver tissues. This research demonstrates that all tissues show decreased lipid content with decreasing fish condition, but not all tissues responded in the same way and compares the different in lipid concentration of maiden (pre-spawning) fish and mature, stripped (post-spawning) fish. After spawning fish show markedly decreased lipid levels, with lipids from some tissues having been drawn down in preference to others. Low-lipid levels reduce the likelihood of Atlantic salmon completing an iteroparous lifestyle.

In Chapter 4, the focus moves to the assays of a specific class of lipid, triacylglycerides (TAGs). TAGs are a primary energy store (rather than comprising a structural reserve) and were found to be preferentially conserved in the red muscle post-spawning. But diminishing TAG levels after the spawning migration show how energetically costly the migration can be.

The final chapter of this thesis (Chapter 5) summarises the general outcomes gained from the research formed in the previous chapters. It aims to critically examine the usefulness and accuracy of measuring the condition of Atlantic salmon through metrics of quality; condition factors, lipid contents and triacylglyceride concentrations. It then suggests future directions for research.

Appendix 1 focuses on post-spawning Atlantic salmon, and an investigation of the total lipid and triacylglyceride content of their eggs. Salmon provision their eggs prior to spawning and during vitellogenesis, and this work clearly demonstrates that lipids are a key component of this provisioning process.

2. Atlantic salmon, condition factor and lipid calibration readings

Introduction

Declines in the abundance of wild Atlantic salmon (*Salmo salar*) have been shown across their geographical range and have been correlated with declines in abundance (ICES 2012). The migration of Atlantic salmon (*Salmo salar* L.) for it's over wintering period to sea has been shown to be increasingly expensive in terms of fish survival. Catch numbers of returning wild Atlantic salmon in both North America (Condron *et al.* 2005) and the North East Atlantic (Europe) (Beaugrand & Reid 2003) have been declining since the late 1980s and have increasingly become a cause for concern (Friedland *et al.* 2003b; ICES 2012). Estimates suggest a decrease of approximately 3 million individuals between 1980 and 2008 (ICES 2009; Hindar *et al.* 2010). These declines have led to the introduction of fishing quotas upon fisheries and the restriction of the Greenland fisheries for local use (ICES 2012).

Due to these fishing quotas, estimating the extent of these reductions from current catch data has become increasingly difficult, hence scientists have sought alternative measures of fish success. Studies now show that not only have fish numbers declined, but that in Scotland there also is a trend in diminishing condition factor (as a measure of individual "quality") of those that survive to return to their natal rivers (Todd *et al.* 2008). These declines in quality and numbers are of concern to fishery managers because they suggest that not only are fish dying and thus not returning, but also that those that die as a result of environmental change presumably are of even poorer quality.

The use of quality to measure population success is not a new concept but there is little consensus as to how this is best measured (as discussed in Chapter 1).

Many studies have used a range of morphological factors to determine success, including individual mass, length and condition factors (Blackwell *et al.* 2000; Weber *et al.* 2003).

Population and stock abundance estimates for Atlantic salmon may derive either from angling catch records or from sampling commercial net fisheries. In routine monitoring of salmon it is common practice for fish to be recorded for a few standard characteristics, including weight, length, and a visual assessment of gender based on secondary sexual characteristics. The river age (smolt age) and sea age of the fish can subsequently be derived from microscopic examination of circuli on the scales. Given the length and weight of an individual fish its somatic condition factor can be calculated– either by assuming a fixed length:weight allometry as the comparator, or by determining a population- or stock-specific length:weight relationship for that particular river. Condition factors therefore provide a measure of the quality ("plumpness" or "skinniness") of the fish and such data can be especially informative if they can be aligned to, for example, further data on stock abundance. There are a wide variety of mathematical condition factors that can be, and have been, calculated by using the length-weight relationship of fish (Blackwell *et al.* 2000; Marshall *et al.* 2004; Froese 2006).

Fulton's condition factor

The traditional salmonid fisheries method of calculating the relative or predicted weight of a given fish has been to use Fulton's condition factor (K) (Froese 2006), which was (and still is to some extent) widely applied (Blackwell *et al.* 2000; Bacon *et al.* 2009). Fulton's Condition factor is given as:

1. $K = (W/L^3) \times 100{,}000$ (Blackwell *et al.* 2000; Froese 2006)

where W is weight in grams, L is length in millimetres and 100,000 is a constant used for scaling purposes. Fulton's K assumes that weight increases as the cube of length (isometric growth) (Marshall *et al.* 2004), and that the shape of a fish does not change as it grows, and is calculated as the ratio between the observed weight and the expected weight for a fish of that length (Blackwell *et al.* 2000). If the fish species does not conform to a cube relationship then Fulton's K will be length dependent in a marked manner. Thus, one needs to derive an appropriate comparator to address the problem of length dependence.

W_R condition factor

Most measures of condition are vulnerable to length dependence (Blackwell *et al.* 2000), thus finding a measure which is less susceptible to bias is essential when comparing populations. W_R, condition factor uses a method where one can derive a population-specific standard curve to address this length-dependence and apply this in the calculation of condition factor.

Condition factor W_R can be calculated as:

2. $W_R = W/W_S$ (Blackwell *et al.* 2000; Brenden *et al.* 2003)

where W is the observed weight and W_S is the standard weight for a fish of that length. The standard weight (W_S) can be calculated by the method first proposed by Murphy et al. (1990). This applies a regression-line-percentile (RLP) technique, based upon the 75^{th}-percentile weights and not the 50^{th} percentile (Murphy *et al.* 1990; Blackwell *et al.* 2000). The 75^{th} percentile is typically used in managing fish populations because the average condition (the median) is perhaps too low a target in encouraging management improvements and reduces the influence of outliers in determining the W_S curve (Brenden *et al.* 2003). The data for weight and length are $\log_{10}$ transformed and regressed

weight on length. These data are then considered as a statistical population to be modelled, the predicted $\log_{10}$ weights at 1 cm length intervals at each year measured are back transformed to weight and the 75th percentile of these weights, at length, is calculated (Brenden *et al.* 2003).

The 75th percentile weights are then $\log_{10}$ transformed and regressed against the $\log_{10}$ lengths to give the W_S (standard weight) equation (Blackwell *et al.* 2000):

3. $\log_{10}W_S = m\log_{10}L + c$ (in the form $y = mx + c$) (Kolander *et al.* 1993; Blackwell *et al.* 2000; Froese 2006)

determined from the equation of the regression line. Standard weight can then be calculated as in equation 3, and used to calculate the overall condition factor (W_R) of individual fish as in equation 2 (Todd *et al.* 2008). This removes a large amount of the length dependence seen with Fulton's condition factor, but it has to be acknowledged that no condition factor metric is ever truly or totally length-independent.

Although much improved, this method for the calculation of condition factor is still biased (as any would be). The fact that the sex of a fish influences its size is generally accepted. With male fish found to be typically longer and heavier, using the RLP condition factor removes some of this bias by accounting for that length variation, but it still assumes that male and female fish grow in the same pattern. Work by Todd *et al.* (2008) seems to indicate that male and female one-sea-winter (1SW) fish do grow in similar patterns, however, this may not be the case for multi-sea-winter (MSW) fish. It is also true that MSW fish grow differently to 1SW fish (Todd *et al.* 2008) thus a separate RLP condition factor would need to be calculated for MSW to remove this bias. Similarly, in order to remove any

male-female bias, individual condition factors for each sex perhaps should be calculated.

One intractable difficulty arising from this is that fish typically do not grow in uniform shapes, so any comparisons that use Fulton's condition factors will always be limited and applicable only to fish of very similar lengths. For example, male Atlantic salmon within a year class typically are longer and heavier than females (Todd *et al.* 2008), thus in such populations Fulton's condition factor would be subject to bias. Bacon et al. (2009) applied Fulton's and made this key error, two fish; from different rivers, with a different shape; may have actually identical condition but very different Fulton's K values. Thus when Todd et al. (2008) and Bacon et al. (2009) used different calculations the condition factor of the same salmon stocks; Bacon et al. (2009) use Fulton's K and found no influence of increasing sea-surface temperature on these returning fish, whereas Todd et al. (2008) use river specific condition factors and were able to find a correlative effect.

Due to the fishing quotas, estimating the extent of these reductions from current catch data has become increasingly difficult, hence scientists have searched for alternative measures of fish success. Studies now show that, not only have fish numbers declined, but there is also a trend in diminishing quality amongst those that survive to return to their natal rivers (Todd *et al.* 2008; Bacon *et al.* 2009). These declines in quality and numbers are of concern because they suggest that not only are fish are dying and thus do not return, but also that those dying are perhaps of even poorer quality and hence have succumbed to environmental factors.

The inference is that sea surface temperature warming alone can exert an indirect negative effect on salmon growth condition as a result of climate-driven changes in the availability and/or quality of prey. Thus, whilst it remains

problematic to estimate population abundances of salmon from catch data and automated in-river counter records (Thorley *et al.* 2005), mean indices of individual growth condition can provide detailed quantitative indicators of growth success at the entire population or cohort level.

However, fitness in terms of lipid storage does not vary in a simple manner with length, weight or condition factor (Todd *et al.* 2008) and it has been suggested that proximate measures such as protein content (Hillestad & Johnsen 1994), fecundity (Berg *et al.* 2001) and lipid contents (Davidson & Marshall 2010) may offer more ecologically relevant and appropriate information to explain the effects of environment changes (Litzow *et al.* 2006).

This highlights a central issue in understanding the declines in quality of returning Atlantic salmon populations and their possible causal factors; are condition factors an appropriate proxy for individual energy reserves? Due to the complexity of Atlantic salmon life history patterns, the fish sampled in these previous studies (Todd *et al.* 2008; Bacon *et al.* 2009) are yet to complete their spawning migration and their energetic reserves must be sufficient to support them through their journey upstream and the costly spawning process (Chapter 1).

Coastal fisheries, commonly used to capture large numbers of Atlantic salmon, exploit fish returning to their natal rivers to spawn. Thus salmon caught at this life stage are approaching the start of sexual maturation. It is not clear what triggers the start of this maturation process or the possibility of a subsequent return migration (after spawning); it is suggested that a mixture of genetic (growth patterns, condition optima) and environmental (body condition, overall growth, photoperiod) cues are influential (Hutchings 1991; Jonsson & Jonsson 2007; Thorpe 2007) (Hutchings & Jones 1998; Thorpe et al. 1998; Jonsson &

Jonsson 2007). It should be noted that the importance of these environmental cues will vary between populations.

Modelling approaches undertaken by Thorpe *et al.* (1998) on juvenile salmon indicate that there should be a difference in the lipid reserves of maturing and non-maturing fish; thus fish must effectively assess their body condition and have adequate reserves to complete the return migration and spawning process. Upon the start of their return from ocean feeding grounds to spawn Atlantic salmon cease feeding (Kadri *et al.* 1996), thus their lipid stores at river entry must be sufficient to maintain them for their journey upstream and permit the energetically costly spawning process. Atlantic salmon will continue feeding until a lean mass threshold is reached (Kadri *et al.* 1996). This offers a new question; do the declines seen in morphometric condition indices of returning fish translate into the lipid reserves of fish?

It is widely accepted that lipids are the key energy reserve of fish, including the Atlantic salmon (Jonsson *et al.* 1997). Although lipid content varies markedly between species, work on cod (*Gadus morhua*) indicates that both temperature and diet are vital in determining fish condition (Sandeman *et al.* 2008). Silver eels (*Anguilla anguilla* L.) must also perform an energetically costly migration to spawn, returning from river to sea (they are catadromous - the reverse of the Atlantic salmon lifestyle), with the total energetic cost of spawning upon fat reserves being 67% of the total lipid stores (Palstra & Thillart 2010). Work undertaken on herring (*Clupea harengus* L.) indicates that fat reserves and body condition are representative of feeding success (Davidson & Marshall 2010). It has been demonstrated that the lipid content of wild juvenile Atlantic salmon will determine its survival potential (Næsje *et al.* 2006). Extensive studies into the effect of diet on farmed Atlantic salmon demonstrate that fish fed a poor quality diet show reduced lipid contents (Torstensen *et al.* 2000); thus if wild Atlantic salmon face poor diets, in terms of the prey they find at sea, they will also show reduced lipid contents.

To assess the hypothesis that declines in Atlantic salmon condition factor and thus quality is equivalent to fish total lipid content, the relationship between condition and lipid content was assessed over a 6-year period. The fat content of fish was measured using a hand-held device, a Fatmeter. This was calibrated for a sub-sample of the population against the true lipid content of a fish via means of lipid extraction.

This allowed the relationship between condition factors and lipid content to be examined, with the aim of determining if lipid content is a good proxy for fish condition and thus quality.

The hope was this could then be applied to archival datasets (curated by Marine Scotland Science) extending back to 1963 exist for multiple river populations of Scottish salmon, detailing only the weight, length, river age, sea age and sex of fish; there is no biochemical or genetic data available for these fish. A consistent relationship between condition factor and total lipid content was sought in order to perhaps allow hind-casting of energetic stores of historical Atlantic salmon populations.

Methods

Experimental Methodology

Sample Collection and Preparation

Monitoring Data

Wild, adult, one-sea-winter (1SW) maiden (hereafter "pre-spawning") Atlantic salmon were selected from fish caught at the coastal mixed-stock fisheries at Strathy Point, Armadale and Melvich (North Scotland). Fish were sampled from 2006-2011 during a restricted part of the commercial netting season (June to August – weeks 23-33). Table 2.1 includes information on sample numbers.

Year	Monitoring Fish Sampled	Lipid Fish Sampled
2006	345	35
2007	150	36
2008	293	34
2009	441	27
2010	497	28
2011	423	N/A

Table 2.1: Table of sample sizes of fish monitored and fish sub-sampled for lipid extraction in sampling years 2006-2011.

All fish were monitored for fork length (rounded down to 0.5cm), weight (to 0.01kg), gender, disease prevalence (red vent syndrome) and the river (smolt) age and sea age were ascertained for individual fish from scale readings. The individual fish monitored were selected from a sub-sample of the fish caught; this was not done at random but rather the fish were chosen to include the range

of sizes of salmon available. The fish selected for lipid sampling were selected by plotting the ranges of lengths available, in a given season, and selecting fish that spanned this range.

Scale samples were taken from just posterior to the dorsal fin and above the lateral line, the standard region (Jensen *et al.* 2012); after which they were stored and dried in paper packets. Impressions were taken of the scales on acetate strips. Counts and measures were made of all circuli on the scales by Bryce White (Marine Scotland Science) and used to derive the river age (years spent living in a river) and sea age (years spent living at sea) of the salmon. These annuli are identified in the conventional manner from observation of the transitions in spacing between circuli (Shearer 1992).

Fatmeter methodology

The lipid content of an intact fish can be estimated by taking fatmeter readings ("Principle of Operation - Distell.com" 2013) along the lateral line of the body. This technique can be used to measure lipid content of fish non-destructively, which in the present case was essential because the monitored fish were sampled from commercial catches. The reading plate of the hand-held fatmeter is placed in contact with the fish and the meter gives a reading of the microwaves reflected (i.e. not absorbed) and that value is then converted to fat content by calibration with extracted fish lipid.

Fat Reading	Accuracy
2 – 15 %	± 0.5%
16 – 30 %	± 1.0%
31 % upwards	± 2.0%

Table 2.3: Table of Distell Fatmeter Accuracy adapted from Distell 2013 ("Accuracy of Results - Distell.com" 2013).

Lipid content tends to show an inverse relationship with the water content of a tissue; thus, by measuring one of these parameters one can estimate the value of the other if they show a clear relationship. The Distell Fatmeter works by means of this principle to permit the estimation of the fat content of a tissue sample ("Principle of Operation - Distell.com" 2013). The fatmeter uses a 'microstrip sensor' that can measure the absorbance of the emitted microwave radiation of the hydrogen bonds in the water of a sample and then using a standard calibration the meter can convert this into a percentage tissue content of fat. The accuracy of the meter is dependent upon the level of fat content, with the meter being more accurate at low fat levels (see Table 2.3), thus it is necessary to follow a rigorous methodology with many repeats.

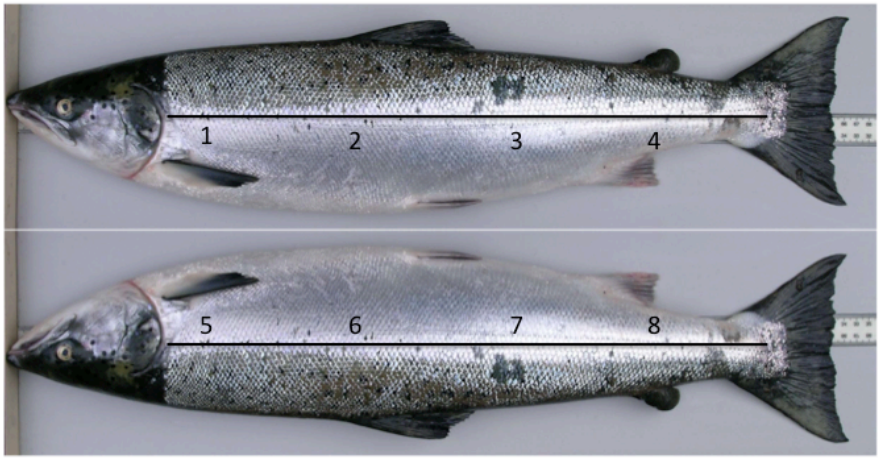


Figure 2.4: A shows both sides of an entire Atlantic salmon, the black line indicates the lateral line of a fish and the numbers the points on the line where fatmeter reading were taken.

Fatmeter readings were taken in eight repeat measurements for each fish, with four along each side of the salmon down the length of the lateral line of the fish (see Figure 2.4). Prior to readings being taken, the fish were wiped to remove

excess mucus and water, to ensure an adequate seal of the meter head on the fish skin, and that no bubbles of air were intervening. Before and after each fatmeter reading session, readings were taken from the standard pads, and then intermittently during sampling, to ascertain that the meter was functioning accurately.

Routine checking of the raw fatmeter readings would indicate any obviously spurious measurements that were deleted. Outliers arose in these few instances either from an accumulation of mucus on the meter plate, or perhaps from air bubbles. These investigations revealed that the 4th and 8th readings were commonly outliers. The 4th and 8th readings gave consistently lower fat meter readings than all of the other reading points. The manufacture, Distell, was contacted and it was suggested that this was an artefact of the narrowing of the fish (the 4th and 8th reading were both taken at the tail-end of the fish). The decision was taken that these readings were not accurate and thus all of these readings taken at the 4th and 8th points were removed from the dataset.

For each sample season the standard pad readings, taken for each day of sampling, were investigated to check for any long-term "drift" in the measurements taken by the fatmeter during that sample season. Figure 2.5 clearly demonstrates that some seasons were more prone to meter drift than others. Fatmeter readings taken over the season were adjusted for Fatmeter drift, by calculating the regression equation of each season's drift of standard readings and the residual between this and the true reading value. The residual then was combined with the difference between the predicted value and the mean standard reading to create an adjustment value for the routine salmon readings.

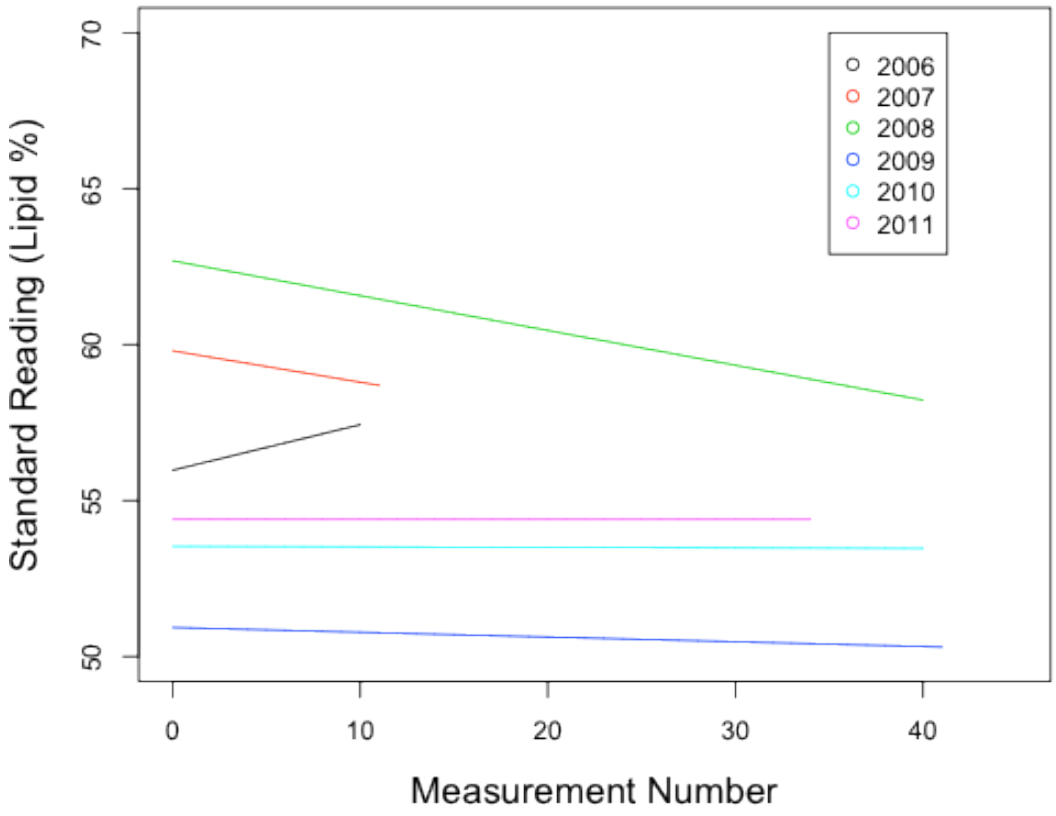


Figure 2.5: Regression lines drawn from the Standard Reading taken from the Fatmeter standard pad over the course of a season (Measurement Number). The year of sampling is indicated by colour.

Various difficulties arose when using the fatmeter over the duration of the study. The meter was recalibrated by Distell ("Principle of Operation - Distell.com" 2013) (the manufacturer) following a battery charging problem in 2008. The same charging problem arose during sampling in 2011, but Distell were able to reset the meter to the same setting. Damage to the fatmeter in 2009 required repair and there was a brief hiatus in the recordings for that season.

Lipid Extraction

Lipid content of the fish was determined from a sub-sample of the monitoring fish, selected to embrace the range of condition factors available (see Table 2.1 for sample numbers). Lipid content was determined gravimetrically from a sample of the homogenised fish using a modified Bligh and Dyer method. Lipid content was measured in triplicate and then calculated as a percentage of the mass of the entire fish. The lipid contents of the sub-sampled fish then were used to calibrate the fat content readings measured for the monitoring fish by using the fatmeter.

Whilst whole body sampling was possible for some of the pre-spawning fish, in order to ensure accurate and direct comparisons between all fish a standard transverse body section (the so-called "Norwegian cut" in the salmon aquaculture industry, Figure 2.1) was taken from each fish by removing the section between the posterior insertion of the dorsal fin and the anus (Todd *et al.* 2008). The Norwegian cuts were either immediately dissected or stored at -80°C until an appropriate sampling opportunity. Those samples that were frozen were vacuum packed before freezing and then allowed to thoroughly thaw before any analyses were performed. All sections were weighed before and after freezing (as necessary), and gravimetric outcomes adjusted accordingly.

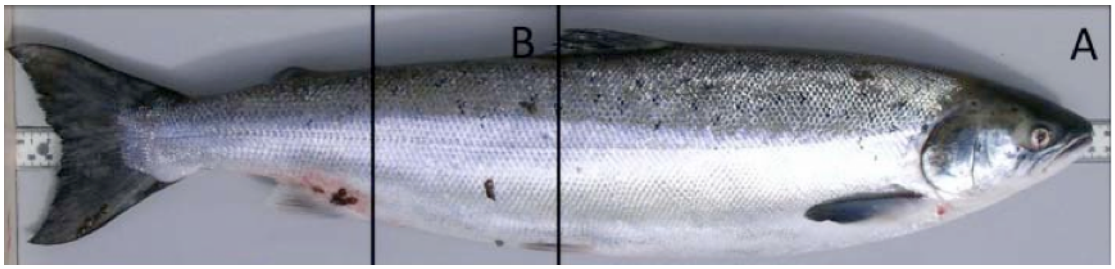


Figure 2.1: A shows an entire Atlantic salmon (A), the black lines indicate the cuts (the first behind the dorsal fin and the second in front of the anus) taken to create the Norwegian cut (B).

Basic lipid extraction

The Bligh and Dyer lipid extraction method was initially developed to permit analysis of fish tissues specifically with low fat content (Bligh & Dyer 1959). This well-established lipid extraction method has been shown to produce consistent, repeatable results for fish with low lipid contents (Ando *et al.* 1996; Bendiksen *et al.* 2003; Todd *et al.* 2008). The methodology is based on the power of chloroform and methanol to isolate lipids from water-rich biological tissues. The method demonstrates that optimal lipid extraction will occur when tissue is homogenised with chloroform, methanol to form a 'monophasic' solution (Bligh & Dyer 1959). The resulting solution can then be diluted with water to produce a 'biphasic' solution in which the underlying chloroform layer contains the lipids and the methanol-water layer the non lipids and the tissue mass (Bligh & Dyer 1959). The present study applied a standard ratio of approximately 40ml chloroform: 40ml methanol: 20ml water per sample of tissue homogenised, with typically ~20g of tissue per sample tested.

The original use of this method by Bligh and Dyer utilised low-lipid tissue from cod. It has been shown, however, that for fish or tissue of a high lipid content (including Atlantic salmon), the lipids often are underestimated by this process, even if their levels are as low as 2-10% by mass (Iverson *et al.* 2001), To ensure all lipid is successfully removed from the tissue, Iverson et al. (2001) recommended an initial round of non-polar solvent extraction (using chloroform only), to be followed by the standard Bligh and Dyer extraction. Iverson et al. also indicate at in very high lipid tissue (like those of the Atlantic salmon) repeated Bligh and Dyer extractions would be more successful than increasing solvent volumes.

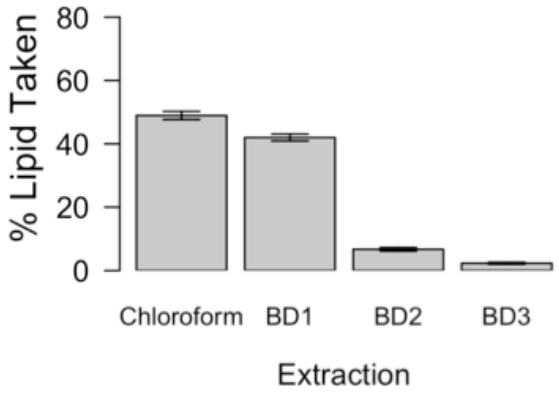


Figure 2.2: Preliminary results of mean percentage lipid taken at different extraction levels. The type of extraction in noted by Chloroform (chloroform), BD1 (Bligh and Dyer 1), BD2 (Bligh and Dyer 2) or BD3 (Bligh and Dyer 3). Standard error bars of the extractions are shown.

Prior to lipid extraction the whole of the sample was homogenised in a food processor. Samples were taken of this homogenised tissue in triplicate and subjected to Bligh and Dyer lipid analysis. Then following the work of Iverson et al. (2001) the preliminary whole fish samples and Norwegian cut tissue were treated to an initial chloroform extraction, followed by three subsequent Bligh and Dyer lipid extractions to ensure all lipid was removed from the lipid-rich tissues. Typically fisheries scientists tend to perform just one Bligh and Dyer extraction on tissue samples, however, as figure 2.2 demonstrates not all the lipid is extracted from the tissue sample in just one extraction. In the basic lipid extraction method four rounds of Bligh and Dyer extractions were performed, to ensure all lipid was extracted from the tissue. However, it is important to note that if too many rounds of Bligh and Dyer extractions are performed it is possible to extract non-lipid components.

A good indicator that the later Bligh and Dyer extractions are extracting lipid is the presence of the lipophillic astaxanthin pigment, which is bright orange; this

pigment is taken up in the extraction with the lipid-chloroform phase and while visible indicates that there is still lipid in the extract. The colouration of the lipid extract fades with each round of Bligh and Dyer performed, and thus when the extract turns colourless can be used as an indicator that all the lipid is extracted.

The lipid extract was measured gravimetrically from the average of triplicate samples; True Lipid Proportion of Fish = (Mass of lipid extracted/Mass of tissue), and then this concentration was converted into a percentage and hence used as a proxy for the entire fish.

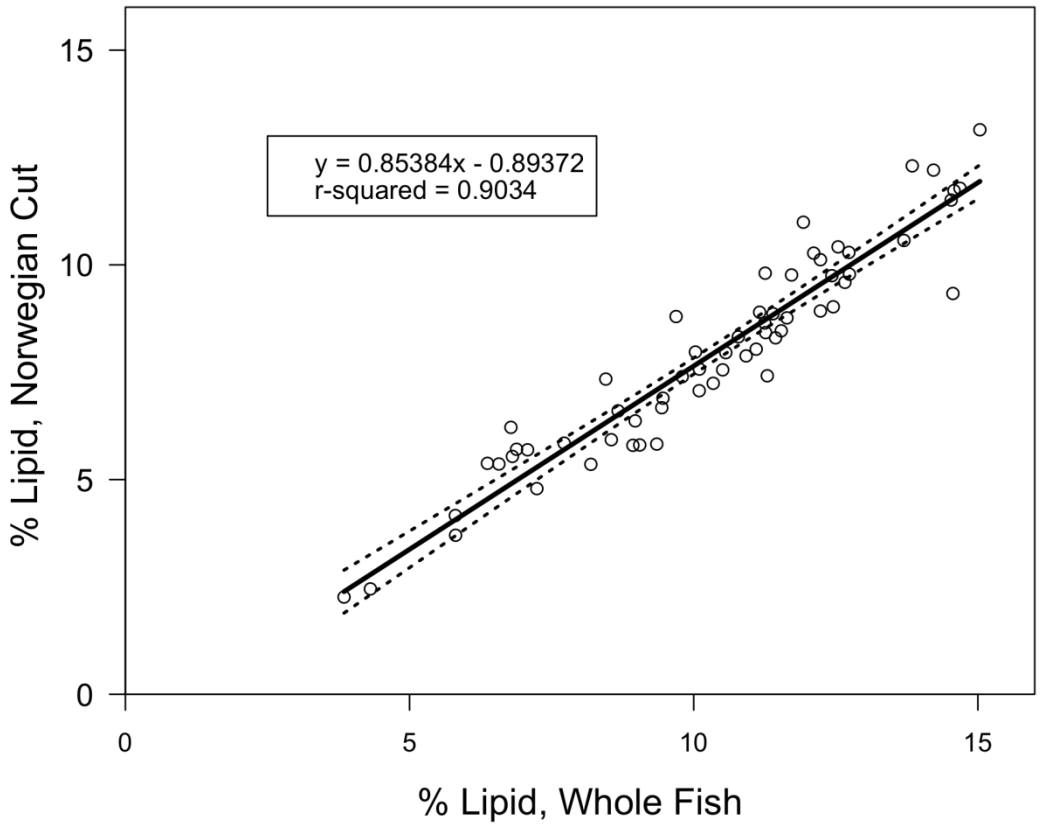


Figure 2.3: Percentage lipid of the whole Norwegian cut of pre-spawning fish plotted against percentage in the whole fish. Individual data points are shown (grey) and the linear model prediction drawn (solid black line), 95% confidence intervals are indicated (dotted lines). The equation of the model line and r^2 value is indicated.

Initially the fish lipid content was estimated from sampling the entirety of an Atlantic salmon. However, in fishery research it is common to use the Norwegian cut (Fig. 2.1). To ensure that the lipid in the Norwegian cut is a good estimate of the lipid in the entire fish, lipid extractions were performed on the entire fish and the Norwegian cut for 60 fish. Figure 2.3 demonstrated the close, linear relationship between the percentage lipid in both the whole fish and Norwegian cut ($y=0.8538x -0.894$, $r^2=0.903$), indicating that the Norwegian cut does provide a suitable, conveniently extractable and reliable means of estimating the lipid content for the entire fish. Thus for reasons of reliability and comparability of the derived data, the lipid extractions undertaken here pertain exclusively to the Norwegian cut unless otherwise stated.

Skin (g)	Bone (g)	**% Component of Norwegian Cut**
14.04	7.73	5.18
11.13	6.47	5.68
11.96	5.49	5.81
11.23	6.36	5.50
12.66	6.94	5.77
11.52	6.23	5.72
12.37	7.59	5.52
14.16	8.87	5.36
15.65	11.90	5.30
24.52	14.45	5.82
		Mean = 5.56% ±0.07

Table 2.2: Table of bone and skin mass in relation of total Norwegian cut mass.

The skin and bone in the Norwegian cut is a significant proportion of the overall weight of the section. Whilst it is easy to remove the skin, it did not prove possible to adequately dissect away all the tissues from the skeletal bones in the Norwegian cut, without destruction of the tissue. The easiest method of removing the tissue from the bones was to boil the flesh, which corrupts any

lipids stored in the tissue. Preliminary studies indicate that approximately 5.56% (±0.07) of the total weight of the Norwegian cut is bone and skin mass (see table 2.2), and thus all Norwegian cut weights were adjusted by 5.56% to ensure that lipid was only gravimetrically assessed against tissue weight.

Fatmeter calibration

Fatmeter readings alone can provide only an indication or estimation of the actual lipid store in a fish. The meter is not able to read the adipose fat as it is relying on an indication of the water content of the musculature along the region of the lateral line. In order to achieve a more 'real' meaning these readings require validation (Davidson & Marshall 2010). Accordingly, the lipid extractions of which the methodology was detailed earlier in this chapter were used to calibrate the Fatmeter readings against true lipid values, thus a 'real' lipid value, here after called the 'Predicted Entire Fish Lipid Content' could be given to all monitored fish.

The calibration equations were calculated by performing geometric mean regression upon the fatmeter reading for a given 'calibration' fish and the observed percentage lipid content extracted from the Norwegian cut. The geometric mean was used in preference to a standard arithmetic mean because this relationship changes multiplicatively rather than additively (it follows a logistic pattern) (Crawley 2007a), and this being a case of y on x regression, whereby the response variable is dependent on set x values (Ricker 1973; Warton *et al.* 2006); and where both x and y are measured with error (Crawley 2007a). Conventional y on x regression assumes x is measured without error; hence for regression of log fish weight on log fish length Ricker (1973) showed that the geometric mean is appropriate over the conventional y on x regression.

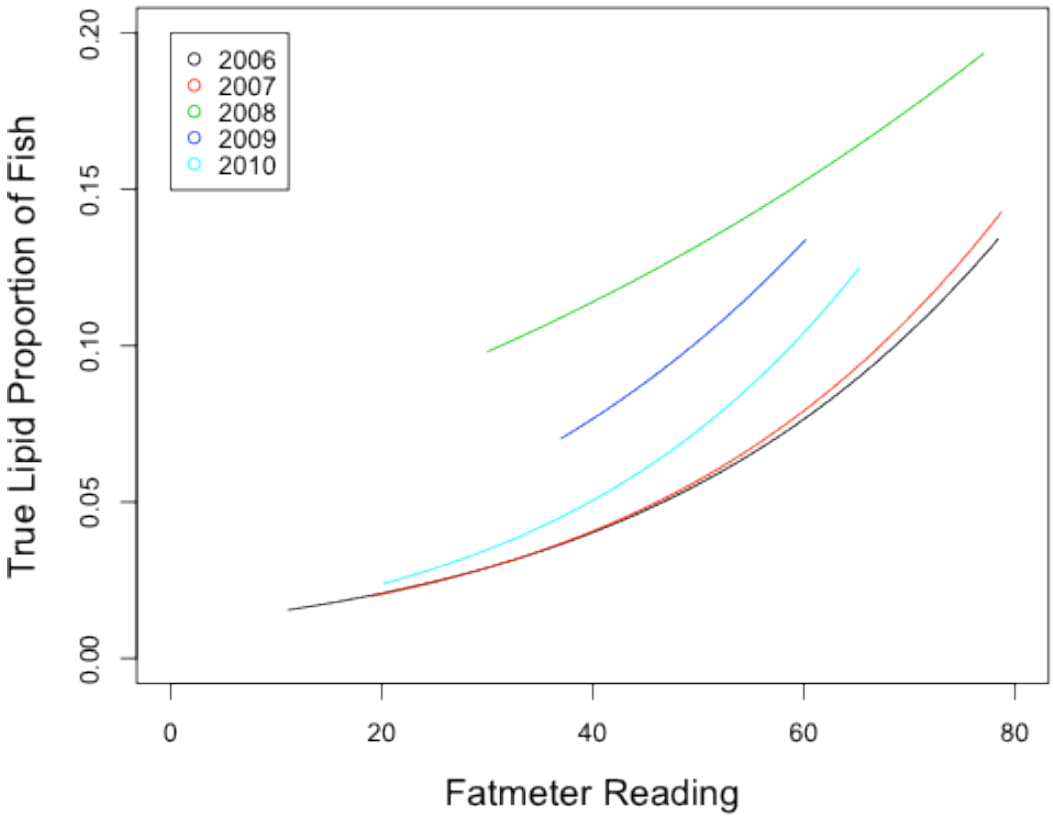


Figure 2.6: Logistic Regression lines drawn to calibrate Fatmeter readings with true extracted lipid proportions. The year of sampling is indicated by colour.

Figure 2.6 demonstrates that the calibration relationship between the Fatmeter reading and the True Lipid Proportion of the Fish (the concentration of lipid in a fish's tissues) varied between years and thus it was necessary to calibrate the fatmeter with real extracted lipid values. This figure demonstrates the need to validate the Fatmeter readings as there is clear variation between the years of study.

These calibration equations (see Table 2.4 for details of the equations) were then used to convert the Fatmeter readings taken in each year to be a more accurate representation of the lipid content of the fish in question. Thus hear after all

Fatmeter reading have been converted to show this and are now called Predicted Entire Fish Lipid Content (meaning that they are the predicted lipid content calculated from the applying the calibration equations to the Fatmeter readings).

Year	Fatmeter Calibration Equation
2006	log Y = 0.0155X + 0.0423
2007	log Y = 0.0155X - 0.0428
2008	log Y = 0.0132X + 0.0378
2009	log Y = 0.0138X + 0.3168
2010	log Y = 0.0167X + 0.0397

Table 2.4: Table of the calibration equations applied to the Fatmeter readings to convert them into True Lipid Proportions of the Fish (%), the equation for each year of sampling is shown. Fatmeter reading is annotated as X and Lipid Proportion is annotated Y. The 2010 equation was also applied to the Fatmeter readings taken in 2011.

Application of W_R condition factor

Following on from the work of Todd *et al.* (2008), this study sampled the same fishery and thus population of fish. Therefore the equation to calculate W_RRLP condition highlighted in Todd *et al.* has been used in this work:

4. $\log_{10}W_S = 3.084\log_{10}L + - 5.068$

The result of this can then be applied to equation 2 (see introduction) to calculate the relative mass index of a fish or its W_RRLP condition factor (Blackwell *et al.* 2000).

5. $W_R = W/10^{(3.083\log10L - 5.068)}$

Statistical methodology

In an ideal world data are easily manipulated and follow the 'linear' or 'normal' pattern upon which most statistical methodology is based; problems arise when data do not conform and cannot be (or should not be) transformed to do so. The data used in this thesis concerned a variety of continuous and categorical explanatory variables to describe a percentage response variable. To accurately consider the effect of all explanatory variables upon the response variable, it is not possible to use normal statistical approaches. All data analysis completed in this thesis was performed in R (Core Development Team 2013).

Generalised linear modelling

Generalised linear models (GLMs) derive from basic regression models, which consider the relationship between two continuous variables, but GLMs are designed specifically for analyses where variance is not constant (Crawley 2007b). GLMs have been developed to allow for other response distributions, as well the normal distribution (e.g. poisson, binomial), and to accommodate datasets with a degree of non-linearity within the model structure. GLMs differ from regression analyses because a link function (which transforms the data so they can be analysed using a linear structure) and the distribution family must be identified prior to analysis.

GLMs are a parametric form of statistical analysis as they fit the model smooths in line with an existing error structure. This is a power way of performing a statistical test, however, the ability to apply them is dependent on the data confirming to this criteria.

Generalised additive modelling

Generalised additive modelling (GAM) is based upon generalised linear modelling (GLM) (Wood 2006; Crawley 2007c). GAMs also involve the use of different error structures and a link function in analysing count or proportional data. However, GAMs do not assume linear relationships and thus the shape of the relationship between the response and explanatory variable can take any form, they are non-parametric. This type of modelling is particularly useful where more than one continuous explanatory variable is being used to describe a response variable, and where there is no reason to select a particular model shape over another.

GAMs can use non-parametric 'smoothers' at the same time as parametric forms, allowing for a range of parameters to be tested in the same model. These smoothers can be formatted to a range of error families (Crawley 2007c). A wide range of error structures can be used with GAMs (binomial, Poisson, gamma, etc.). In the present study the 'quasibinomial' error structure was frequently applied, because the percentage data (which had to be tested as proportional data within R) were constrained to the range from 0-100% (0-1 the binomial structure); 'quasi' indicates that the data recorded only fill a small range of this structure (Wood 2006) and no biological tissue will comprise 100% lipid.

Once a GAM was been run it can then be tested, much like other models, using various tests including ANOVA. Terms within the model are tested using smooths; smooth terms are represented using penalised regression splines (Crawley 2007c). Various non-parametric smooths are available for use depending on the data applied; s-smooths are used for independent data, c-smooths for cyclic data or te-smooths for two dependent x-variables of different units (Wood 2006; Crawley 2007c).

GAMs can be simplified to the most parsimonious model structure using the generalised cross validation (GCV) criterion (similar to using AIC) (Wood 2006;

Crawley 2007c). Given a model structure, specified by a GAM formula, attempts to find the appropriate smoothness for each applicable model term using prediction error criteria or likelihood based methods. The prediction error criteria used are Generalized Cross Validation (GCV) (Wood 2006).

GCV scores become smaller as the model becomes a better fit for the data. A GAM model can have an improved fit by removing non-significant terms from it, if doing so reduces the GCV score. The fit of the models used in this work was improved by model simplification by monitoring the GCV score.

GAMs are occasionally subject to being over-fitted, perhaps resulting in a significant outcome where there is in fact none, but this can be controlled by setting the gamma function to 1.4 (Wood 2006). The gamma function acts to control the degrees of freedom within the model, as the size of a dataset increases, so do the degrees of freedom. By setting gamma to 1.4 the penalty that a GAM model applies per degree of freedom increases, reducing the possibility of over-fitting without compromising the model's performance.

The analysis in this work focused on the relationship between condition factor and lipid content. Both of these metrics are measured and show variation, thus neither a true x value. However, this work seeks to demonstrate the difference between these metrics and the ability of lipid content to be a more informative metric than condition factor. Thus the variation of lipid is considered with condition factor represented on the x-axis.

The GAM model was applied in this form to analyse the data:

model<-gam (Predicted Lipid Content ~s(Wr Condition Factor , bs="ts") +s(Day of Return, bs="cc") +te(Day of Return Length, bs="ps") +te(Wr Condition Factor, Day of Return, bs="cc") +te(Wr Condition Factor, Length, bs="ps") +River Age+ Sex+ Year+ Location, family=quasibinomial)

The non-parametric, non-linear terms were analsyed using the GAM model smooths s and te. With the s-smooths being applied to the independent data and the te-smooths to considered the relationship between two dependent x-variables of different units. Parametric (GLM) smooths were applied to the categorical variables to assess their impact on lipid content.

Results

Salmon W_R (condition factor) ranged from 0.63 (2010) to 1.14 (2006) and predicted entire fish total lipid content (which are the Fatmeter readings calibrated to predict the real lipid content of a fish) ranged from 1.18% (2010) to 18.53% (2007), demonstrating the diversity in fish monitored. Predicted entire fish lipid content increased significantly with W_R (GAM, F=55.960 p<0.001) (Figure 2.8), and showed an interaction with fork length (GAM, F=4.406 p=0.001). Sex, fishery, red vent prevalence and the river age of the fish had no significant influence on predicted total lipid content and were dropped from the model.

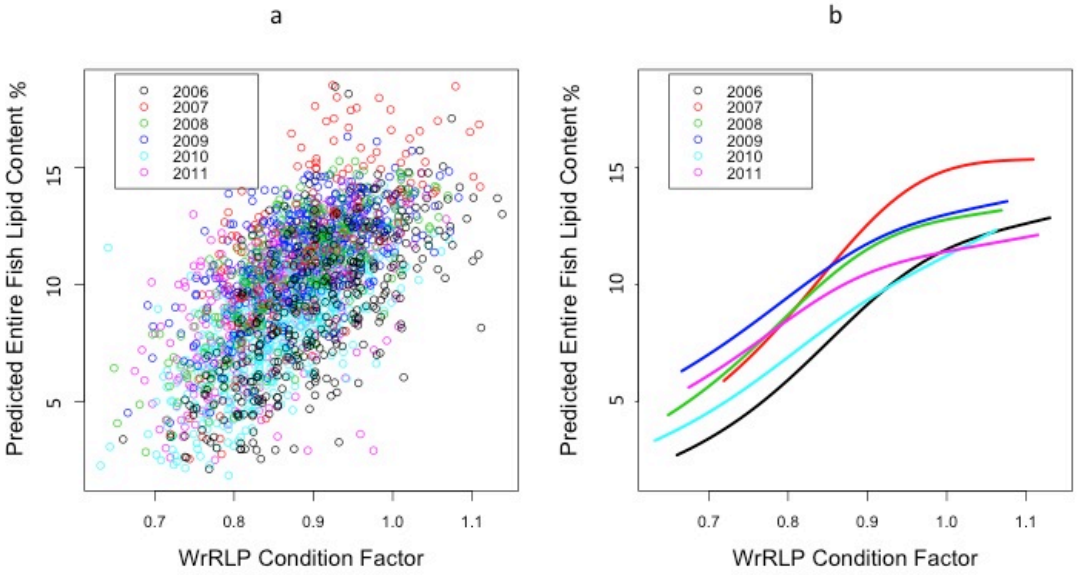


Figure 2.8: Variation of predicted entire fish total lipid content (proportion) with W_R (condition factor), spilt by year of return. Individual data points are shown (a) and GAM model predictions shown (b).

Predicted total lipid content varied significantly with capture date (GAM, F=5.460 p<0.001) (Figure 2.9). This variation also was manifested in complex

interactions between fork length and day of year (GAM, F=8.361 p<0.001), due to fish that return later having had more time to grow. No significant direct effect of fork length was found to influence fish total lipid content.

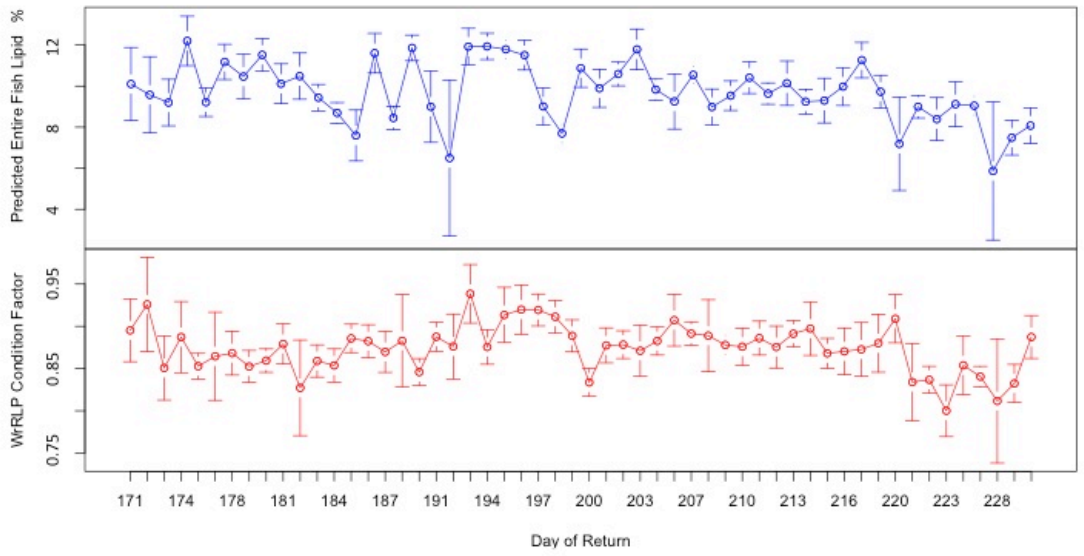


Figure 2.9: Variation of predicted entire fish total lipid content (proportion) and W_R (condition factor) with day of return of fish. Mean lipid content and W_R (condition factor) are shown for each day with standard error bars.

GAM analysis indicated that there was a significant effect of year upon fish total lipid content (GAM, t=-414, p<0.001), with significantly different patterns amongst years (Figure 2.8b). To compare the difference between the year's pairwise comparisons of the individual GAM model for lipid content for each year was undertaken. This indicated that, with the exceptions of 2006 *vs.* 2011, and 2007 *vs.* 2009, the lipid content of a fish differed significantly with year (Table 2.5, Figure 2.10). This variation highlights 2010 as the year with fish of lowest mean lipid content and 2007 as the year with fish of highest mean lipid content; all subsequent years falling somewhere in between.

Year	2006	2007	2008	2009	2010
2007	<0.001*	-	-	-	-
2008	<0.001*	<0.001*	-	-	-
2009	<0.001*	0.384	0.001*	-	-
2010	<0.001*	<0.001*	<0.001*	<0.001*	-
2011	0.085	<0.001*	<0.001*	<0.001*	<0.001*

Table 2.5: Table of pairwise comparison of GLM model p-values of whole fish total lipid content (%) and year of study, comparisons are corrected using Bonferroni adjustments. Significant results are highlighted with *.

When W_R (condition factor) was tested as the response variable it was significantly influenced by year (GAM, t=572.6 p<0.001); there were, however, fewer distinctions between years compared to the results for total lipid content. To compare the difference between the year's pairwise comparisons of the individual GAM model for W_R for each year was undertaken. (Figure 2.10; Table 2.6). As a result of these yearly variations in relationship it is clear that it is not possible to apply an accurate or reliable method of hind-casting of lipid content based on length/mass data for individual fish in the historical datasets.

Year	2006	2007	2008	2009	2010
2007	1.000	-	-	-	-
2008	<0.001*	0.060	-	-	-
2009	0.442	1.000	0.083	-	-
2010	<0.001*	<0.001*	<0.001*	<0.001*	-
2011	<0.001*	<0.001*	0.095	<0.001*	1.000

Table 2.6: Table of pairwise comparison of GLM model p-values of W_R (condition factor) and year of study, comparisons are corrected using Bonferroni adjustments. Significant results are highlighted with *.

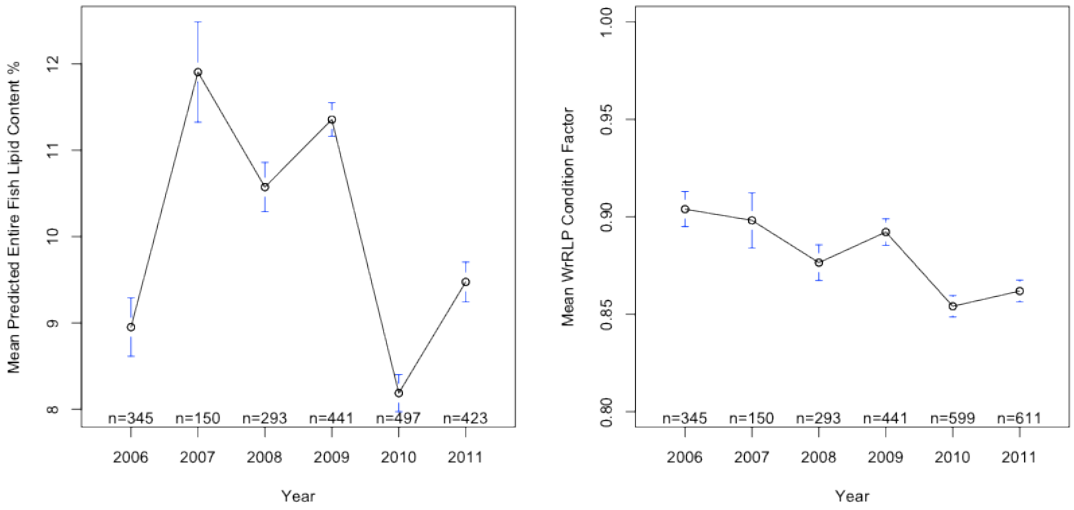


Figure 2.10: Variation of mean predicted entire fish total lipid content (%) and mean W_R (condition factor) with year of capture. Mean lipid content and W_R (condition factor) are shown for each day with standard error bars.

W_R is influenced by fork length (GAM, F=3.737, p=0.005) because statistical measures of condition factor all have a greater or lesser tendency to be length dependent. W_R varied significantly with the day of capture (GAM, F=6.154 $p<0.001$) and, as was noted for the predicted lipid content (Figure 2.9), this variation also manifested itself in complex interaction effects between fork length and day of year (GAM, F=2.916 p=0.009).

Discussion

This chapter considers the relationship between a morphometric measure of quality, W_R (condition factor) and a physiological metric, predicted entire total lipid content (%) of wild Atlantic salmon, to assess whether or not there is a difference in the predictions of fish quality attributable to the two methods and whether or not the relationship is consistent.

Total lipid as a proxy for environmental change

This study clearly demonstrates that there is a positive significant relationship between the condition factor of a fish and its total lipid content. As indicated by the preliminary results reported by Todd *et al.* (2008), a clear non-linear relationship is evident, with poorer condition 1SW fish having disproportionately low lipid levels. In view of the widely reported continuing decline in wild salmon populations, in terms not only of individual quality (Todd *et al.* 2008; Bacon *et al.* 2009) but also numbers of returning adults for spawning (Beaugrand & Reid 2003; Condron *et al.* 2005; Hindar *et al.* 2010; ICES 2012) this is worrying.

Salmon obtain lipids through their diet, and thus any population decreases in lipid content imply that salmon are limited in food supply or are unable to feed to a sufficiently high level to maintain a 'healthy' or optimal body condition. Because salmon cease feeding on their migratory return to spawn (Kadri *et al.* 1995), they must rely on stored lipids to complete their migration and maturation processes. In female (and male) salmon reproduction is a costly process (Jonsson & Jonsson 2003) and these remaining stores of lipids are vital for the maturation and provisioning of eggs (Jonsson *et al.* 1996). They must also be used to complete the migration process from coast to river breeding ground.

Fluctuations have been recorded amongst phytoplankton and zooplankton in one of the key feeding grounds of Atlantic salmon (Beaugrand *et al.* 2002; Beaugrand & Reid 2003). These changes in plankton populations have been correlated with the variation in sea surface temperature, and in turn have been linked to declining salmon populations (Beaugrand & Reid 2003; Todd *et al.* 2008). Plankton are not only important as a food source, but also play a key role in nutrient cycling (Richardson & Schoeman 2004). Recent declines in salmon populations have been attributed to the period of time spent at sea rather than time in freshwater as fewer adult salmon are returning and disproportionately less is known about the life of salmon at sea (Beaugrand & Reid 2003; ICES 2012). These shifts may have serious consequences for the already exploited fisheries found in the North Atlantic; the resilience of such systems to climate change is unclear.

Figure 2.8 demonstrates that there is no overall constant relationship of total lipid content and W_R (condition factor), and that although some years do not significantly differ in pattern from the null model, the majority do. This makes it impossible to accurately hind-cast the relationship between condition factor and lipid content of fish. Thus, in order to be sure of the true quality and energetic condition of an individual Atlantic salmon both the lipid content must be measured (or estimated – e.g. with a Distell fatmeter) and the somatic condition factor calculated every season.

A pattern that is apparent both for W_R (condition factor) and total lipid content is that fish of better quality tend to return earlier in the netting season. Todd et al (2012) modelled the time series delay in the return time of salmon in relation to declining W_R and find that the return time is related to fish condition. This could be that these better quality fish are being caught earlier in the season so have yet to use up so much of their stores or that the poorer condition fish are remaining at sea longer, delaying migration and the suspension of starvation, to try achieve better somatic quality (Todd *et al.* 2012). Thorpe (2007) discusses the triggers of

maturation in Atlantic salmon and indicates that salmon should assess the magnitude of their reserves prior to being the maturation process.

The difference between using condition factor and lipid content

Whilst this study has demonstrated the similarities of lipid content and condition factor as proxy measures of individual quality, it also highlights the disparity between the two techniques. Figure 2.10 demonstrates that whilst condition factor measurements are capable of resolving large changes or discrepancies in fish quality, lipid levels are a more reliable and informative measure of subtle changes or differences amongst individuals. Work by Litzow *et al.* 2006 indicates that lipid variations may offer accurate and reliable information when considering the intricacies of environmental change.

Work by Bennett and Janz (2007) on Northern Pike (*Esox lucius*) indicates that whilst morphological measures of condition may not vary the lipid content of individuals can vary significantly, rendering it the more ecologically relevant measure of energy (Bennett & Janz 2007). The present work indicates that whilst condition factors are useful and applicable measures of a fish's quality, they cannot be extrapolated to provide a direct proximate measure of a fish's energy reserves (Litzow *et al.* 2006).

Conclusions

This study of energy reserves of individual fish has focused exclusively on total lipids (meaning all the lipids possible to extract from fish tissue) estimated for the entire body of the fish. Whilst fish store lipids throughout their bodies, there are key storage sites or depots and different body tissues have very different lipid concentrations (Zhou *et al.* 1996). The influences of varying quality and recent declines in salmon population are not yet established upon them.

Some studies have suggested a recovery of salmon populations in Scotland, at least in terms of numbers (ICES 2012). The present study does not support that outcome, but rather indicates that although fish are continuing to return they are doing so in increasingly poor condition. This work has focused on the total lipids held throughout the body (specifically the Norwegian cut) of the fish. To obtain a clearer and more informative understanding of the importance of the variation in the lipid stores as measures of fish quality it is necessary to examine the individual tissues of the fish.

Acknowledgements

The author would like to thank the salmon teams at Strathy, Armadale and Melvich for catching the fish; in particular J. Paterson for selecting the sub-samples for analysis. The lipid extractions were performed by a team of laboratory workers in St Andrews, without whom getting such a large amount of data would be impossible.

3. A comparison of pre-spawning and post-spawning Atlantic salmon, condition factor and the distribution of lipids between the body tissues

Introduction

Considerable work has been performed examining the variation in lipid content of tissues of the Atlantic salmon. However, this research tends to be focused on the aquaculture industry (considering optimal feeds and improving taste, flesh texture and flesh colour), rather than examining the natural diet of the wild salmon. Commonly studies have assessed lipid contents of one specific tissue or reported on the total lipid levels within the fish, but research indicates that the different tissues of the Atlantic salmon have different functions with regard to lipid storage, mobilisation and metabolism. A few studies have examined the relationship between total lipid content of Atlantic salmon, the effect this has on the different lipid depots found within the body and how this might influence future survival.

The major lipid storage sites in fish are the mesenteric tissue, muscle, liver and sub-dermal fat (Zhou *et al.* 1996); however, in Atlantic salmon most of the lipid is stored in the muscle tissues, rather than for example, the liver which is the major storage site in gadoid fish (Grant & Brown 1999). Teleost fish myotomes include two kinds of muscle fibres (Hudson 1973); white muscle used for sudden bursts of swimming, such as escape behaviour, and red (or dark) muscle used for sustained swimming behaviour, such as migration and horizontal balance (Hudson 1973; Zhou *et al.* 1996; Anttila & Mänttäri 2009). In view of these functional contrasts the red muscle generally is expected to be more lipid-rich than the white muscle (Zhou *et al.* 1996). In teleost fish the body mass is supported by muscle fibres rather than the skeleton (Zhou & Ackman 1995) and thus the muscle tissues are key to maintaining body structure as well as overall somatic condition.

Different species of fish have different white muscle to red muscle ratios (Hudson 1973) and store their energy reserves (lipids) in different places in the body; for example, Atlantic Cod (*Gadus morhua)* store high levels of lipids in their liver (Grant & Brown 1999), whereas Atlantic salmon store lipids in multiple tissues throughout their bodies (Zhou & Ackman 1995; Katikou *et al.* 2001). The Atlantic salmon liver is relatively low in lipids (~4%), compared with other body tissues (Torstensen *et al.* 2000).

Lipids are an important part of the salmonid diet and it has been estimated that 30% of the salmon diet should be lipid for them to be healthy (Torstensen *et al.* 2000); lipids are key in terms of salmonids maintaining high energy levels. Torstensen et al. (2000) suggest that the food source will affect the quality of farmed salmon and go on in future work to state that the red and white muscles are most compromised, in terms of lipid content by poor diet (Torstensen *et al.* 2004).

The main lipid stores of the Atlantic salmon are the adipose tissues, found in deposits amongst the viscera and along the sub-dermal dorsal midline and ventral region (or belly flap) of the fish (see Fig. 3.1) (Zhou *et al.* 1996; Nanton *et al.* 2007). Farmed fish fed high fat content diets have high lipid amounts in the body, especially in the fat deposit regions (adipose tissues) (Nanton *et al.* 2007). Nanton et al. found that the dorsal adipose tissue was ~80% fat, the ventral adipose tissue was ~49% and that the red and white muscle both show comparably lower fat levels.

Studies of farmed fish have noted a relationship between the increasing size of salmon and higher fat contents, with the composition of somatic tissues (white muscle) tending to become correspondingly more fat-rich as fish grow larger (Jobling & Johansen 2003). The adipose tissues were more greatly affected by the

treatments studied (a high-fat ~ 39% lipid diet; and a low-fat ~ 28% lipid diet) and were thought to show a more immediate response to changing diet. The adipose tissues were found to hold 19-25% of the total fat content of the fish irrespective of diet. The fillet (musculature) of Atlantic salmon increases in importance as a fat deposit with increasing fish weight. Jobling and Johansen (2003) note that only limited information is available about this relationship for wild salmon, and that little is known about the changes of body fat distribution during marine growth.

Atlantic salmon cease feeding prior to their re-entry of the river for spawning and therefore fast for an extended period of several weeks to perhaps many months in freshwater. As a consequence, they rely totally upon stored lipid reserves for energy metabolism and final provisioning of oocytes (eggs) with lipoproteins (Fleming *et al.* 1997; Berg *et al.* 2001). Thus, even if a length-independent measure of condition factor (such as the Relative Mass Index, W_R) is applied, that gravimetric measure of fish condition might not adequately reflect the "quality" of the fish if, for example, lipid reserves do not show a simple linear relationship with W_R (Chapter 3).

As salmon complete the final provisioning of their eggs and prepare to spawn they mobilise stored lipids. At reproduction, the ovaries of Atlantic salmon contain ~30% of the total energy content of the fish (Jonsson *et al.* 1997), but it is not clear from where in the body these lipids are mobilised. Given that Atlantic salmon are potentially iteroparous it is apparent that a trade-off between current and future offspring production might be selectively advantageous (Tierney *et al.* 2009).

Survival rates of Atlantic salmon returning for a second spawning typically are <10%, but this does vary widely between populations (Fleming & Einum 2010); for example, return spawners in the River Teno (northern Finland) may

comprise as high as ~5% of the total spawning population (Niemelä *et al.* 2006). From model analyses, Thorpe *et al.* (1998) showed the importance of genotype x environment interactions influencing the accretion and deployment of lipid reserves preparatory to the maturation process, and that this will be further influenced by the potential for a fish to be iteroparous (Thorpe *et al.* 1998). The foregoing all point to the importance of Atlantic salmon not expending all their lipid reserves during spawning, but which lipid depots are exploited, and to what extent, remains unclear.

The pre-spawning (or maiden) fish studied in this thesis were caught at coastal netting stations on their return from the Norwegian Sea. The pre-spawning fish were intercepted at the near-completion of their marine phase of their spawning migration. They would have entered their natal river between July and September but would not have spawned until November-December. Had they survived spawning, the kelts would likely have remained in-river until March or April the following year. This means these fish are not the final comparison point before spawning and instead would have had to remain, not feeding in rivers for a number of months. This will have led to a reduction in the lipid content of the fish studied. Maternal investment in offspring has been suggested to be influenced by body condition and that female salmon can adjust their fecundity when experiencing poor conditions (Jonsson *et al.* 1996; Burton *et al.* 2012).

The post-spawning fish sampled were caught after spawning maturation had been completed, just before they laid their eggs. This allowed for the eggs, as well as the fish, to be examined (see Appendix 1). Studies have shown that the lipid content of the Atlantic salmon can decrease by ~60% in the white muscle (Vuorinen *et al.* 2014) during the spawning migration. The red muscle is also affected by the spawning process, and observations of decreases in body weight by up to 20% will lead to marked reductions in total red muscle lipid content (Bombardier *et al.* 2010).

This chapter examines the lipid storage in Atlantic salmon during their spawning migration by comparing the differences between pre- and post-spawning maturation.

Pre-spawning fish of varying condition factor were assessed to see how the storage tissues for lipids differed. If fish of varying condition factor had the same total lipid content, then the partitioning of the lipid would be comparable for differing condition factor. Chapter 2 demonstrated that lipid content varies with condition factor, thus this Chapter will demonstrate how wild salmon use and store lipids in different tissues.

This research used a miniaturised version of the Bligh and Dyer method (Chapter 2) to determine total lipid content in the standard Norwegian cut tissue as well as specifically in red and white muscle, dorsal adipose and liver tissues for that standard experimental section of the body of the Atlantic salmon.

This chapter will show that Atlantic salmon used the lipid in storage tissues (e.g. the adipose tissues) preferentially to the lipid in the musculature of the fish that will be required for future migration. Because Atlantic salmon enter a fasting period prior to spawning, it is expected that there should be declines in body lipid content during this spawning migration (Jonsson *et al.* 1997).

The objective of this chapter was to identify how the spawning process differentially influences the storage depots of Atlantic salmon. The study will demonstrate that salmon draw down their lipid reserves to spawn and the lipid concentration of the key body tissues is significantly reduced. Lipid concentrations in the relatively small mass of the body adipose tissue are high, but following the completion of spawning it can be shown that the adipose

tissues were preferentially exploited compared to the deposits in the red and white muscle.

Methods

Sample collection and preparation

Atlantic salmon were sampled from two locations in Scotland. Three year-classes (2008-10) of returning pre-spawning adult one-sea-winter (1SW) maiden (hereafter "pre-spawning") fish were sampled throughout the summer commercial netting season (weeks 23-33) from coastal trap nets at Strathy Point, Melvich and Armadale, Scotland, UK. The second sample of 1SW salmon was obtained from sexually mature manually stripped (hereafter "post-spawning") fish trapped over a four-day period (30 November-3 December 2009) in the River Blackwater, Ross-shire, Scotland, UK. Although the Blackwater is named as a separate river it is, in effect, the upper reaches of the River Conon (Burton *et al.* 2012).

Year	Lipid Fish Sampled
2008	34
2009	27
2010	28
River Conon (post-spawning)	28

Table 3.1: Table of sample sizes of pre-spawning fish sampled for lipid extraction in sampling years 2006-2011 and those post-spawning fish selected from the River Conon in 2009.

Fish selected for tissue lipid analysis in this Chapter do not comprise a random selection of these populations, but rather were specifically chosen to offer a complete range of lengths and condition factors available during sampling of these year-classes. All fish were measured for fork length (rounded down to 0.5 cm) and weight (to 0.01 kg). River age – that is, the age of the juvenile smolt at river emigration in the year previous to adult capture and sea age were

determined from scale reading. The sex of the fish was confirmed at dissection and only females were sampled at the River Conon, which was obvious, as fish had completed spawning maturation.

Scale samples were taken from just posterior to the dorsal fin and above the lateral line, the standard region (Jensen *et al.* 2012); after which they were stored and dried in paper packets. Impressions were taken of the scales on acetate strips. Counts and measures were made of all circuli on the scales by Bryce White (Marine Scotland Science) and used to derive the river age (years spent living in a river) and sea age (years spent living at sea) of the salmon. These annuli are identified in the conventional manner from observation of the transitions in spacing between circuli (Shearer 1992).

Fish dissection

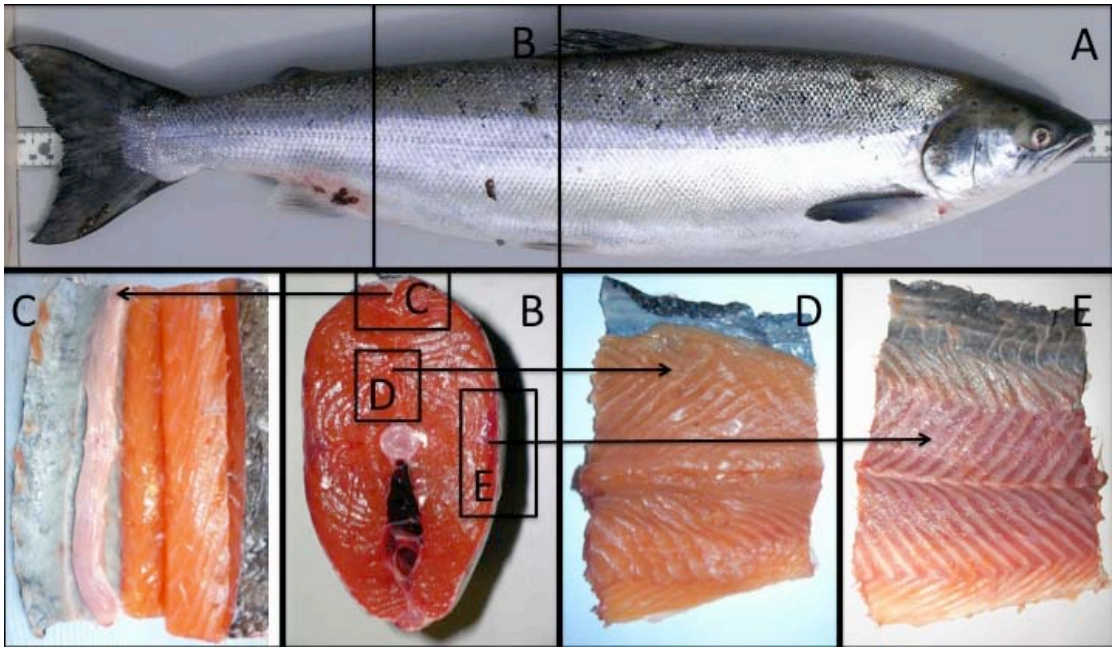


Figure 3.1: A shows an entire Atlantic salmon, the black lines indicate the cuts (the first behind the dorsal fin and the second in front of the anus) taken to create the Norwegian cut (B). B shows a cut Norwegian section, with the dorsal adipose (C), white muscle (D) and red muscle (E) highlighted. C, D and E show the dissected dorsal adipose, white muscle and red muscle respectively.

The entirety of the red muscle and dorsal adipose tissues were removed from the section (where possible) and weighed. These two tissues are easily separated by scalpel dissection of the Norwegian Cut with the result that only the white muscle and skeletal bones remain. It was not possible to remove the entire white muscle from the section, as this constitutes the majority of tissue in the Norwegian cut. It also proved impossible to remove the ventral adipose tissue (from the belly flap area of the fish) from the white muscle as they were too closely entwined. The dorsal adipose tissue is a distinct and removable tissue (see Fig. 3.1C), however the ventral adipose tissue is intimately associated with the musculature and connective tissue of the fish. Therefore the ventral adipose tissue is not a discrete tissue that can be isolated by dissection.

Miniaturisation of lipid extraction

Dorsal adipose, red muscle and white muscle tissue samples were isolated from the Norwegian Cut of the fish (Figure 3.1) and separate tissue samples were also taken from the liver of the fish. Lipids were taken extracted from the tissues following the method outlined in Chapter 2, however, to broaden the scope of lipid content analysis to small tissue samples (e.g. adipose tissue) in this study the Bligh and Dyer lipid extraction method required miniaturisation. The modification of the method was completed with assistance from B. Todd a masters student (Todd 2009).

It was necessary to utilise smaller samples than are traditionally applied in the Bligh and Dyer method due to the available amount of tissues from the Norwegian cut. As stated in Chapter 2, the traditional method utilises approximately 20g of tissue for each replicate, whereas the miniaturised method used samples of no larger than 0.5g per replicate vial. These samples were too small to be homogenised in a food processor, so instead were subject to sonication (Hielscher UP200S Probe Sonicator) in order to disrupt cell membranes and to permit efficient extraction of lipids.

The miniaturised Bligh and Dyer method applied a standard ratio of approximately 2ml chloroform: 2ml methanol: 1ml water per gram of tissue tested. Due to the small quantities of reagents used the chloroform layer (containing the lipids) was removed from the sample via pipetting. This method was prone to error and tend to lead to over-estimation of lipid, due to tissue mass being pipetted with the chloroform; the over-estimation led to stringent outlier analysis, using coefficients of variation (as detailed later in this chapter).

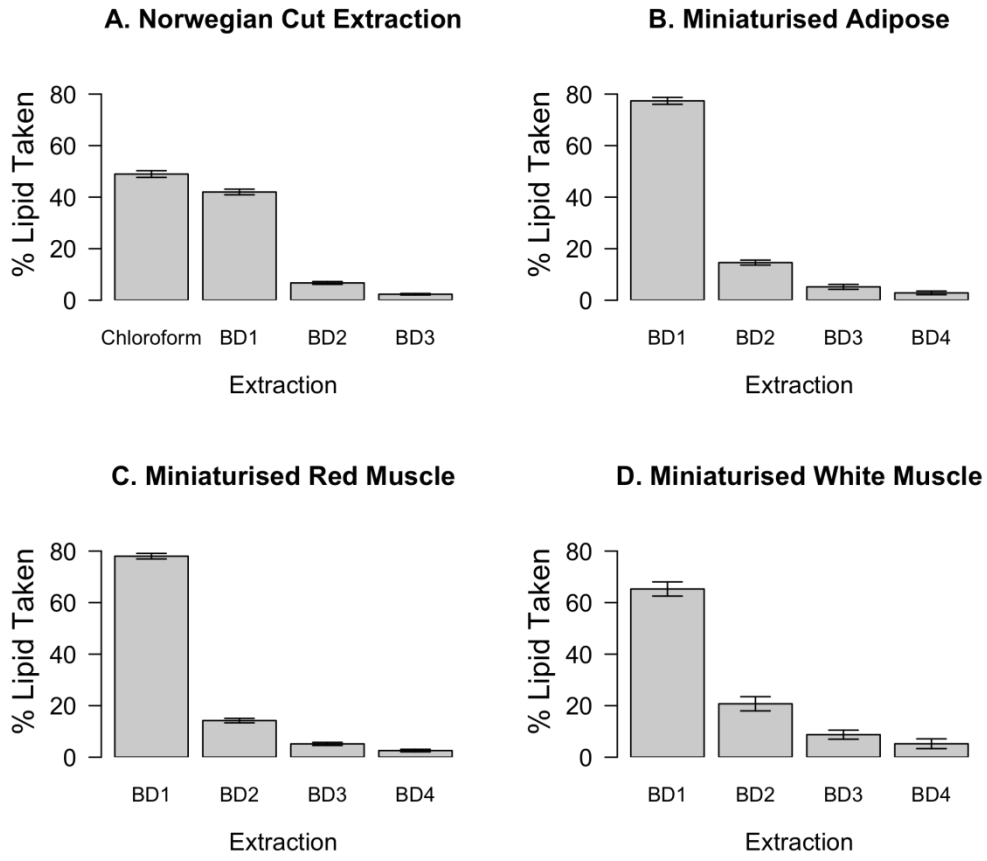


Figure 3.2: Preliminary results of mean percentage lipid taken at different extraction levels. The type of extraction in noted by Chloroform (chloroform), BD1 (Bligh and Dyer 1), BD2 (Bligh and Dyer 2), BD3 (Bligh and Dyer 3) or BD4 (Bligh and Dyer 4). Standard error bars of the extractions are shown. Fig 3.2A shows the results of the 'standard' Bligh and Dyer extractions of the Norwegian Cut (as report in Chapter 2) and Figs. 3.2B, C and D show the 'miniaturised' Bligh and Dyer extractions of the Dorsal Adipose, Red Muscle and White Muscle, respectively.

Preliminary studies indicated that an initial chloroform extraction was unnecessary (as used for the basic lipid extraction method) for efficient lipid extraction of the sonicated sample material and further preliminary studies (Figure 3.2 B, C and D) demonstrated that only 3 rounds of Bligh and Dyer extractions in the vials were necessary to facilitate the removal of the majority of the lipid. Lipid content in the tissue was measure gravimetrically, with three repetitions of each Bligh and Dyer extraction performed. All extractions were undertaken in triplicate.

Data Analysis

The miniaturised extraction technique resulted in some variation in percent lipid extracted amongst triplicates. Assessment of statistical outliers was by inspection of the coefficient of variation (CV) (for more detail see Chapter 2) amongst triplicates; tissues with a CV >30 were deleted from the present analyses and if the triplicates for more than one tissue had a CV >30 the fish was removed from the study. Thus for dorsal adipose tissue 7 out of the 129 fish were deleted, red muscle 8 out of the 129 fish were deleted and white muscle 26 out of the 129 fish were deleted. It should be noted that 18 out of 26 outliers of the white muscle samples were all in the first year of study and if this year is excluded the white muscle outliers show the same error rate as the other tissues.

The variation of the tissue total lipid proportion, being non-linear, were analysed for each tissue by means of a generalised additive model (GAM), compared with both the lipid in the whole Norwegian cut and the W_R condition factor, calculated as in Chapter 2. Separate models were used to test each tissue type.

The GAM model was applied in this form to analyse the data:

Model<-gam (Tissue Lipid~ s (Norwegian Cut Lipid) +s (Length) +s (Weight) +s (Tissue Weight) +te (Weight, Tissue Weight) +te (Length, Tissue Weight) +year

+River Age +Sex-1, family=quasibinomial)

In each model the percentage total lipid content of the Norwegian cut, fish length, weight, W_R condition factor and total tissue weight were modelled as smooth, non-parametric functions and with the year-class, fish sex and river age being included in the model as parametric factors. The quasi-binomial error structure was used due to the limitations of the response variable, proportions of total lipid (so could only range between 0 and 1), and to control for the over-dispersion (the range of proportions although varying fits into only a limited range). Gamma was increased to 1.4 to control for over-fitting within the model. The model was simplified to find the most parsimonious setup and the validity and utility of this model was tested using the general cross validation (GCV) method.

Reconstruction of lipid in Norwegian cut

To estimate the relative proportions of lipid found in the Norwegian cut tissues, the total lipid in the Norwegian cut was estimated from the lipid contents found in the individual tissues sampled. The dorsal adipose tissue and red muscle were fully dissected from the Norwegian cut and weighed prior to lipid extraction. Thus an estimate of the total amount of lipid within these tissues could be made as a percentage of the tissue weight. As it was not possible to fully dissect the white muscle from the Norwegian cut (as it is the main constituent), this was estimated from the difference between the extracted Norwegian cut lipid weight and that of the dorsal adipose and red muscle tissues.

However, this gave a much higher estimate than was possible, as indicated by the lipid concentration of the white muscle via extraction (which is very low). This is explained by the fact that the tissue remaining in the Norwegian cut, once the dorsal adipose and red muscle tissues have been removed, is not just white muscle. There is additional adipose tissue found in the ventral region of the fish,

the ventral adipose tissue or belly flap, which cannot easily be dissected out as it is so closely entwined with the white muscle. The lipid content of this ventral adipose tissue was then estimated as the difference between the total lipid in the Norwegian cut and the lipid in the white muscle, red muscle and dorsal adipose tissue. The difference between these estimated lipid contents in the individual tissues and their spawning state was then analysed using a generalised linear model (as discussed in Chapter 2) with a binomial distribution.

Results

Condition factor

The Norwegian cut percentage lipid content of Atlantic salmon increases non-linearly with W_R condition factor (F=47.07, p< 2e-16, GCV= 0.0040063; Figure 3.3). This pattern did not vary between year-classes (t=-0.225, p=0.823, GCV= 0.0040063) so all the years studied were pooled; however this is contrary to the yearly variation demonstrated in Chapter 2, the lack of pattern is probably an artefact of the sample size and would probably be seen in larger samples.

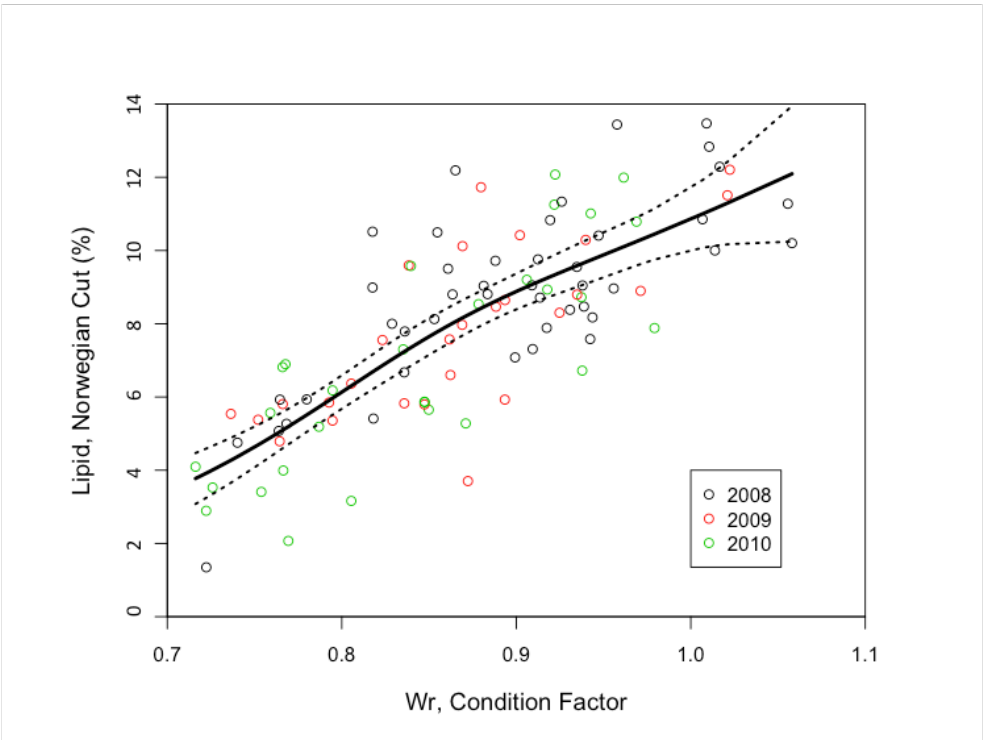


Figure 3.3: Proportion lipid of the whole Norwegian cut of pre-spawning fish plotted against W_R condition factor. Year of sampling was pooled, but the individual data points for each year are shown (coloured open circles) and the GAM model prediction drawn (solid black line) with 95% confidence intervals as indicated (dotted lines).

This concurs with the preliminary results shown by Todd et al. (2008), which affirmed the non-linear relationship between condition factor and total lipid content of individual Atlantic salmon. Such a relationship is, perhaps,

unexpected, because it is likely that fish of higher condition factor (which have a high mass: length relationship) will contain more storage products, including lipids.

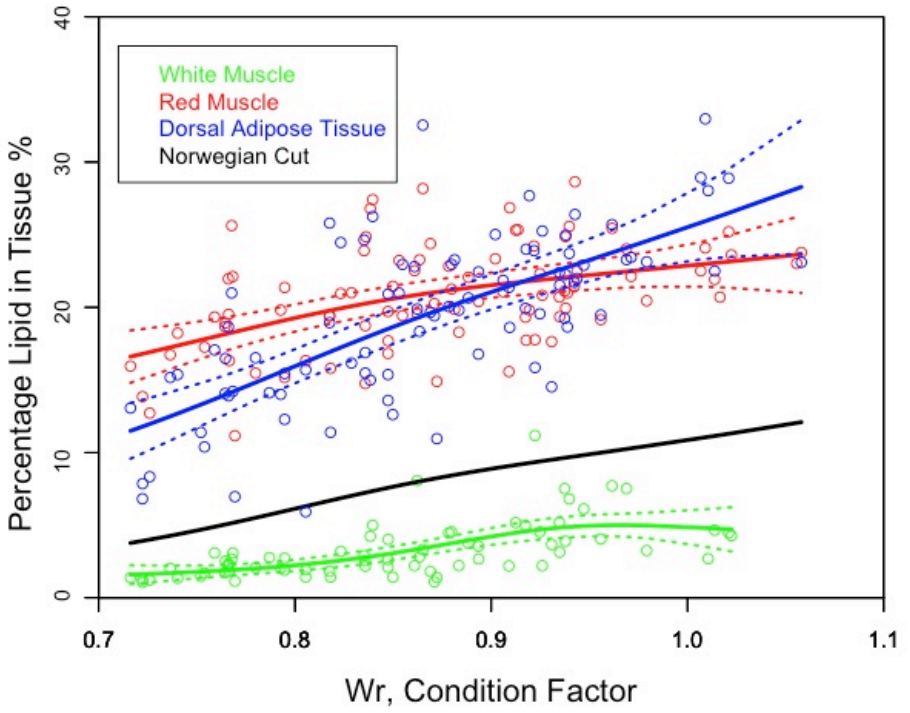


Figure 3.4: Proportion lipid of White Muscle (green), Red Muscle (red), Dorsal adipose tissue (blue) and for the Norwegian cut (black; as per Figure 3.3) plotted against W_R condition factor. Year of sampling was pooled. Individual data points are shown for the three tissue types and the GAM model prediction drawn, 95% confidence intervals are indicated (dotted lines).

The lipid contents of all the tissues were significantly related to the W_R condition factor of the fish (Table 3.2). In view of the results discussed in Chapter 2 this was to be expected and follows the pattern seen here (Figure 3.4) between percentage lipid content of the Norwegian cut and W_R condition factor. The implication is that whilst W_R condition factor cannot be utilised as a direct metric for lipid content, it does still offer a useful qualitative measure of overall somatic condition. Slender, under-weight fish are expected to have low lipid levels, but

this is not a linear relationship; emphasising further the inaccuracy of hind-casting lipid information on to past data sets (Chapter 2).

	White Muscle		Red Muscle		Dorsal Adipose		Liver	
Non-Parametric	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value
W_R	16.95	<0.001	8.12	<0.001	35.95	<0.001	6.10	<0.001
Tissue Weight	np	np	4.77	<0.001	10.30	<0.001	ns	ns
Parametric	t-value	p-value	t-value	p-value	t-value	p-value		
Year: 2008	ns	ns	ns	ns	ns	ns		
Year: 2009	ns	ns	ns	ns	ns	ns		
Year: 2010	2.52	0.014	ns	ns	ns	ns		

Table 3.2: Model terms from the GAM performed between tissue total lipid and W_R condition factor, F- and p-values are given for non-parametric smoothed terms and t- and p-values for parametric terms. Those terms that are not significant are annotated "ns" (and were removed from the model so no value is shown for them) and those not possible "np".

It would not be effective to calculate a condition factor for the post-spawning fish sampled in this chapter. The post-spawning fish have been fasting for a period of months and thus will automatically have low condition factors. Therefore the pre- and post-spawning fish considered here are contrasted in terms of lipid reserves in their tissues.

Tissue lipid content

The individual tissue's percentage lipid concentration (white muscle, red muscle and dorsal adipose tissue; sampled from the Norwegian cut) of both the pre- and post-spawning fish show a positive, non-linear relationship with percentage total lipid concentration of the Norwegian cut (Table 3.3, Fig. 3.5, Fig. 3.6). As only one year-class of post-spawning fish was studied it was not possible to test for a year effect. Individual percentage tissue lipid content was not influenced by river age of the fish in question. Neither the weight nor the length of the entire fish had

any effect on the percentage total lipid content of any of the individual tissues. In all tissues post-spawning fish generally had lower percentage total lipid in their tissues than pre-spawning fish (Fig. 3.6).

	White Muscle		Red Muscle		Dorsal Adipose	
Non-Parametric	F-value	p-value	F-value	p-value	F-value	p-value
NC Lipid Content	35.18	<0.001*	15.46	<0.001*	43.18	<0.001*
Tissue Weight	-	-	3.32	0.039*	25.90	<0.001*
Parametric	t-value	p-value	t-value	p-value	t-value	p-value
Spawned	-0.144	0.886	-2.36	0.021*	-7.22	<0.001*

Table 3.3: Model terms from the GAM performed between Norwegian cut total lipid (%) and individual tissue total lipid concentration (%) in pre- and post-spawning fish. The F statistic and p-values are given for non-parametric smoothed terms and the t- and p-values for parametric terms. Those terms that are significant are annotated "*".

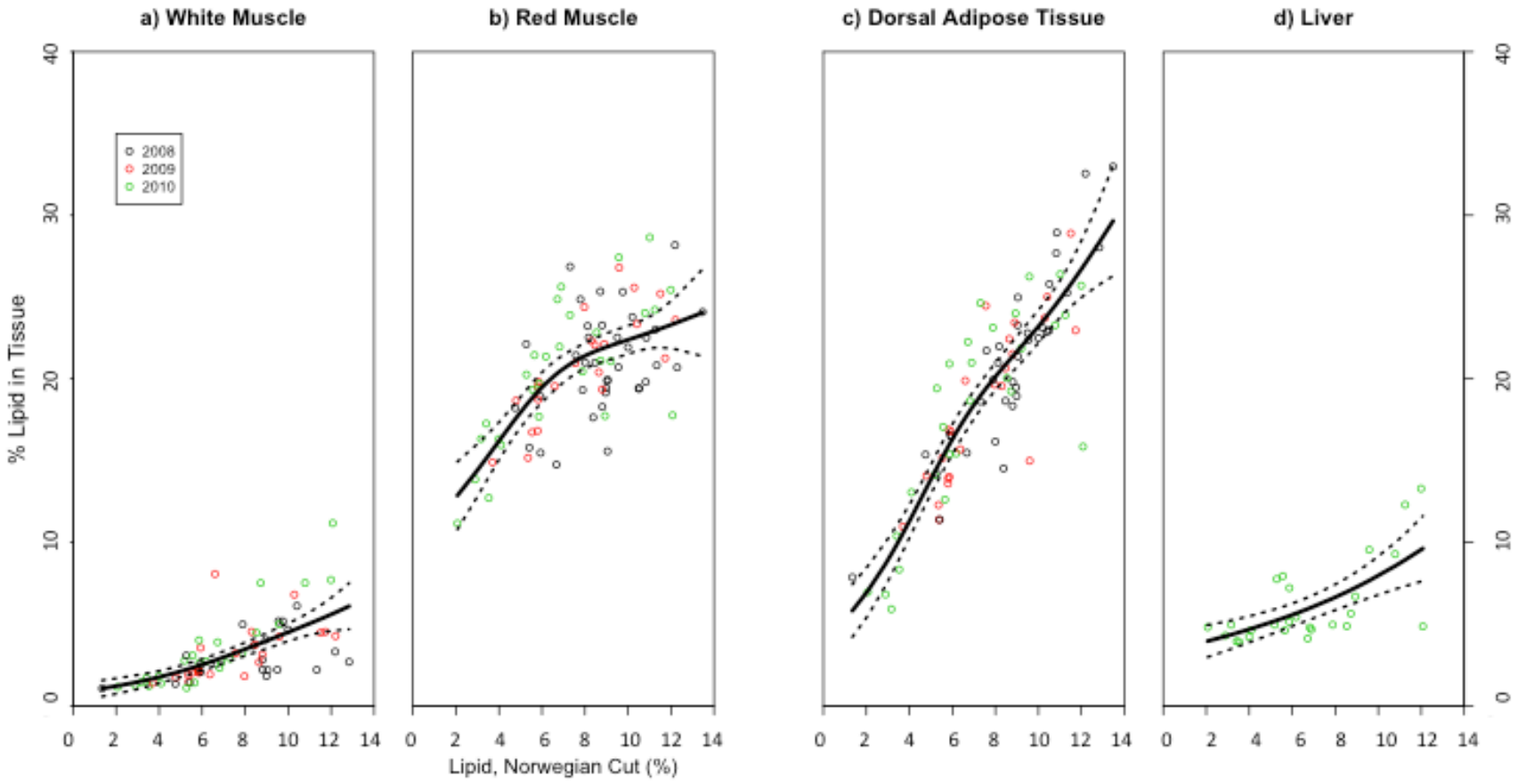
The various tissue lipid content for individual fish was not influenced by river age of the fish (t=-0.225, p=0.823, GCV=0.0046472), indicating that the marine phase of the Atlantic salmon lifecycle may be key in influencing the lipid content of these fish. The sex of the salmon studied had no impact upon the percentage lipid content of all tissue types (t=-0.197, p=0.845, GCV=0.0046472). The weight of the entire fish had no effect on the proportional total lipid content of any of the individual tissues (F=0.415, p=0.522, GCV=0.0046472).

Pre-spawning Fish

White Muscle

In pre-spawning fish the white muscle percentage lipid content was consistently lower than that of either the red muscle or dorsal adipose tissue (Figure 3.4). However, the white muscle is the main constituent (by weight) of the Norwegian cut, so whilst the percentage lipid content of this tissue is lower than the other tissues, the lipid content of all the white muscle within the Norwegian cut will by

Figure 3.4: Percentage lipid content of the tissue (as indicated) plotted against percentage lipid of the whole Norwegian cut, for pre-spawning fish only. Year of sampling was pooled, however individual points as coloured by year of study. The GAM model prediction is drawn (solid black line), 95% confidence intervals are indicated (dotted lines).



far exceed that of the red muscle or dorsal adipose tissue. GAM modelling indicated that the percentage lipid content of the white muscle increases with the percentage lipid content of the Norwegian cut (Table 3.3).

Red Muscle

The red muscle GAM modelling of pre-spawning fish indicates that percentage lipid content for that tissue also increases with the percentage lipid content of the Norwegian cut (Table 3.3) and is also influenced by year-class of the fish in a manner similar to the white muscle. However, the red muscle also showed increases in percentage lipid content with increasing red muscle tissue mass found in the Norwegian cut, indicating that fish with more tissue mass not only have more red muscle lipid but a higher concentration of lipid within that tissue itself. However, in the red muscle this seems to reach a threshold level at approximately 21% lipid within the tissue (Figure 3.5).

Dorsal Adipose Tissue

The dorsal adipose tissue percentage lipid content in pre-spawning fish is also influenced by the percentage lipid content of the Norwegian cut (Table 3.3) and year-class of the fish. However, it showed a much stronger relationship with the percentage lipid content of the Norwegian cut, ranging from fish with 5.92% lipid in the dorsal adipose tissue to 38.61%. The dorsal adipose tissue is also slightly influenced by the total length of the fish (Table 3.3), and related to increasing adipose tissue mass (Fig, 3.5). Thus fish of a given length with more tissue mass not only have more dorsal adipose tissue lipid but also more lipid within the tissue itself, as was shown for the red muscle. However, unlike the red muscle, this does not seem to attain a threshold level, suggesting that fish can potentially store more lipid in the dorsal adipose tissue than was recorded in the present study.

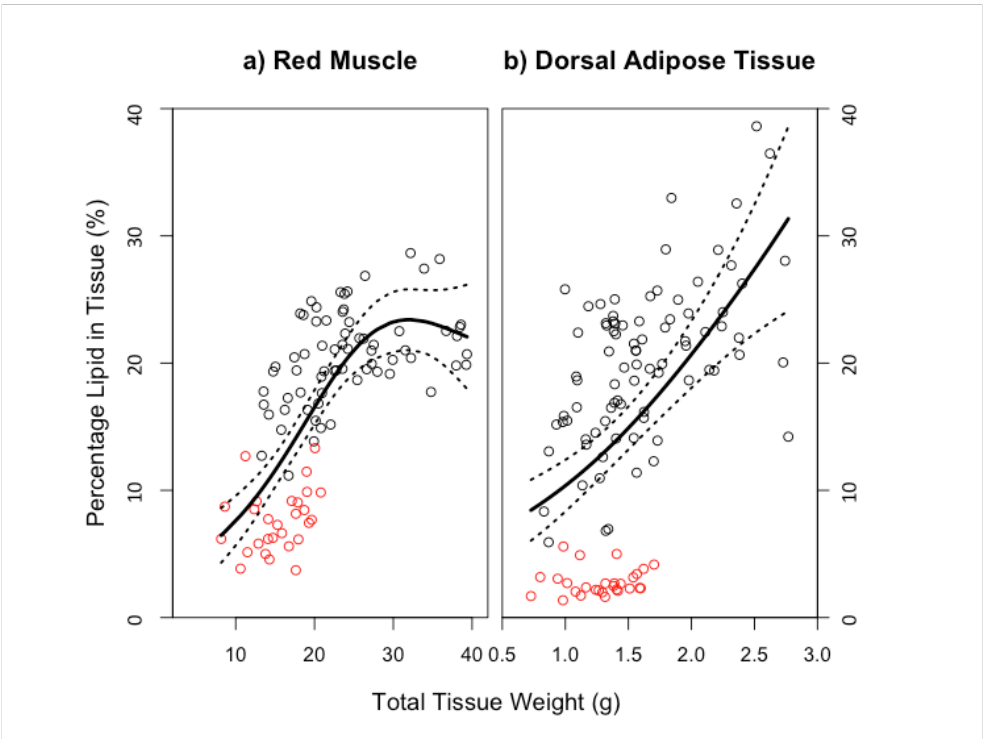


Figure 3.5: Percentage lipid concentration of the red muscle and adipose tissue (as indicated) plotted against the weight of the whole tissue, year of sampling was pooled. Pre-spawning fish are shown in black and post-spawning fish shown in red. The GAM model prediction is drawn for pre-spawning fish (solid black line), and the 95% confidence intervals are indicated by dotted lines. Year of sampling was pooled.

Liver

The liver tissue percentage lipid content is also influenced by the lipid content of the Norwegian cut ($F=10.02$, $p=0.003$), but shows no effect of total liver weight or length of fish. The liver was sampled in two years only and showed no influence of year. The lipid concentrations within the liver tissue were similar to that of the white muscle and thus the Norwegian cut as a whole. This is not unexpected in salmon because unlike gadoid species they do not store their lipids in the liver.

Comparison of pre- and post-spawning fish

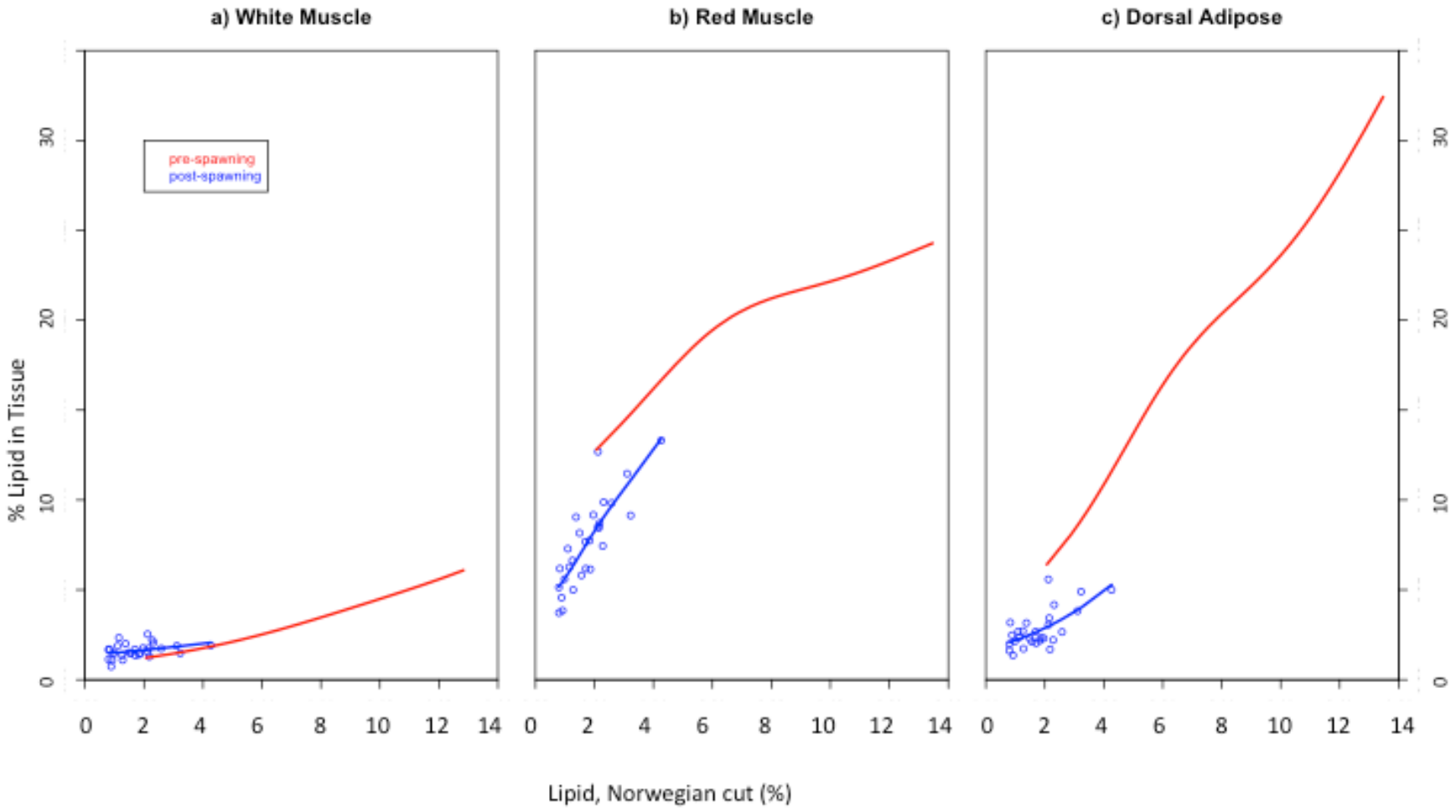
White Muscle

The percentage lipid content of the white muscle and the percentage lipid content of the Norwegian cut in pre- and post-spawning fish were not significantly different from each other (Table 3.3, Fig. 3.6). Whilst post-spawning fish generally had a lower lipid proportion in the white muscle than pre-spawning fish, they followed the same pattern. This implies that fish continue to draw lipid from the white muscle in the same way after spawning and that salmon appear to maintain a low level of lipid in the white muscle.

Red Muscle

The relationships between percentage lipid concentration of the red muscle and the percentage lipid concentration of the Norwegian cut in pre- and post-spawning fish were significantly different from each other (Table 3.3, Fig. 3.6). Post-spawning fish showed a significantly lower lipid percentage in the red muscle than pre-spawning fish, and this followed a rather different pattern to that of pre-spawning fish (Fig. 3.6). The red muscle also increased its percentage lipid content with increasing red muscle tissue mass found in the Norwegian cut (Table 3.3, Fig. 3.6). It is notable that this follows the same pattern both in pre- and post-spawning fish. There was no significant interaction effect between tissue weight and spawning state influencing the percentage lipid concentration of the red muscle. This suggests that spawned fish have lower lipid concentrations in the red muscle and that the muscle tissue itself is smaller, but has been reduced in proportion with the body size of the pre-spawning fish.

Figure 3.6: Percentage lipid content of the tissue (as indicated) of post-spawning fish is add to the model lines drawn in Fig. 3.5, plotted against percentage lipid of the whole Norwegian cut. Individual data points are shown as a red line for pre-spawning fish and blue for post-spawning fish. The GAM model prediction is drawn (solid black line), 95% confidence intervals are indicated (dotted lines).



Dorsal Adipose Tissue

The dorsal adipose tissue percentage lipid concentration and Norwegian Cut percentage lipid concentration relationship was significantly different for pre- and post-spawning fish (Table 3.3, Fig 3.6). This difference was more strongly significant than that for the red muscle, and seems to be a step-like function, indicating a large decrease in the percentage lipid concentration of the adipose tissue of post-spawning fish. The dorsal adipose tissue also showed increases in its percentage lipid concentration with increasing dorsal adipose tissue absolute mass found in the Norwegian Cut (Table 3.3, Fig. 3.6). This was significantly different between the pre- and post-spawning fish (F=-2.36, p=0.021). Unlike the red muscle tissue mass, the adipose tissue differed significantly, with post-spawning fish showing a low lipid concentration and the tissue itself also was reduced in relation to body length.

Reconstruction of lipid in the Norwegian cut

The analysis of total lipid amount within the tissues of the Norwegian cut allowed an estimate of the size and thus total amount lipid in the white muscle to be calculated. From this an estimate of the partitioning of total lipids within the Norwegian cut could be made for each fish studied (see Fig 3.7).

The generalised linear model analysis of total lipid percentage of the tissues within the Norwegian cut identified that there was a significantly greater proportion of the total lipid in the Norwegian cut stored in the white muscle (40.76 to 64.29%, z=1.96, p=0.04) of post-spawning fish, whilst the ventral adipose tissue (38.53 to 6.71%, z=-2.67, p=0.01) contained significantly less in terms of percentage post-spawning. The dorsal adipose tissue (1.10 to 0.89%, z=-0.10, p=0.92) and the red muscle (18.65 to 28.11 %, z=1.03, p=0.30) showed no significant change in lipid proportion of the total lipid in the Norwegian cut from pre- to post-spawning (see Fig. 3.7 A and B).

When percentage lipid content of the Norwegian cut is compared with the lipid concentration in the tissue (see Fig. 3.7C), the reverse is seen. This is due to the relative size of the tissues studied. Although low in lipid concentration, the white muscle comprises the largest absolute reserve of lipid extracted from the Norwegian cut (and thus the whole fish). Thus when considering the whole fish, white muscle is the major lipid store. However, this shows no significant variation between pre- and post-spawning fish (Fig 3.7 C).

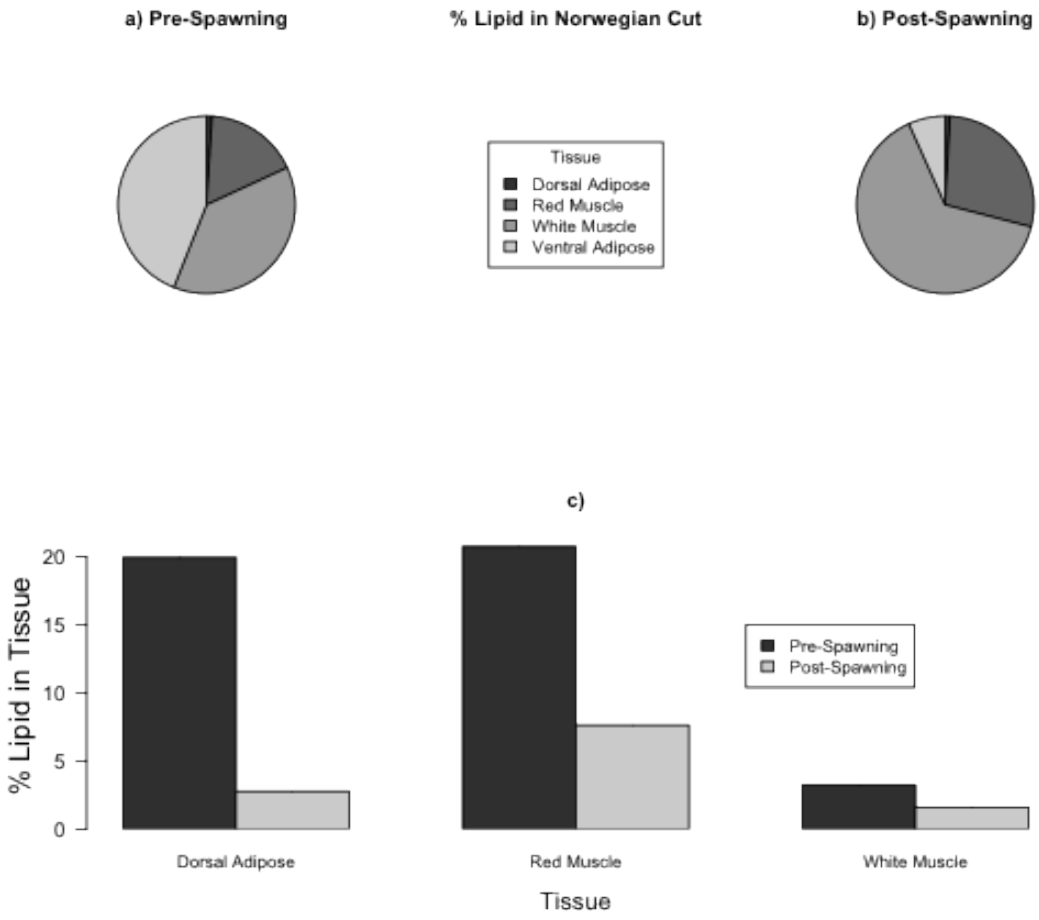


Figure 3.7: Percentage lipid composition of the Norwegian cut estimated from the lipids of the tissues studied. Figure a) shows the estimated ratio of lipid in the different tissues pre-spawning, b) shows the estimated ratio of lipid in the different tissues post-spawning and c) shows the relationship between the measured lipid concentration of the tissue at both pre- and post-spawning. Year of sampling was pooled for pre-spawning fish.

Discussion

Although previous studies have reported lipid content values for Atlantic salmon, the majority of these reports have focused on one tissue type (Katikou *et al.* 2001; Bogevik *et al.* 2009; Johnston & Bergeron 2010). The present study refined an already established methodology for lipid extraction to accommodate tests on smaller, more specific tissue samples. In this study this process focuses on a specific standard sample cut of the fish (the Norwegian cut) and is miniaturised, yet the method still results in accurate total lipid content measurements when compared with use of the existing methodology, Chapter 2. Understanding the relationships between the various levels of lipid storage among tissue types is critical to quantifying the quality of returning adult salmon and how they may be influenced by varying environmental conditions in the open ocean feeding areas. This study demonstrates that Atlantic salmon store lipids in different quantities throughout their tissues and that these stores are necessary for their spawning migration. The results from Chapter 4 can be summarised as:

1. Fish with a higher condition factor tend to show higher lipid concentrations and therefore total lipid content
2. The dorsal midline adipose tissue and red muscle depots contain the highest percentage concentration (by tissue fresh weight) of total lipid
3. The white muscle, although of low percentage lipid concentration, is the major lipid storage site for the fish due to its large mass
4. After spawning maturation the lipid stores of Atlantic salmon have been severely compromised.

This study has shown that Atlantic salmon store lipids in different quantities throughout their tissues and that these stores are used differentially in their upstream migration and maturation for spawning. It can be seen that that salmon preferentially maintain lipid levels in the musculature through the spawning process, and in particular in the low-lipid white muscle. Following the completion of spawning the adipose tissues are preferentially exploited compared to the lipid deposits in the red and white muscle.

Difference in total of lipids in low and high condition fish

The total lipid concentration of the Norwegian cut is significantly related to the condition factor of the fish, indicating that fish of a higher somatic condition have a higher lipid concentration than those of poor condition. Lipid dynamics, the storage and quantity of body lipids, are key to assess fish condition, and have been shown to be particularly descriptive for fish of poor quality because little lipid is present (Litvin *et al.* 2011). In weakfish (*Cynoscion regalis*) increasing lipid levels with size indicated accumulated levels of energy with growth.

It is highly plausible that the different tissues of wild Atlantic salmon will not only contain different quantities of lipid types but also lipid molecules or classes that are exclusive to the individual tissues. Naesje *et al.* (2006), for example, indicate that a fish's chance of survival is dependent upon its lipid class content (Næsje *et al.* 2006); fish are limited by essential fatty acid availability and if dietary supplies are disrupted or lacking this can lead to reorganisation of fish communities and even regime shifts (Litzow *et al.* 2006).

Relationship between total lipid and condition factor

Condition factors are widely used in fisheries science, management and conservation to describe fish quality (Blackwell *et al.* 2000; Froese 2006). A study performed by McPherson *et al.* on herring indicated that whilst condition factors can be highly descriptive, they offer a more accurate measure of individual quality when used in combination with an independent measure of lipid content (McPherson *et al.* 2010). Todd *et al.* (2008) illustrated that low condition salmon will have low lipid contents. This relationship is non-linear, and thus the lipid content of a low condition fish is disproportionately low (Todd *et al.* 2008). The present study confirms this (Figure 4.5) and further demonstrates the non-linear relationship between condition factor and lipid

content; high condition fish will have disproportionately higher lipid content than a fish's condition factor would indicate.

Figure 4.5 demonstrates that when fish draw lipids from the red and white muscles the lipids are taken in amounts that maintain the ratios of lipids with and between the tissues. Whilst lipid content is drawn in large quantities from the adipose tissue, it is being moved to be used in the metabolic processes of the fish and to ensure that the fish's muscle tissues have a constant energy source. The various lipid deposits of a fish are all inter-connected.

Different tissue store lipids in different quantities

Adipose tissue

The dorsal adipose tissue often is described as the primary lipid store of the Atlantic salmon (Zhou & Ackman 1995; Zhou *et al.* 1996; Nanton *et al.* 2007) because it stores high concentrations of lipids. Data for farmed Atlantic salmon show that the adipose tissue contains between 19-25% of the salmon's fat, irrespective of diet (Jobling 2003). The present work demonstrates that the capacity for the dorsal adipose tissue to store total lipid differs from that for the red and white muscle tissues (Figure 4.2), it does not seem to have reached a threshold level for the total amount of lipid that can be stored. These high concentrations of lipid are probably due to the ability of the adipose tissue to store lipid, not only in adipocyte cells, but around them in lipid droplets (Zhou *et al.* 1996).

This is not unexpected because the adipose tissue is, by definition, a storage tissue and thus it follows that for fish of a high lipid content it is likely to contain a large quantity of stored lipid, whereas in low lipid fish this store will either have been used for metabolic functions or never have been filled. It is assumed

that the class of lipids stored in the adipose will differ between high and low lipid fish.

Red Muscle

Work by Torstensen *et al.* (2000) on farmed Atlantic salmon considered the effect of different diets upon salmon tissue provisioning. They showed that poor quality diets adversely affected the lipid content of tissues, with the red muscle showing the biggest impact of diet in terms of lipid content; however they did not examine the dorsal adipose region (Torstensen *et al.* 2000).

Whilst the present study shows the adipose tissue is significantly different from the white muscle tissue in terms of lipid content, it has a striking similarity in lipid density to the red muscle (Figure 4.3). This supports previous work indicating that a high lipid concentration is necessary to power sustained swimming in the red muscle of wild Atlantic salmon (Hudson 1973; Zhou *et al.* 1996; Anttila & Mänttäri 2009). Although red muscle and adipose tissue share similar lipid concentrations it is clear from the GAM model that the red muscle seems to increase towards a threshold level, whilst the adipose tissue shows no obvious indication in these data of a threshold or maximum percentage lipid content. Thus further underlines the difference between a specialised storage tissue and the body musculature. It is relevant to note in this context that lipid-rich farmed fish can store even higher levels of lipid in their adipose tissue (Hamilton *et al.* 2005) than have been observed here for wild fish.

White Muscle

The white muscle comprises the bulk of the body of an Atlantic salmon and is used for bursts of swimming behaviour (Hudson 1973; Zhou & Ackman 1995; Anttila & Mänttäri 2009). This study shows that white muscle has a much lower lipid concentration than both the red muscle and the adipose tissue, in

concurrence with previous results for farmed salmon (Nanton *et al.* 2007). The white muscle itself is the dominant tissue by weight of the Norwegian cut (and the fish itself). Because of its relatively large mass the white muscle contains the greater part of lipid found in the salmon, with approximately 50% of all lipid within the Norwegian cut being attributable to the white muscle (Figure 4.4). In terms of white muscle comprising an energy reserve or storage site, it is suggested that the primary energy source of the white muscle is not lipid, but is in fact glycogen (Zhou *et al.* 1996). This might explain the low level of lipid found in the tissue compared with the red muscle.

Comparison of pre- and post-spawning fish

This chapter confirms previous findings that the decreased condition and body weight attributable to spawning lead to reductions in the lipid content of the musculature of the salmon (Bombardier *et al.* 2010). Work by Vuorinen et al. (2014) indicated that lipid decreases of 60% in the white muscle can occur during the spawning process. This chapter also found an effect on the concentration of lipid in the white muscle tissue; however, this effect was even more pronounced when assessing the estimates of total lipid content of white muscle tissue for the entire fish.

This chapter demonstrates that post-spawning Atlantic salmon are in much poorer condition, compared with the pre-spawning fish sampled. It is expected that fish should have their peak condition prior to maturing to ensure the highest level of reproductive success (Kadri *et al.* 1996). Kadri et al. (1996) found that maturing fish do have better body condition than non-mature fish and indicate that a threshold level must be reached before a fish will mature. Returning salmon in recent years have been recorded as being in poor condition (see Chapter 3) (Todd *et al.* 2008; Bacon *et al.* 2009), yet these fish are making the migratory journey to mature and reproduce.

Potential for iteroparity

The Atlantic salmon is a potentially iteroparous species, and a small proportion of maiden spawners will survive reproduction, emigrate to sea once again, and perhaps return to spawn on one (or rarely) two further occasions. From model analyses, Thorpe *et al.* (1998) showed the importance of environmental interactions influencing the use and storage of salmonid lipid reserves in preparation for the maturation process. The preparations a fish makes will be influenced by its lifestyle; if the fish is iteroparous it should save some of its reserves (Thorpe *et al.* 1998). Thus this highlights the importance of Atlantic salmon not expending all their lipid reserves during spawning.

This chapter clearly indicates that although successfully spawned salmon do draw down their lipid reserves, no fish studied had completely used all of its lipid stores. The dorsal adipose stores were not entirely drawn down, which is key as these are the major lipid storage sites in the salmon. Survival rates of Atlantic salmon returning for a second spawning typically are <10%, but this does vary widely among populations (Niemelä *et al.* 2006; Fleming & Einum 2010) and will – perhaps to some extent - be dependent on lipid stored. Spawning leaves low lipid reserves remaining in the body and must diminish a fish's future chance of survival, and hence its capacity to be iteroparous. However, none of the stripped fish had fully exhausted their lipid reserves that could be an indication of a semelparous life history. The lipid stores the spawning fish will provide to the eggs are key for offspring survival (Berg *et al.* 2001).

Conclusions

A variety of different lipid physiological metrics can be used to assess condition, but triacylglycerides are an informative indicator of energy reserves (Litzow *et al.* 2006). The present study has focused solely upon fish total lipid content, without considering the different constituent lipids that may be contained with

the fish and its tissues. Farmed Atlantic salmon has been shown to differ from wild salmon in terms of their fatty acid profiles, to such an extent that fish can be identified as farmed or wild fish simply from their diet-derived fatty acid compositions (Jonsson *et al.* 1997; Megdal *et al.* 2009). Distinctions also can be made between the different tissues of farmed fish by observing the composition and relative levels of fatty acid classes and components (Nanton *et al.* 2007). In Chapter 4 the relationship between triacylglycerides and lipids will be examined more closely.

Because salmon expend time and energy in ascending rivers to spawn, and remain in freshwater perhaps for weeks or months following spawning, it is possible that this may lead to fish draining the majority of the lipids stored in the adipose tissue, but retaining the lipid levels in their muscles. Work by Berg *et al.* (2001) and Einum and Fleming (2002) indicate that there is an optimum size for salmon eggs, so there is a threshold of lipid provision for eggs. Perhaps all fish try to provision eggs to this point and those that have lipid reserves remaining will survive, migrate to sea once again and return to spawn another year. This supports the Atlantic salmon lifestyle, they are iteroparous, and indicates that if conditions are favourable salmon will attempt to return to spawn again.

The study highlights the fact that fish that have spawned show severely reduced lipid concentrations when compared with maiden fish (Fig. 5.1); however, work by Jonsson and Jonsson (2003) indicates that approximately 30% of the total energy content of a female fish is found in the ovaries at reproduction, whilst males use their energy stores for visual cues (e.g. colouration) and to compete for and maintain territories on the spawning redds.

This work has focused on the total lipid held in the body tissues of Atlantic salmon; however, this measure includes a wide variety of different lipid classes, and fatty acids are perhaps of especial importance in energy metabolism and

vitellogenesis of Atlantic salmon. Triglycerides or triacylglycerides (TAG) are generally accepted to be the major energy in fish (Bennett & Janz 2007). Studies of chum salmon (*Oncorhynchus keta*) indicate depletion in TAG levels in fish found at spawning sites (Ando *et al.* 1996). Since TAGs are so energetically important, a level of maintenance would be expected in an iteroparous species to ensure future success.

Acknowledgements

The author would like to thank B. Todd for helping to develop then miniaturisation process. The data used in this chapter from 2008 was collected by B. Todd and R. Hough for dissertation projects, as well as by A. Howe; this is the first time this work has been analysed together. The post-spawning fish sampled on the River Conon were generously collected and donated by T. Burton and the River Conon team. The post-spawning fish data used in this chapter was partially collected by J. Forrest a dissertation project, as well as by A. Howe; this is the first time this work has been analysed together.

4. A comparison of pre- and post-spawning Atlantic salmon with triacylglyceride distribution

Introduction

The term lipid is used to encompass a wide array of compounds. Lipids are diverse and are used by organisms for a variety of functions; they can be used to maintain cellular structure and can be metabolically important (Cowey & Sargent 1975). It has been convention to assess lipid extracts as measures of the total body lipid content using the long-established Bligh and Dyer method (1959), and this has been commonly applied by fish biologists. However, the accuracy of this method has been questioned (Bennett & Janz 2007), and it has been suggested that the assessment of specific lipids, rather than measuring total lipids, may be a more appropriate physiological metric of fish bioenergetics, both from an ecological and physiological standpoint (Bennett & Janz 2007). Triacylglycerides, or TAGs (formerly known as triglycerides), are a common lipid class that are used by organisms primarily for energy supply.

Variations in lipids or other dietary components can be influenced by environmental changes but their ecological impacts rarely are direct, because of the structure of complex ecosystems, and because particular species are influenced both by their prey (abundance and nutritional quality) and predators (Litzow *et al.* 2006). Litzow *et al.* (2006) suggest that fluctuations in essential fatty acid (EFA) production by phytoplankton can influence marine ecosystems, as summarised in their so-called 'EFA limiting hypothesis'. This is reliant upon the restrictions of trophic structure (species are dependent on each other), assuming that all EFAs are obtained through diet. Salmonids are not just directly affected by fluctuations in sea surface temperature, they are also limited by their prey (Beaugrand & Reid 2003) indicating that the salmon body condition may be affected by diet.

Triacylglycerides (TAGs) are an important group of fatty acids, and are named tri-acyl due to the three fatty acids, attached to a glycerol molecule, that comprise the lipid molecule (CBN 2014). TAGs are energetically important due to these three bonds between the glycerol molecule and the fatty acids, because when this bond is broken energy can be released (Sheridan 1988). TAGs have been suggested as the predominant storage mechanism for energy in salmon and that these stores are mobilised during periods of starvation (Rainuzzo *et al.* 1997). TAGs are mobilised by lipase enzymes and must be broken down in order for them to move between tissues because they cannot be transported in their normal state (Sheridan 1988).

Although between-species variation of TAG storage is common (Bennett & Janz 2007), it appears justifiable to assume that TAG analysis is an appropriate measure of energy stores for *Salmo salar* L., and a range of triglyceride assays have been tested in numerous fish species including Pike (*Esox lucius*), Burbot (*Lota lota*), Slimy Sculpin (*Cottus cognatus*) and Spottail Shiners (*Notropis hudsonius*) (Bennett & Janz 2007; Bennett *et al.* 2007; Kelly & Janz 2008) as well as for Atlantic salmon (Zhou & Heras 1997). It also has been suggested that these triacylglyceride assays could be applied to specific tissues (e.g. the gonads) (Bennett *et al.* 2007), however this is dependent upon the lifecycle stage of the salmon. These types of lipids are of especial ecological importance due to their limiting capabilities upon fundamental processes (Litzow *et al.* 2006), and the fact that triglycerides are the major energy store in salmon (Zhou *et al.* 1996).

Once salmon return to the rivers to spawn they stop feeding. Thus these stores are likely to provide an informative indicator of not only the energetic quality of the salmon but also will offer some insight into the future likelihood of a salmon's survival as it travels up-river, as these fish are not being sampled at their end of their migration.

The results of the previous chapters in the thesis have focused on the overall, or total, lipid content of the tissues of Atlantic salmon, but in order to place the results in an appropriate ecological context it is necessary to consider these variations at a biochemical level. The extracted total lipid and triglyceride levels will be analysed with respect to their relationship with the somatic condition factor of the salmon, with a view to highlighting any differences between the two measures.

Methods

Triacylglyceride extraction

Triacylglycerides (commonly known as triglycerides), or TAGs, have been suggested as a more appropriate measure of fish bioenergetics than is provided for total lipids extracted by the Bligh and Dyer methodology (Bennett & Janz 2007). Triacylglycerides are the main metabolic energy store in fish (Zhou & Ackman 1995).

Triacylglyceride analysis was performed on a selection of the already extracted lipid samples (stored under nitrogen), obtained by the miniaturised Bligh and Dyer method; but these particular samples were taken only from vials of the first round of Bligh Dyer extraction. The lipid to be extracted for TAGs was placed in glass vials and evaporated from the chloroform under a constant stream on nitrogen then sealed and place in a 4°C fridge for storage. This ensured the extracted lipid remain inert until testing. The majority of lipids are extracted in the first of the three Bligh & Dyer washings (see Chapter 2) and, by restricting the TAG analysis to lipids from that first lipid extraction, this approach ensured that no structural lipids were included in the TAG assay samples (Bennett *et al.* 2007).

In preparation for TAG analysis the lipid samples then were dissolved in 500 µl isopropanol and assayed using a Sigma-Aldrich Serum Triglyceride Determination Kit (TR0100). This technique includes a free glycerol reagent and a triglyceride reagent to determine the true triglyceride content of a sample. The test involves enzymatic hydrolysis by lipase of the triglycerides to glycerol and free fatty acids, hence the need to test the glycerol level of the sample prior to triglyceride determination. The glycerol produced is measured by an enzymatic reaction that produces a quinoneimine dye that shows an absorbance maximum at 540 nm. The increase in absorbance at 540 nm is directly proportional to the concentration of triglyceride in the sample.

The samples were set on a 96-well plate reader, with deionised water as the absorbance zero reading and a range of glycerol standards (from 0 to 2.5 mg/ml). The glycerol and total triglyceride readings were calculated from the geometric mean regression of the standard curve, with the true triglyceride reading being the difference between these two values. Four replicates of each sample were tested and appropriately diluted to ensure that the sample concentrations accorded to the standard curve.

TAG analysis was performed on pre-extracted lipid from the fish studied in Chapters 2 and 3, taken from the first round of Bligh and Dyer extractions to ensure clean samples (Bennett *et al.* 2007). Lipid extractions were stored prior to testing following the method outlined in Chapter 2. All extractions were undertaken in quadruplicate.

Year	Sample Size
2008	14 (2 fish deleted)
2009	16
2010	16
Post-Spawning (2009)	16

Table 4.1: Numbers of fish sampled for TAG analysis in the years of sampling.

Statistical Modelling

The TAG assaying technique resulted in some variation in the TAG concentration recorded amongst quadruplicates. Assessment of statistical outliers was by inspection of the coefficient of variation (CV) amongst quadruplicates; tissues with a CV >30 were deleted from the present analyses and if more than one tissue had a CV >30 the fish was removed from the study.

The variation in TAG concentrations of pre-spawning and post-spawning fish for separate tissues (red muscle, white muscle, adipose) were non-linear and thus were analysed using a generalised additive model (GAM), compared with the concentration of Norwegian Cut total lipid and also with W_R condition factor in the case of pre-spawning fish. Separate models were used to test each tissue type and to consider the effect of spawning on the TAG concentrations of the various tissues. For each model the percentage of total lipid content of the Norwegian cut, fish length, weight, W_R condition factor and total tissue weight were modelled as smooth, non-parametric functions.

The GAM model was applied in this form to analyse the data:

Model<-gam (Tissue TAG~ s (Tissue Lipid) +s (Length) +s (Weight) +s (Tissue Weight) +te (Weight, Tissue Weight) +te (Length, Tissue Weight) +year +River Age +Sex-1, family=quasibinomial)

The year-class, sex and river age also were included in the model as parametric factors. The quasi-binomial error structure was used due to the limitations of the response variable (total lipid) – concentrations of which could range only between 0 and 1 – and to control for over-dispersion (the range of proportions, although varying, fits into only a limited range). Gamma was increased to 1.4 to control for over-fitting within the model. The individual models were simplified to find the most parsimonious setup, and the validity and utility of this model tested using the General Cross Validation (GCV) method.

Results

There is a positive relationship between the triacylglyceride concentration of Atlantic salmon tissue and a fish's concentration of total lipid. Figure 4.1 demonstrates this relationship ($y=2.1283x - 0.4842$, $r^2=0.4407$) for pre-spawning fish. However, there is a large degree of variation in TAG concentrations that cannot be explained by increasing lipid concentration of the fish. There was no significant effect of year on this relationship so all years were pooled. Post-spawning fish contain significantly less TAG in the Norwegian cut tissue than did pre-spawning fish ($t= -4.491$ $p<0.001$, see Fig 4.1).

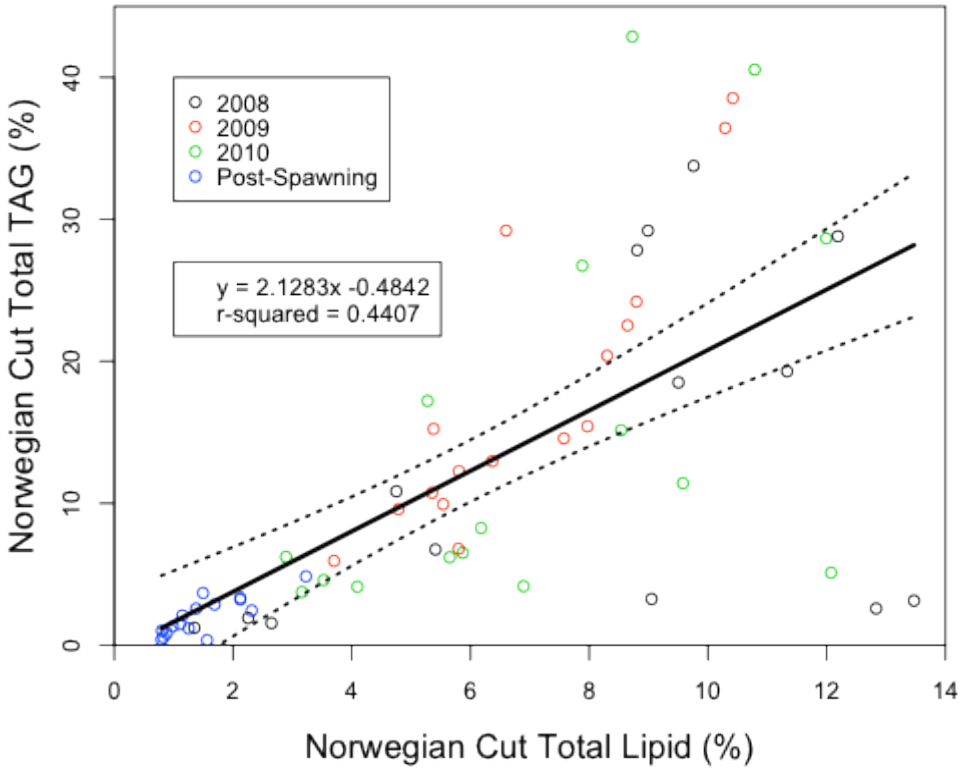
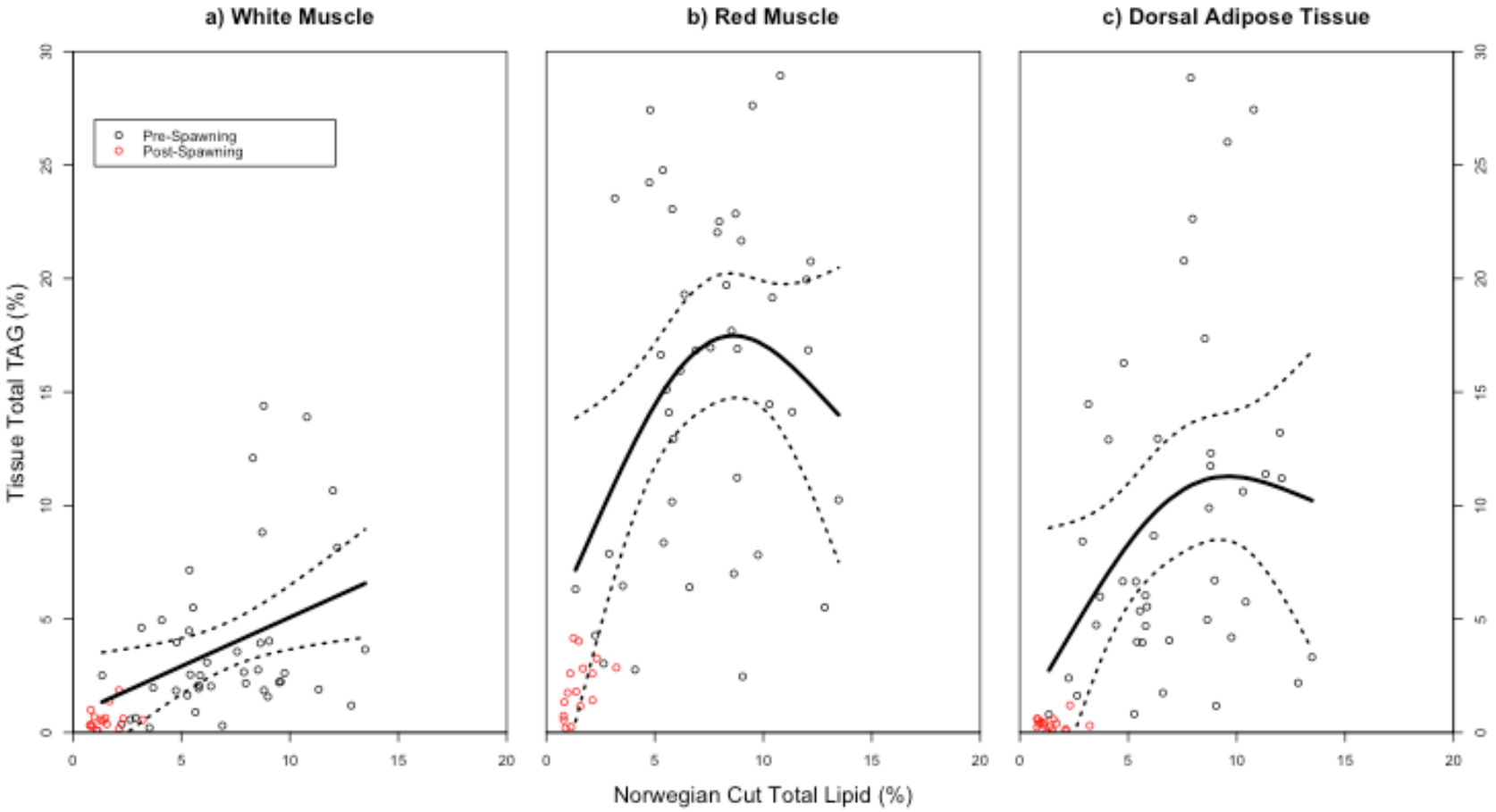


Figure 4.1: Concentration lipid of the Norwegian cut (%) of pre- and post-spawning fish plotted against concentration of TAG in the Norwegian cut (%). The linear model is drawn (solid black line), 95% confidence intervals (dotted lines), demonstrate the relationship in pre-spawning fish only. The equation of the model line and r^2 value is indicated. Individual data points are coloured by sampling year and indicate spawning state.

Figure 4.2: Concentration of TAG in the tissue (%) (as indicated) plotted against lipid concentration (%) in the Norwegian cut. Individual data points are shown in black for pre-spawning fish and red for post-spawning fish. The GAM model prediction for pre-spawning fish is drawn (solid black line), 95% confidence intervals are indicated (dotted lines).



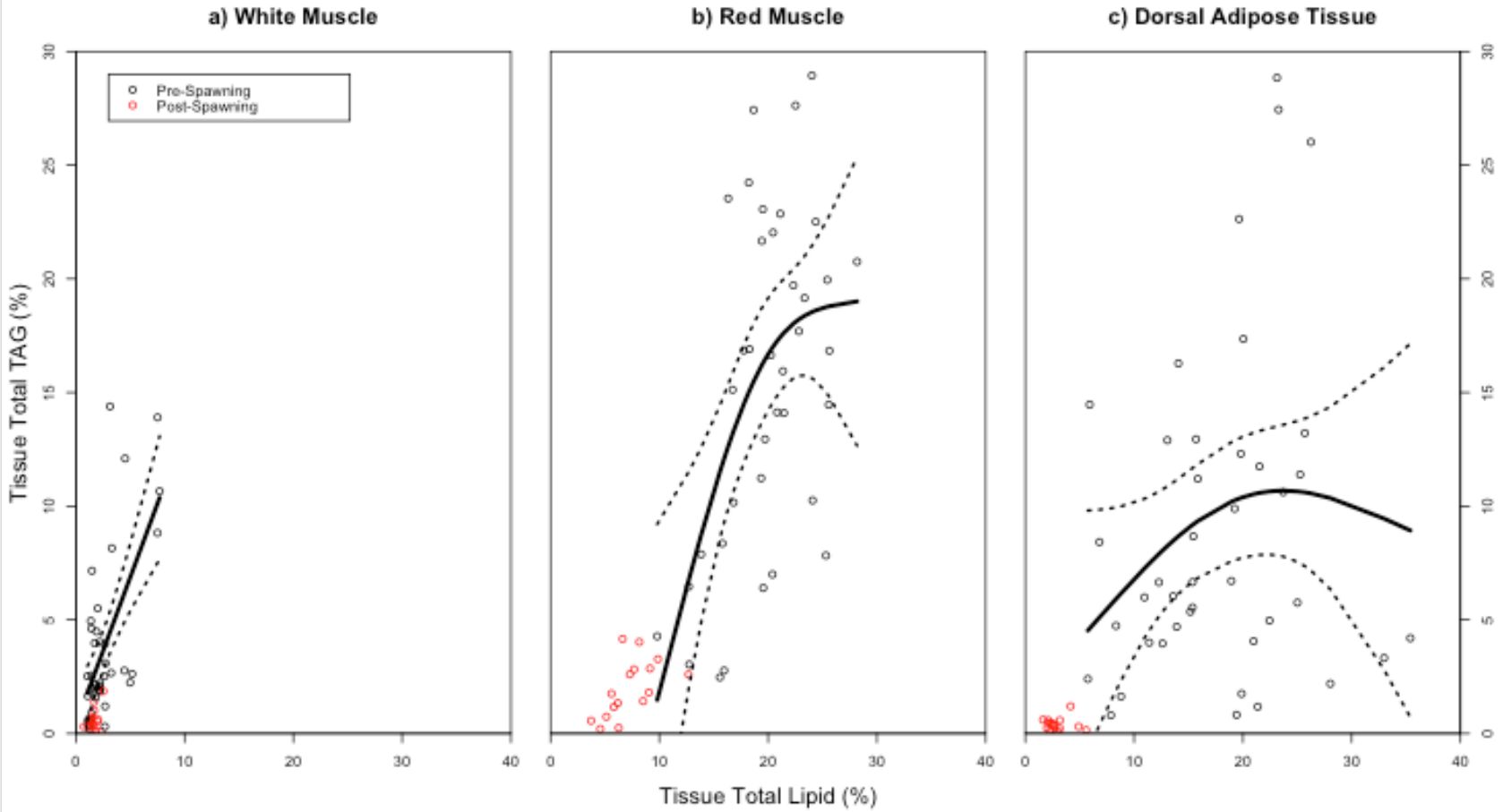
Individual tissue TAG content was not influenced by river age of the fish either for pre- or post-spawning salmon, indicating that the at sea stage of the Atlantic salmon lifecycle may be key in influencing the observed TAG concentration of these fish. The sex of the pre-spawning fish had no impact upon the TAG content of all tissue types, however sex was not studied in post-spawning fish as only females were sampled. Neither the weight nor length of the entire fish had any effect on the percentage TAG concentration of any of the individual tissues. There was no significant influence of year on TAG concentration.

The percentage TAG concentrations of the white muscle and dorsal adipose tissues in pre-spawning showed positive, non-linear relationships with lipid concentration of the Norwegian cut (Fig 4.2, Table 4.2). The red muscle TAG concentration was not influenced by variation in Norwegian cut total lipid and this muscle tissue seems to maintain a relatively constant TAG concentration (Fig. 4.2).

	White Muscle		**Red Muscle**		**Dorsal Adipose**		**Liver**	
Non-Parametric	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value
NC Lipid (%)	0.222	0.640	3.216	0.184	8.709	<0.001*	1.082	0.383
Tissue Lipid (%)	35.200	<0.001*	2.537	0.001*	7.458	0.002*	4.129	0.067
Tissue Weight g	-	-	1.000	0.929	7.856	0.001*	1.082	0.383
Parametric	t-value	p-value	t-value	p-value	t-value	p-value	t-value	p-value
Spawned	-1.5378	0.001*	-0.4368	0.004*	-4.3751	<0.001*	-	-

Table 4.2: Model terms from the GAM performed between the individual tissue TAG Concentration (%) and the Norwegian cut TAG Concentration (%); total tissue lipid concentration (%); tissue weight (g); and whether the fish had spawned. The F statistic and p-values are given for non-parametric smoothed terms and t value and p-values for parametric terms. Those terms that are significant are annotated "*".

Figure 4.3: Concentration of TAG in the tissue (%) (as indicated) plotted against lipid concentration (%) of the tissue. Individual data points are shown in black for pre-spawning fish and red for post-spawning fish. The GAM model prediction for pre-spawning fish is drawn (solid black line), 95% confidence intervals are indicated (dotted lines).



The white muscle TAG concentration is lower than that of either the red muscle or dorsal adipose tissue (Fig. 4.2, Fig. 4.3). However, the white muscle shows the strongest relationship between TAG and total lipid concentration (%) of the tissues studied (Fig. 4.3, Table 4.2).

The red muscle GAM modelling indicates that its TAG concentration also increased with the lipid content of the red muscle (Fig. 4.3, Table 4.2). However, red muscle showed no significant relationship with tissue weight, suggesting that the red muscle maintains a consistent TAG concentration in the tissue of pre-spawning fish (Fig. 4

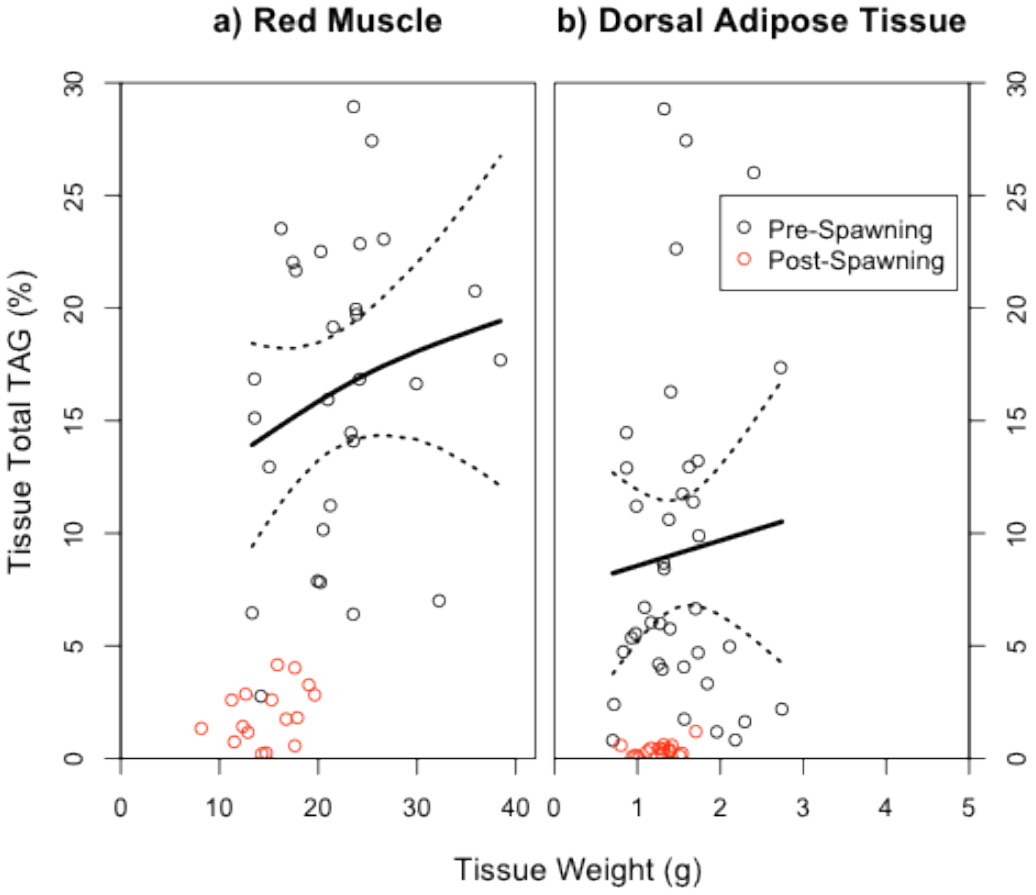


Figure 4.4: Concentration of TAG in the tissue (%) (as indicated) plotted against the tissue weight of the tissue studied. Individual data points are shown black for pre-spawning fish and red for post-spawning fish. The GAM model prediction for pre-spawning fish is drawn (solid black line), 95% confidence intervals are indicated (dotted lines).

The dorsal adipose tissue TAG concentration also is influenced by the by its tissue lipid concentration (Table 4.2, Fig 4.3), increases with higher levels of total lipid. However, it shows a much weaker relationship than the white muscle. It also shows a significant relationship with tissue weight, reflected as a slight increase in TAG concentration with the amount of adipose tissue in the fish (Fig. 4.4).

Post-spawning fish

The results demonstrate that the TAG concentration in all tissues is significantly lower in post-spawning fish (Table 4.2, Figs. 4.1, 4.2, 4.3, 4.4). This follows on from the work in Chapter 3, indicating that the triacylglyceride are markedly compromised by post-spawning.

Liver

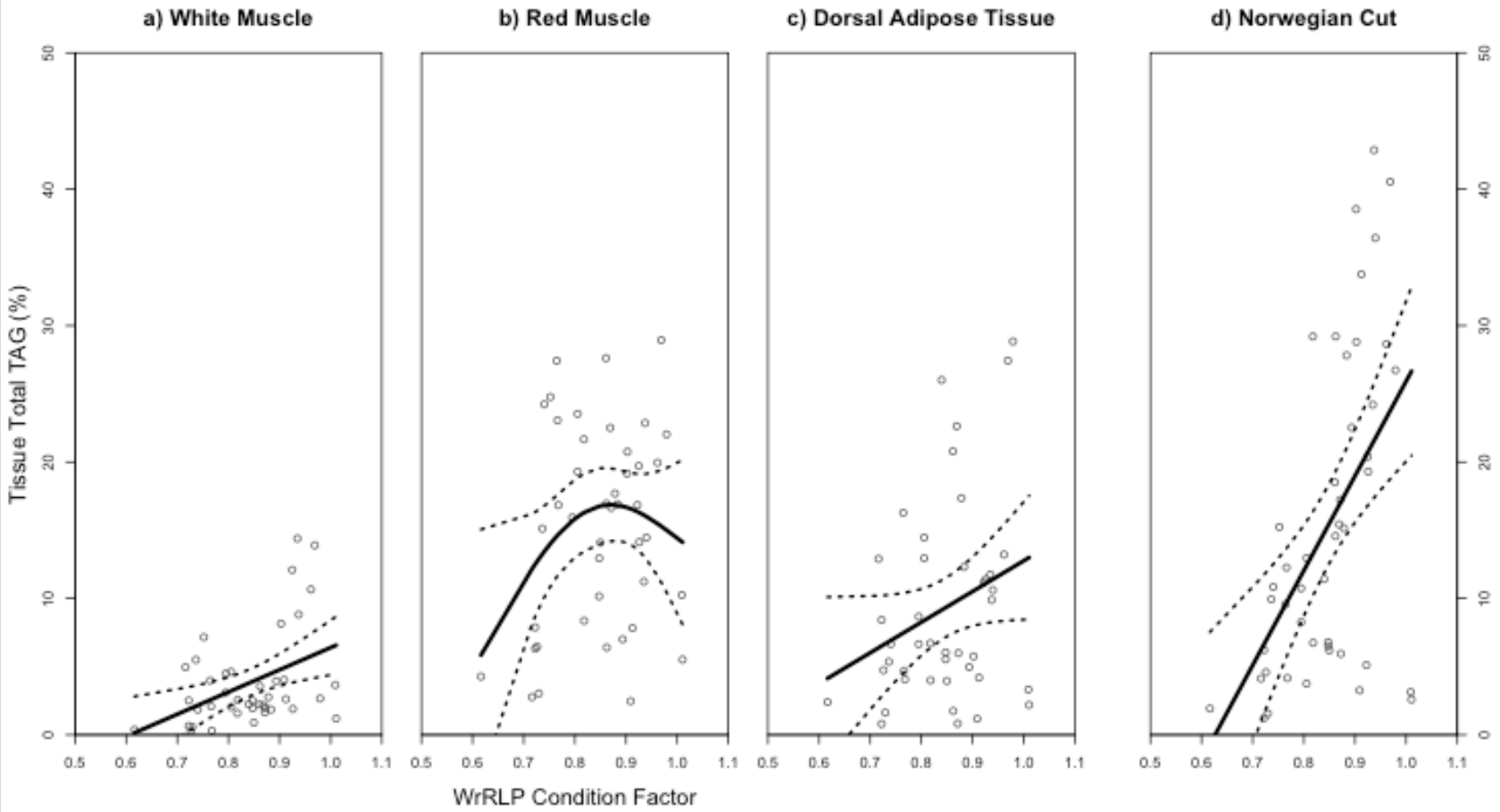
The liver tissue TAG concentration (%) showed no relationship with increasing lipid concentrations in the entire fish, the tissue itself nor condition factor. The liver stores TAG in very small amounts; the TAG levels within the liver are the lowest of all the tissues studied.

Condition factor

	White Muscle		Red Muscle		Dorsal Adipose		Norwegian Cut	
	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value
W_R	5.482	<0.001*	3.181	0.008*	3.522	0.068*	5.353	<0.001*

Table 4.3: Model terms from the GAM performed between tissue TAG concentration and W_R condition factor, F- and p-values are given for non-parametric smoothed term. Those terms that are significant are annotated "*".

Figure 4.5: Concentration of TAG in the tissue (%) (as indicated) plotted against W_R RLP condition factor, in pre-spawning fish. The GAM model prediction for pre-spawning fish is drawn (solid black line), 95% confidence intervals are indicated (dotted lines).



The TAG concentrations of the white muscle, red muscle dorsal adipose tissue and the overall Norwegian cut are significantly related to the W_R condition factor of the fish (Table 4.3, Fig. 4.5). This is expected and follows the pattern seen between percentage lipid content of the Norwegian cut and W_R condition factor (see Chapter 2). However, there was considerable variation in the TAG concentrations recorded and, although there is a relationship between W_R condition factor and TAG concentration, it is not strong, except for that of the white muscle and the overall Norwegian cut.

Discussion

Previous studies have been directed at the total lipids found in Atlantic salmon, and have focused on measuring all lipids stored within salmon body tissue (Jonsson *et al.* 1997; Katikou *et al.* 2001). This chapter applied triacylglyceride (TAG) analysis to lipid extractions of the Norwegian Cut and separate tissues thereof in order to allow testing of the variation of energetic stores within individual salmon. Understanding the importance of TAGs to fish is key to accurately measuring the quality of returning salmon and how the spawning process affects them. This study demonstrates that Atlantic salmon deplete their TAGs reserves during spawning, but prior to this they store TAGs in different quantities throughout their tissues. It can be shown here that:

1. Fish with higher lipid concentrations in the white muscle and adipose tissues have higher TAG concentrations.
2. The red muscle conserves TAG, even after spawning.
3. TAG concentration in the white muscle, adipose tissue and the overall Norwegian cut (but not the red muscle) increases with somatic condition factor.

TAG variation

Work on juvenile smolts in the River Alta (Norway; (Næsje *et al.* 2006)) indicates that TAGs are the principle energy store in Atlantic salmon. This demonstrates that juvenile survival at sea may be dependent not just on total lipid content, but also on lipid class composition. Depleted TAG stores lead to a high risk of energy-related mortality. Naesje et al. (2006) found that TAGs varied more amongst individuals than did other lipid groupings (e.g. fatty acids, cholesterol) and that most of the variation in total lipid concentration could be explained by this TAG variation. Fish mortality increases when TAG is low or absent (Næsje *et al.* 2006). This is perhaps of concern to fishery managers as the results found in this chapter indicate that Atlantic salmon have severely depleted their TAG reserves post-spawning.

Naesje et al. (2006) indicate that total lipids do provide a good description of TAG variation except at very low TAG levels. This may explain the large variation found here in assaying TAG concentrations. There was considerable variation among the fish sampled, but this variation was consistent with repetition and replication of the TAG assays and cannot be explained by testing for outliers. Individuals with no or little TAG still have measurable quantities of other lipids in their tissues; therefore total lipid cannot be used to predict TAG in low energy fish (Næsje *et al.* 2006). It has been suggested that because TAGs are important in terms of fish growth and little TAG is found in poor condition fish, it is an excellent measure of fish condition (Litvin *et al.* 2011).

Despite this variability in the TAG results, it still seems appropriate to utilise them as a measure of fasting or starvation in salmon because they are readily recognised as the key energy store (Rainuzzo *et al.* 1997). The usefulness and accuracy of measuring total lipids has been discussed (Bennett *et al.* 2007) and it has been suggested that the assessment of specific lipids, rather than measuring total lipids, may be a more appropriate measure of fish bioenergetics, both on an ecological and physiological level (Litzow *et al.* 2006; Bennett *et al.* 2007).

TAG in the red muscle

The red muscle seems to be the key tissue in terms of triacylglycerides; it conserves its concentration of TAG and shows no effect of spawning upon its storage pattern, unlike the white muscle and adipose tissue that showed reduced TAG concentration post-spawning process. Past studies have indicated that the red muscle holds a higher concentration of TAG than does the white muscle and that TAG is the prominent lipid within the red muscle (Zhou & Ackman 1995).

It has been suggested that during periods of fasting, for example the spawning migration, other lipids will be hydrolysed into TAG from storage deposits and for energetic purposes (Zhou *et al.* 1996). This could explain both the reduction of total lipids and the reduction of TAG in the adipose tissue. In farmed fish (pre-spawning) the adipose tissues (in both the dorsal and ventral regions) have been found to have significantly higher TAG concentrations than the muscle tissues (Nanton *et al.* 2007).

Conclusions

TAG analysis of Atlantic salmon reproductive tissues could be applied to highlight key changes in the salmonid lifecycle (Bennett *et al.* 2007), however this is dependent upon the availability of tissues. These types of lipids are useful to study due to their limiting capabilities upon fundamental processes (Litzow *et al.* 2006) and the fact that triglycerides are the major energy store in salmon (Zhou & Ackman 1995). The presence of TAG in fish tissue is extremely important for completion of metabolic processes, providing energy for the fish. It is not surprising, that TAG levels are significantly reduced after spawning (Ando *et al.* 1996), as the process is so energetically costly.

This work indicated that TAG measurements might be a more sensitive metric for measuring fish quality; except in the red muscle, which maintains a TAG concentration. TAG concentration increase with W_R condition factor in the white muscle and adipose tissue. The spawning process markedly draws down the TAG content of Atlantic salmon.

Acknowledgements

The author would like to thank T. Smith for his support, advice and knowledge of triacylglycerides.

5. General discussion

This thesis examined the quality of Atlantic salmon by using various metrics of condition; condition factor, total lipid, tissue lipid, triacylglyceride concentration, fecundity. Repeated use of the same fish to test multiple tissues, with different measures allowed the results of this work to be placed in the context of the usefulness of different metrics. This then allowed the work to contrast the difference between pre-spawning fish and post-spawning fish. In this discussion I describe these contexts and evaluate the use of lipids as a proxy for condition and thus their usefulness to measure the impact of environmental change on Atlantic salmon. I use the research performed to evaluate existing work and indicate the possibilities for future research.

Lipids as a metric of fish quality

This thesis clearly demonstrates that lipids can be used as a metric for fish quality. Chapter 2 demonstrates that the lipid content of a fish is significantly related to its condition factor. Lipid levels can be very informative when assessing fish size, weight and condition; lipids have been found to be especially valuable in describing the variation in quality of poor condition fish (Litvin *et al.* 2011).

In addition to analyses of total lipid content, any further information on particular lipid classes or molecules can be even more informative in assessing variation in fish condition. Appendix 1 indicates that triacylglycerides are not an appropriate measure of quality of salmon eggs; however, Chapter 4 indicates that TAGs are an informative proxy for somatic condition as manifested in variations of lipid content of the white muscle and adipose tissue. Different lipids can be used to assess condition in different functions (Litzow *et al.* 2006). Studies indicate that salmon survival is dependent on its lipid class content (Næsje *et al.* 2006) and that growth can be limited by the availability of the right class of lipid (Litzow *et al.* 2006).

Morphological measures of somatic condition, such as condition factors, are often subjective and under pressure of the assumptions of their model structure (see Chapter 2). Lipids offer the accuracy that these measures can lack (Bennett *et al.* 2007; Davidson & Marshall 2010; McPherson *et al.* 2010). However, measuring the lipid content of fish is a costly procedure; the fish must be killed to be studied, equipment used is expensive and often toxic reagents are used in determining lipid amounts. Determination of the condition factor of an individual fish is not invasive and can, for example, be calculated for live fish. When used in combination with fatmeter readings these morphological metrics can offer an informative estimate of fish condition (Chapter 2). However, they are prone to error (Froese 2006; Stevenson & Woods 2006; Davidson & Marshall 2010).

This work implies that the use of morphometric measures of condition is a good initial step in assessing quality. However, to understand the complexities of the energy reserves of a fish a more detailed physiological measure is required, like using the lipid content of a fish. If undertaken as part of a lifecycle study lipids can be very informative in demonstrating the stage of maturation of a fish through its pattern of lipid storage.

The variation in the relationship between condition factor and lipid content over the years of study (Chapter 2) indicates the importance of having a way to benchmark morphometric measures against a physiological measure. This varying relationship indicates how much more the use of physiological metrics can tell us, and the restrictions of just using condition factors alone.

The inverse water-lipid relationship

It is commonly accepted the water content in biological tissue is, to an extent, inversely related to the fat content. The principle of operation of the fatmeter used here to estimate the lipid content of monitored fish (see Chapter 2 for fatmeter details) exploits this relationship. The meter emits low-power microwave radiation that is partially absorbed by the water molecules in the tissue. The reflected, or non-absorbed, radiation is detected by the meter and this permits an estimate of the water content (and hence the fat content) of the fish tissue (Kent 1990). It has been suggested that variations in condition factor measures are often not as pronounced as measures of actual lipid content, because fish are able to replace declining fat content with water (Sutton *et al.* 2000).

Work on herring (*Clupea harengus* L.) indicates that, due to the inverse relationship of lipid and water, calculations of condition factor based on measures of fish weight (such as W_R used in this thesis) may consistently over estimate fish quality (Oskarsson 2008; Davidson & Marshall 2010). Oskarsson (2008) and Davidson & Marshall (2010) both suggest that as herring lose lipid from their tissue they replace it with water. This means that the herring show smaller decreases in body weight than if they just lost all their lipid (water weighs less than the same volume of lipid). This would indicate that lipids are a much more representative and ecologically relevant measure of fish condition than simple morphometric condition indices, including W_R.

This replacement of lipid by water could lead to a size bias effect on measures of condition factor (Davidson & Marshall 2010); this might extend to, for example, the non-linear relationship between W_R condition factor and total lipid concentration as reported in Chapter 2. Water can replace the fat in the fish musculature of individuals of poor condition, rendering condition factors an inconsistent measure of lipid content (Oskarsson 2008).

Studies of Atlantic salmon parr showed that condition factors (in this case Fulton's K) were more significantly influenced by water weight, rather than the fat reserves of a fish (Sutton *et al.* 2000). In fact Sutton et al. (2000) found that there was no significant relationship between Fulton's K and the fat weight of salmon parr. This demonstrates further the importance of measuring fat reserves in addition to morphometric condition factors in order to more fully assess the causes and consequences of environmentally-driven changes in individual quality of fish within a given stock or population. Work by Berg *et al.* (2001) and Einum and Fleming (2002) indicates that there is an optimum size for salmon eggs, so there is a threshold of lipid provision for eggs. It is possible that all Atlantic salmon will try to provision their eggs to this level, however, due to this size optimum it is possible that poor condition fish may provision eggs with water as a lipid replacement.

Semelparous vs. iteroparous lifestyle

The pre-spawning salmon sampled for analysis in Chapters 2, 3 and 4 were caught at the end of their first stage of spawning migration (from sea to the home coast) (Hansen *et al.* 1993). Thus they should still have lipid reserves remaining to complete the second phase of spawning migration from river mouth to spawning tributary (Hansen *et al.* 1993).

The post-spawning salmon sampled in Chapters 3 and 4 demonstrate that at the end of the spawning migration salmon have heavily reduced, but not completely drawn down, their lipid reserves. For Atlantic salmon to be truly iteroparous fish they would of course need to retain sufficient lipid to fuel their return journey to sea and the energetically costly process of moving from river to sea (Thorstad *et al.* 2010), although animals can catabolise protein to provide energy, this causes muscle wasting so it is metabolically expensive. Hence lipids provide the primary energy source in fish.

The post-spawning fish studied had not completely drawn down their lipid reserves, and the expected extremely low levels were recorded. This would affirm that Atlantic salmon are capable of completing an iteroparous lifecycle. However, the salmon studied in this thesis were stripped of eggs and thus did not complete the energetically costly process of spawning, finding a mate or build a redd (Fleming & Einum 2010). Most of the salmon studied here would not have the energy reserves to do this and then return to sea.

Male salmon were not considered post-spawning. However, the process is also costly for them. Although they do not provision eggs, as the female does, they still make the same spawning migration and use their 'extra' energy for visual cues and to establish and defend spawning territories (Jonsson & Jonsson 2003; Fleming & Einum 2010). Male salmon pre-spawning showed the same levels of lipid as female salmon (Chapters 2 and 3).

Report rates of Atlantic salmon completing a second spawning cycle are low; they are not typically greater than 10% (Fleming & Einum 2010). Some populations show even lower rate; the River Teno for example has an iteroparity level of about 5% (Niemelä *et al.* 2006). Careful provisioning of lipids will allow salmon to be iteroparous (Thorpe *et al.* 1998), however, with recorded levels of body lipid so low in salmon and a continuing downward trends of somatic condition (Todd *et al.* 2008) it seems unlikely that many salmon will achieve this. The reality of these low lipid levels means the majority of fish will be functionally semelparous.

The difference between farmed and wild salmon

In completing this work it is clear from the published literature that the majority of studies performed on Atlantic salmon lipid content are undertaken on farmed fish. Although wild fish and farmed fish are the same species (*Salmo salar* L.),

they experience very different growth conditions and have radically different lifestyles. More importantly they have different diets.

Work by Torstensen *et al.* on farmed Atlantic salmon considered the effect of different diets upon salmon tissues. They showed that poor quality diets adversely affected the lipid content of the tissues, with the red muscle showing the biggest impact of diet in terms of lipid content; however they did not examine the dorsal adipose region (Torstensen *et al.* 2000). Diet of farmed salmon has an impact on lipid content of the body tissues, since it is impossible to know exactly what wild fish eat this helps increase our knowledge of the influence of diet on the red muscle.

Farmed Atlantic salmon have been shown to differ from wild salmon in terms of their fatty acid profile and this is a consequence of their differing diets. Research indicates that this difference is so great that a fish can be identified as farmed or wild fish from just its fatty acid compositions (Jonsson *et al.* 1997; Megdal *et al.* 2009). Distinctions can also be made between the different tissues of farmed fish by observing the composition and relative levels of fatty acids (Nanton *et al.* 2007).

It is highly plausible that the different tissues of wild Atlantic salmon will not only contain different quantities of lipid types but also lipids unique to the individual tissues. Naesje *et al.* (2006) indicate that a fish's chance of survival is dependent upon its lipid class content; fish are limited by the availability of essential (i.e. diet-derived) fatty acids. If supplies are disrupted this can lead to reorganisation of fish communities and even regime shifts (Litzow *et al.* 2006).

It is logical to assume that the class and composition of the lipids stored in the adipose tissues will differ between high- and low-lipid fish. Work on farmed

Atlantic salmon suggests that the adipose tissue contains between 19-25% of the salmon's fat, irrespective of diet (Jobling 2003). This is not unexpected because the adipose tissue is a storage tissue and for fish of low lipid content this store will have either been used for metabolic functions or never have been filled during the marine growth phase. Lipid-rich farmed fish can store even higher levels of lipid in their adipose tissue than is observed for wild fish (Hamilton *et al.* 2005), indicating that the adipose tissue can store even more lipid than seen in Chapter 3.

Impact of salmon on their surrounding environment

Species often are studied in isolation. To achieve an unambiguous picture of what influences a species its whole ecosystem must be studied and this should include both abiotic and biotic factors. However, this is complex to achieve, and a balance must be found between simplicity and realism (Cury et al. 2008). Arguably, the Atlantic salmon is an ideal candidate species for monitoring the North Atlantic pelagic ecosystem condition because this fish is a top opportunistic, generalist consumer of both zooplankton and fish (Hansen & Quinn 1998).

No species exists in isolation of others within the ecosystem, and Atlantic salmon themselves are influenced by prey abundance, predator impacts, fisheries activity and the physical environmental conditions (Beaugrand & Reid 2003; Todd *et al.* 2008; Beaugrand & Reid 2012). The influence of the food chain has been demonstrated between plankton (a salmon prey item and key member of the food chain) and sea temperatures (Friedland *et al.* 2003b), however, plankton are not only important as a food source but also play a key role in nutrient cycling (Richardson & Schoeman 2004) and may exhibit 'bottom-up' control over this trophic system. Varying salmon condition has repeatedly been suggested to be influenced as a result of variation in the availability and/or quality of the salmon's prey (Beaugrand & Reid 2003; Richardson & Schoeman 2004; Stokstad 2004; Todd et al. 2008). This implies that this system is subject to

a form of bottom-up control, whereby a species is regulated by the species found at lower trophic levels (Cury et al. 2008).

The linking of prey variations to population level trends is not uncommon. But there are still few studies linking climate regime shifts to body lipid contents (Litzow et al. 2006). Lipid concentrations can be influenced by climatic fluctuations, so this relationship needs to be considered. It is problematic that the shifts seen are very rarely direct, because they are part of complex ecosystems and often are influenced by either bottom-up or top-down control (Litzow et al. 2006).

Variations of phytoplankton and zooplankton populations have been recorded in Atlantic salmon feeding grounds (Beaugrand & Reid 2003). These changes have been correlated with the variation in sea surface temperature, a trend that has also been shown in salmon (Beaugrand & Reid 2003; Todd et al. 2008). Recent declines in salmon populations have been attributed to the period of time spent by salmon feeding at sea rather than time in freshwater (Mills 2000), because despite freshwater numbers and smolt production apparently being more or less maintained, fewer adult salmon are returning (Beaugrand & Reid 2003). This thesis aimed to help uncover more about the influence of this at-sea period and finds that it has caused marked decreases in Atlantic salmon body condition and lipid reserves. However, disproportionately less is still known about the life of salmon at sea. Any changes in salmon prey populations may have even more serious consequences for the already exploited fisheries found in the North Atlantic (Beaugrand et al. 2002), and the resilience of such systems to climate change is still unclear (Richardson & Schoeman 2004).

Salmon are affected by their prey, but in turn they also influence their surrounding environment. Few studies exist of the relationship between European salmon and their predators, work by N. Hanson has used stable

isotope analysis to document the relationship between Atlantic salmon and grey seals (*Halichoerus grypus*); suggesting that grey seal declines may be due to human overexploitation of salmon fisheries (Hanson 2011). Some work has been completed describing the relationship between blue whiting and pilot whales, but this showed a bottom-up influence of plankton on both these predators (Hátún *et al.* 2009).

Studies in North America have considered the relationship between North American brown bears (or grizzly bears; *Ursus arctos*) and Pacific salmon (*Oncorhynchus spp.*) (Hilderbrand *et al.* 1996; 1999); black bears (*Ursus americanus*) and chum salmon (*Oncorhynchus keta*) (Reimchen 2000); beavers (*Castor canadensis*) and chinook salmon (*Oncorhynchus tshawytscha*) (Gleason *et al.* 2005); and wolves (*Canis lupus*) with Pacific salmon (*Oncorhynchus spp.*) (Darimont & Reimchen 2002; Darimont *et al.* 2003). These studies noted various behaviours of the predators to eating these salmon species. For example wolves were noted to only eat the lipid-rich salmon heads (Darimont *et al.* 2003) and black bears were recorded as preferring to eat the head, ovaries and other lipid-rich tissues (Reimchen 2000). This indicates that species dependent on salmon for a food source also would be influenced by decreasing fish condition; this is perhaps especially relevant given the low lipid concentrations reported in this thesis.

There has been little documented work examining the relationship between Scottish terrestrial predators of Atlantic salmon, perhaps due to their general lack. Anecdotal evidence exists of otters raiding fish farms, and with the reintroduction of beavers to Scotland and conservation plans to increase otter numbers in the UK environmental managers should perhaps take into account the declining quality of wild Atlantic salmon as a food source.

Salmon are not only a food source to other species, but can also be an important nutrient source for the wider ecosystem. *Oncorhynchus spp.* studied in North America, tend to be abundant and semelparous (unlike Atlantic salmon) and thus the successful spawning migration of these fish ends with death and decay. If salmon are of sufficiently good condition to achieve this migration, they add a significant influx of (marine-derived) nutrients to the surrounding (terrestrial and freshwater) ecosystem (Darimont *et al.* 2010).

Although potentially an iteroparous species, the majority of Atlantic salmon are effectively semelparous, and die after spawning. However, the lipid reserves of post-spawning fish are not completely drawn down (see Chapter 3). Typically less than 10% of Atlantic salmon will display iteroparity and re-spawn in subsequent years, but this does vary widely among populations (Fleming & Einum 2010). In years of fish with increasingly poor condition, it is therefore likely that yet more Atlantic salmon will die post-spawning, increasing the level of nutrients added into the surrounding ecosystem. However, fewer salmon are returning, so it is important to take into account the wider ecological benefits of salmon species being able to return to spawn (Price *et al.* 2008; Casselman 2009; Moore & Moore 2009; Darimont *et al.* 2010; Casselman 2011).

Body size shrinkage

Condition factor measures are, in effect, measures of body size (Blackwell *et al.* 2000), thus studies that reveal a decline in condition factor over time are effectively reporting body size shrinkage. Both Bacon et al. (2009) and Todd et al. (2008) reported decreasing weight and length measurements in Atlantic salmon. This has been correlated with increasing sea-surface temperatures (Todd *et al.* 2008) and with prey variations (Beaugrand & Reid 2003)

Studies of other fish indicate that body shrinkage is affected by temperature. It has been suggested that decreasing body size is an ecological response to climate change (Sheridan & Bickford 2011). Oxygen content of water is also a key driver of body size (Cheung *et al.* 2012) of water-breathing species. Cheung et al. (2012) suggest that body size shrink in fish of 14-24% should be expected by 2050, due to a combination of increasing temperatures, lower oxygen content in water and reduced food availability.

Warmer waters will lead to increasing metabolic rates of organism; thus a species must either consume more to meet raised energy demands or shrink (Sheridan & Bickford 2011). However, a species response to temperature change is complex and highly variable. As Atlantic salmon are a relatively long-lived species it would take a considerable amount of time for populations to adapt.

Future work

Fatty acid analysis

This thesis has focused upon fish total lipid content and triacylglycerides, without considering the different constituent fatty acids that may be contained within the fish and which comprise the different classes of lipids. It is highly plausible that different tissues will not only contain different quantities of lipid types but also lipids unique to the tissue itself.

Farmed Atlantic salmon have been shown to differ from wild salmon at a fatty acid level to such an extent that fish can be identified as farmed or wild fish from their fatty acid content (Jonsson *et al.* 1997; Megdal *et al.* 2009). Preliminary work performed on the fish studied in this thesis indicate that wild salmon contain all of the common classes of phospholipids; however the relative ratios and fatty acid compositions observed in the various tissues reflects their different roles (unpublished data). Naesje *et al.* (2006) indicate that a fish's

chance of survival is dependent upon its lipid class content. Greater study of fatty acids would improve the descriptive power of lipid analysis and help reveal more about the condition of fish.

DNA analysis

Genetically healthy populations are a key driver in achieving the long-term stability and sustainability of species. Life history patterns are expected to be influenced by genetic factors (Amstutz *et al.* 2006). Atlantic salmon genetic diversity in river populations is a key indicator of environmental variation; a good example being genetic bottlenecks, which are an easily detectable sign of reductions in population size (Small *et al.* 2006). Advances in genetics now allow a population to be studied at individual level in order to gauge the true level of variation within the population (Paterson *et al.* 2004).

Studying microsatellites in Atlantic salmon populations has a strong basis within scientific literature and they have been widely tested. A concerted effort is being made by a group of salmon scientists, SALSEA (Salmon at Sea-The International Atlantic Salmon Research Board), to use a standard set of microsatellite markers (the 'Virginia Panel') for genetic stock analysis (Verspoor & Hutchinson 2008). This would allow the creation of an international database detailing the genetic variation of salmon populations (Verspoor & Hutchinson 2008). The usefulness of the ability to type salmon to such a database is great; this leaves considerable scope for the analysis of interceptory fisheries.

Microsatellites have already been used to distinguish between various populations of Atlantic salmon at considerable levels of exactness - e.g. fish found in North America and Europe (O'Reilly *et al.* 2006) and fish found within particular rivers, e.g. in Spain (Borrell *et al.* 2008). This arises from the very low levels of gene flow between salmon populations (Youngson *et al.* 2003), probably attributable to the homing precision of salmon to their natal rivers. This homing

ability, although not perfect, exacts a level of reproductive isolation on the various populations of salmon. Nonetheless, some straying between rivers does occur, both naturally and artificially (Gilbey *et al.* 2005), and straying has been historically important in permitting salmon to colonise new rivers. Colonisation of new, ice-free rivers in Russia is, for example, a predictable response of Atlantic salmon to recent climate change.

Difference between 1SW and MSW fish

Greater investigation into the lipid levels in salmon gives a more direct and clear measure of overall condition, as well as providing indicators of a fish's fecundity and likelihood of future survival. The use of grilse (1SW fish) can give a clear picture of the year by year variation of ocean conditions, because the salmon in question remain at sea feeding grounds for a clearly defined and easily measurable time period. This should provide data that can be simply correlated with fluctuations in sea surface temperature, and thus easily related to the abundance of planktonic prey. Multi-sea winter (MSW) fish are also of interest and are of especial conservation value because they are large and particularly prized by anglers. However, the expectation is that any effect of SST variation will be less clear for MSW fish, due to the longer period spent at sea perhaps occluding the effect of 'bad years' in terms of ocean surface temperature and food availability. This will make an interesting comparison point with the 1SW fish. However, MSW fish are returning in decreasing numbers (ICES 2012).

Whole lifecycle analysis

To clearly understand the pressures of the life of Atlantic salmon a whole lifecycle study of a wild population would need to be completed. One constraint in this thesis is the use of separate populations to study fish at different life stages (Chapters 3 and 4). Work has been performed on herring over the whole spawning period (van Damme *et al.* 2009) and has shown that poor body condition led to the 'down-regulating' of fecundity.

Some studies have been performed on captive fish, but this may not be extended in the case of Atlantic salmon because wild and farmed fish are so different. The cost of performing a whole lifecycle analysis on wild fish would be expensive, but it could help explain much of the variation this thesis found in fish.

The declines seen in the Atlantic salmon populations, in both size and condition are of concern to fishery managers and conservationists. However, there are many different areas that still need to be studied which will inform understanding of this problem and perhaps be a guide to achievable and sustainable solutions.

Summary

This thesis has demonstrated that lipids and triacylglycerides can be used as metrics of fish condition. The changing levels of fats in wild Atlantic salmon indicate the effects of environmental change and the strong response of salmon to declining conditions. The use of different body tissues (red muscle, white muscle, adipose tissue, liver, eggs) indicates the complexity in testing the lipid variations in fish and the importance of examining all body tissues for change.

Careful consideration in selection of the correct metric to assess fish quality is required in Atlantic salmon. Whilst morphometric condition factors do have some descriptive power, the use of physiological measures, like lipid content, can tell us much more about an individual fish's quality and thus its chance for survival. The use of physiological metrics is necessary to calibrate morphometric measures, however, a careful balance of the cost, time and quality of testing possible needs to be considered. The destructive nature of testing for real lipid contents makes physiological metrics less acceptable for widespread use.

However, this author would recommend careful consideration of the questions asked before apply just morphometric indicators alone.

Future work will help drive this knowledge further, and add a temporal aspect to these lipids relationships, as we see with condition factor. It is hoped that this work can help bridge the gap between quantitative and qualitative studies and give a true indication of the state of wild Atlantic salmon populations.

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Appendix 1

A comparison of the eggs of pre-spawning and post-spawning Atlantic salmon

Introduction

Previous chapters have discussed the potential of Atlantic salmon to spawn repeatedly (they are iteroparous). It is clear from Chapter 3 that post spawning fish have markedly reduced lipid contents compared to those caught in coastal waters at the completion of their marine migration, but prior to river re-entry. However this raises the unanswerable question of whether the fish have had their lipids drained by the energetically costly process of spawning or if they never achieved high lipid levels originally. Berg *et al.* indicate that eggs produced by Atlantic salmon vary with maternal size (condition), with higher condition fish producing larger eggs (Berg *et al.* 2001). However, other work suggests that provisioning of eggs does not always vary with maternal condition, for example the size of sockeye salmon eggs can be independent of maternal effects (Tierney *et al.* 2009).

Egg size and its relationship with maternal effect have been widely studied in salmonids (Berg *et al.* 2001; Einum & Fleming 2004; Forrest 2010; Mylonas *et al.* 2010; Burton *et al.* 2012). However, little attention has been given specifically to the maternal lipid investment in offspring. At reproduction, past studies have indicated that the ovaries of Atlantic salmon contain ~30% of the total energy content of the fish (Jonsson *et al.* 1997), however this must be influenced by the condition of the fish. Previous chapters have illustrated that fish of poor condition have less lipid in their body tissue, thus it seem likely that this will effect their spawning effort.

Salmon can mature at different sea-ages and this seems to be driven within populations to increase the chances of survival and reproductive success (Klemetsen *et al.* 2003). The maturation of female salmon can be distinguished into two periods; the production and growth of gametes, (i.e. vitellogenesis); and the maturation of gametes, egg maturation (Mylonas *et al.* 2010).

Atlantic salmon do not provide parental investment in their offspring post-spawning. The parental care of salmon is delivered in terms of preparation of the redd and the provisioning of eggs with resources to give offspring the greatest chance of survival, hence enhancing a salmon's own reproductive success. Studies indicate that there are optimal levels of nutrients to be provided, including lipid, protein and energy content irrespective of egg size (Berg *et al.* 2001). These provisions affect juvenile success; with fish hatching from large (and thus assumed to be better provisioned) eggs having a higher survival (Hutchings 1991).

Variation in eggs has been suggested to be driven by a number of factors; diameter, mass, protein, fat and energy reserves are all thought to drive offspring survival (Berg *et al.* 2001) . However, most of the variation seen is offspring is linked to parental success so it is likely that there is an effect of condition (Berg *et al.* 2001).

A review of many fish species suggest that there should be an optimum size for eggs, and that if there is no maternal effect on egg size all eggs should meet this optimum (Einum & Fleming 2004). Intra-population variation in egg size was found in salmonids thus variation could occur if the maternal phenotype can influence offspring environment. However, egg size is thought to be under strong stabilising selection compared with body length and fecundity (Einum & Fleming 2004).

Data for sockeye salmon (*Oncorhynchus nerka*) show evidence of a trade-off in reproduction (Tierney *et al.* 2009). Individual salmonids show variation in their reproductive allocation; some produce many eggs whereas others produce less, but better provisioned eggs to maximise reproductive success. The capacity for a female to be able to adjust the number eggs produced will be affected by the environmental conditions that drive salmon success e.g. their freshwater habitat (Armstrong & Nislow 2006).

The analyses in this Appendix investigate the possible effects of this decline in parental somatic condition over the spawning process and how lipid composition of females changes with spawning. Chapters 3 and 4 focused on the comparison between Atlantic salmon that have and have not spawned. The present chapter considers the level of maternal investment by Atlantic salmon in terms of egg lipid concentrations and how this is related to maternal quality. It is likely that in populations declining in condition, fish will either produce fewer eggs or provision eggs with less lipid at maturation.

Methods

Estimating fish fecundity

Whole ovary lipid extraction

In 2008 the ovaries for 18 pre-spawning female salmon were dissected intact and subject to lipid analysis. Lipid extraction was performed using the Bligh and Dyer method (1957) as discussion in Chapter 2.

Egg sampling

The eggs were sampled in batches of six from the post-spawning fish, to reduce the effect of within fish variation (Berg *et al.* 2001). The eggs were measured for both weight and diameter, with callipers. The egg samples studied were the prepared by freeze-drying of the samples and then extracting lipids to remove the influence of the water-lipid relationship (Kennedy *et al.* 2010).

Fecundity of pre-spawning fish

Fecundity counts of pre-spawning fish were produced by J. Forrest for a master's dissertation (Forrest 2010). This work uses the raw values calculated for fish from 2008 and 2010. The fish selected were those that had also been studied for lipid content within this thesis. 30 fish from 2008 and 10 fish from 2010 were analysed.

Both ovaries were removed intact from the fish and either immediately sampled or stored in a -80°C freezer until sampling. The method used to separate the eggs from the ovary was to incubate the ovary in hot water, and by so doing denature the proteins and harden the eggs (Forrest 2010).

The egg count to estimate fecundity was sampled gravimetrically. The two ovaries were weighed, and then two sub-samples (from each ovary) were separated from the centre of the ovary. These samples were weighed and then the eggs within them counted. The counts from the sub-samples then were used to predict the whole ovary egg count, and total counts from the two ovaries were added together to provide an estimate of fecundity for the fish (Forrest 2010).

Fecundity of post-spawning fish

Fecundity counts of post-spawning fish were calculated by T. Burton (Burton *et al.* 2012) for all 28 post-spawning fish studied in Chapter 3 and 4. The present work uses the raw values of fecundity calculated for the post-spawning fish.

The egg count was ascertained by calculating clutch mass by first recording body mass of the salmon, then stripping it of its eggs and draining the fish of ovarian fluid, before reweighing the fish. Clutch mass is the difference between these weights. Burton et al. (2012) then selected ~10 g of eggs from each ovary, and these were then preserved with 5% buffered formalin. Fecundity (number of eggs) was calculated by counting the number of eggs in each preserved sample and extrapolating this value to the total clutch mass of each fish (Burton *et al.* 2012).

Egg lipid extraction and TAG analysis

The Atlantic salmon used in this chapter were taken from two locations. Firstly as adult one-sea-winter (1SW) maiden (hereafter "pre-spawning") used in the previous chapters (2, 3, 4) from coastal trap nets at Melvich and Armadale, Scotland, UK. Secondly as manually stripped mature (hereafter "post-spawning") returning 1SW female salmon used in previous chapters (3, 4) trapped on the River Blackwater (the River Conon), Ross-shire, Scotland, UK, (Burton *et al.* 2012).

The eggs were sampled in batches of six from the post-spawning fish, to reduce the effect of within fish variation. The eggs were measured for both weight and diameter with callipers to the nearest 0.01 mm. The egg samples studied were the prepared by freeze drying the samples and then extracting lipids to remove the influence of the water-lipid relationship.

Lipids were taken extracted from the tissues following the method outlined in Chapter 2. All extractions were performed on 6 eggs per fish, each egg was extracted individually. TAG analysis was also performed on the eggs as per Chapter 4.

Estimating lipid in the ovaries

The total lipid in the ovaries of post-spawning fish, prior to their being stripped, was estimated by multiplying the mean egg lipid content of a fish by egg total as estimated at stripping.

Statistical Modelling

All lipid egg contents were expressed in relation to the extracted tissue dry weight. The miniaturised extraction technique resulted in some variation in percent lipid extracted amongst triplicates. Assessment of statistical outliers was by inspection of the coefficient of variation (CV) amongst the six eggs; eggs with a CV >30 were deleted from the present analyses and if more than one egg had a CV >30 the fish was removed from the study.

The variation in total lipid concentrations and TAG concentrations between pre- and post-spawning fish for separate tissues (red muscle, white muscle, dorsal adipose) taken from Chapter 3 were analysed with the egg lipid and TAG

concentrations. This was done using a generalised additive model (GAM), compared with the length of the fish. The W_R condition factor (Chapter 2) could not be applied to post-spawning fish as the fish studied were not part of the analysis to calculate this, and the spawning process had compromised the fish so all would produce low condition factors.

Separate models were used to test each tissue to consider the effect of spawning on the relative lipid and TAG concentrations of the various tissues. In each model the fish length, weight, fecundity and total tissue weight were modelled as smooth, non-parametric functions. The year-class, sex, river age and fish status ("pre-spawning", "post-spawning") also were included in the model as parametric factors. The quasi-binomial error structures were used due to the limitations of the response variable (total lipid) – concentrations of which could range only between 0 and 1 – and to control for over-dispersion (the range of proportions, although varying, fits into only a limited range). Gamma was increased to 1.4 to control for over-fitting within the model. The model was simplified to find the most parsimonious setup, and the validity and utility of this model tested using the General Cross Validation (GCV) method.

Results

The egg counts of pre- and post-spawning 1SW fish were found to be significantly different (t=-11.510, p<0.001) (Fig. A.1, Table A.1). Fish egg count showed no significant effect of length (being used as a proxy for condition factor), implying that better condition fish do not contain more eggs. Despite the significant difference seen in egg count, the analysed pre- and post-spawning fish were from two different sources and fecundity was measured by different methodologies. However, there is a very large difference that cannot be explained by this alone.

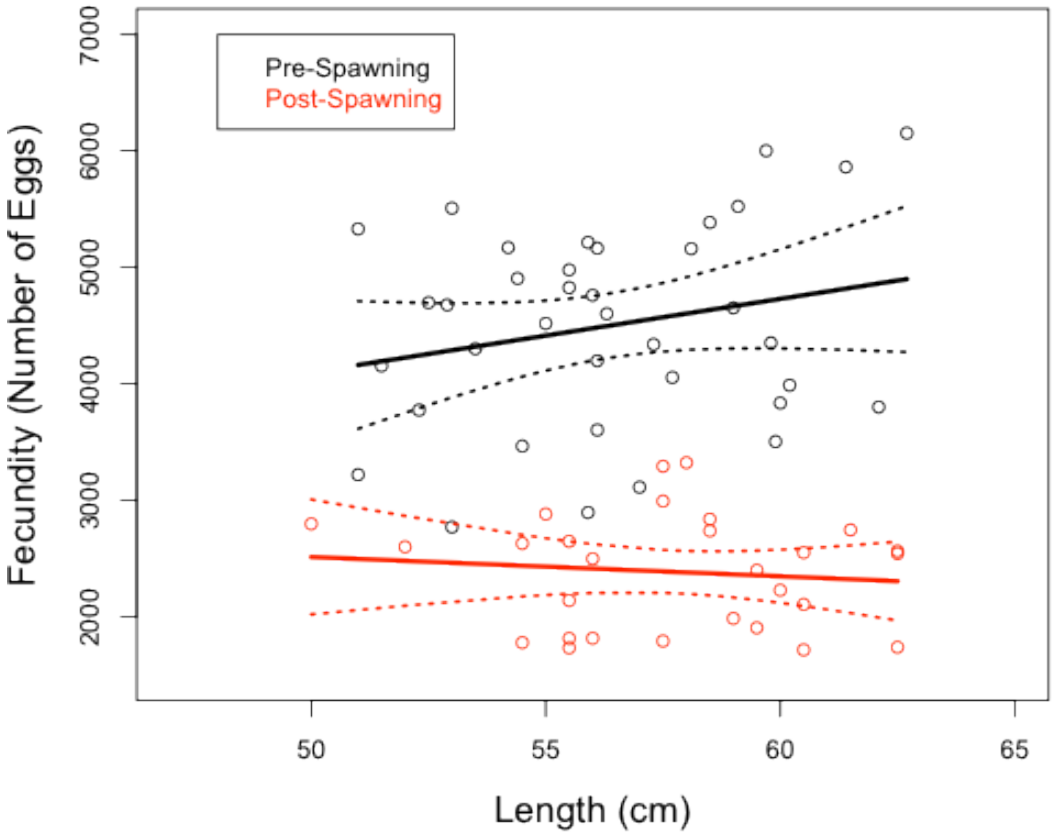

Figure A.1: Egg counts of pre- and post-spawning fish plotted against the fish length (cm). The linear model predictions are drawn (solid line), 95% confidence intervals (dotted lines), demonstrating the relationship in pre- and post-spawning fish. Individual data points are shown coloured by spawning state.

The total lipid concentration of an individual egg did not vary with length, but was significantly different in the pre- and post spawning fish (see Fig. A.2B, Table A.1). When including the results from Chapter 3, this highlights a mobilisation of lipid from the red muscle and adipose tissue into the ovary (Fig. A.2A).

	Ovary (Egg)			
	Spawned		**Length (cm)**	
	t-value	p-value	F-value	p-value
Fecundity	-11.510	p<0.001*	0.997	0.323
Tissue Lipid %	7.551	p<0.001*	2.099	0.078
Tissue Lipid (g)	9.483	p<0.001*	1.963	0.003*
TAG %	-	-	0.870	0.462

Table A.1: Model terms from the GAM performed between egg count; tissue lipid concentration (%); total tissue lipid weight (g); TAG concentration and fish length (cm) in pre- and post-spawning fish, in the ovaries. The F statistic and p-values are given for non-parametric smoothed terms and t value and p-values for parametric terms. Those terms that are significant are annotated "*".

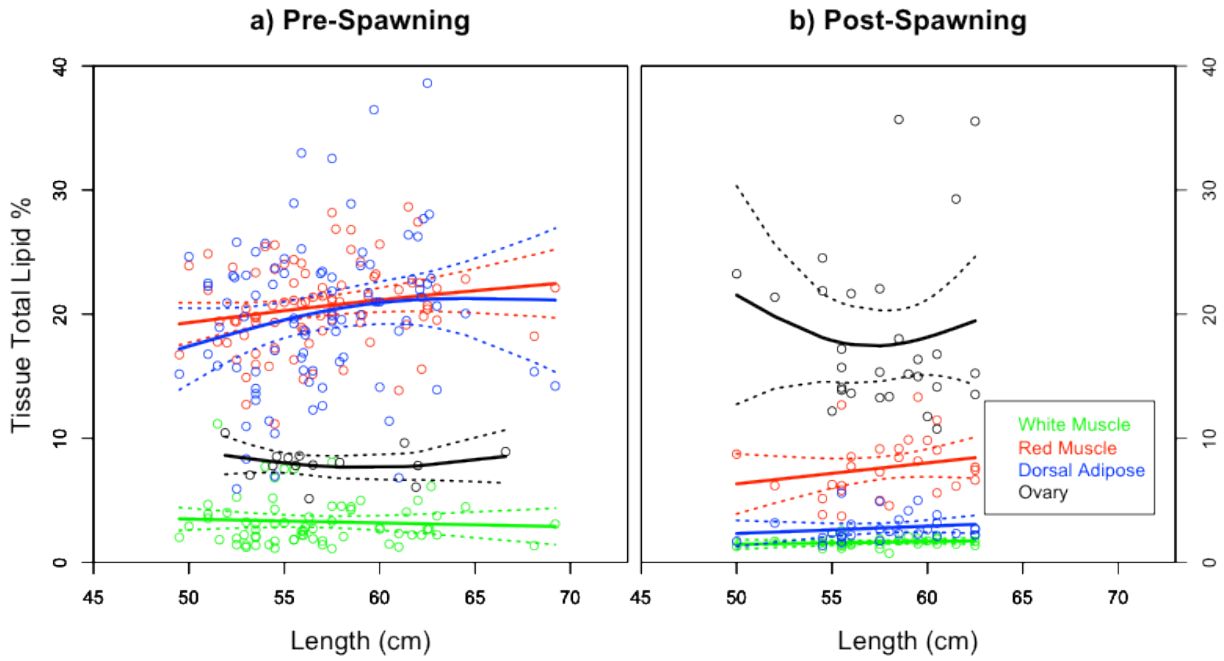

Figure A.2: Length of pre- (a) and post- (b) spawning fish plotted against Concentration of total lipid in the tissue (%). The GAM model prediction drawn (solid lines), 95% confidence intervals (dotted lines), demonstrate the relationship in pre- and post-spawning fish. Individual data points are shown coloured by tissue, as indicated in the legend.

The total lipid weight of the ovaries varied with fish length and showed a significant change between the pre- and post spawning fish (see Fig. A.3B, Table A.1). When compared with the results from Chapter 4, this highlights a mobilisation of lipid from the red muscle and white muscle into the ovary (Fig. A.3A). No significant effect of lipid weight or lipid concentration of eggs was found on egg size.

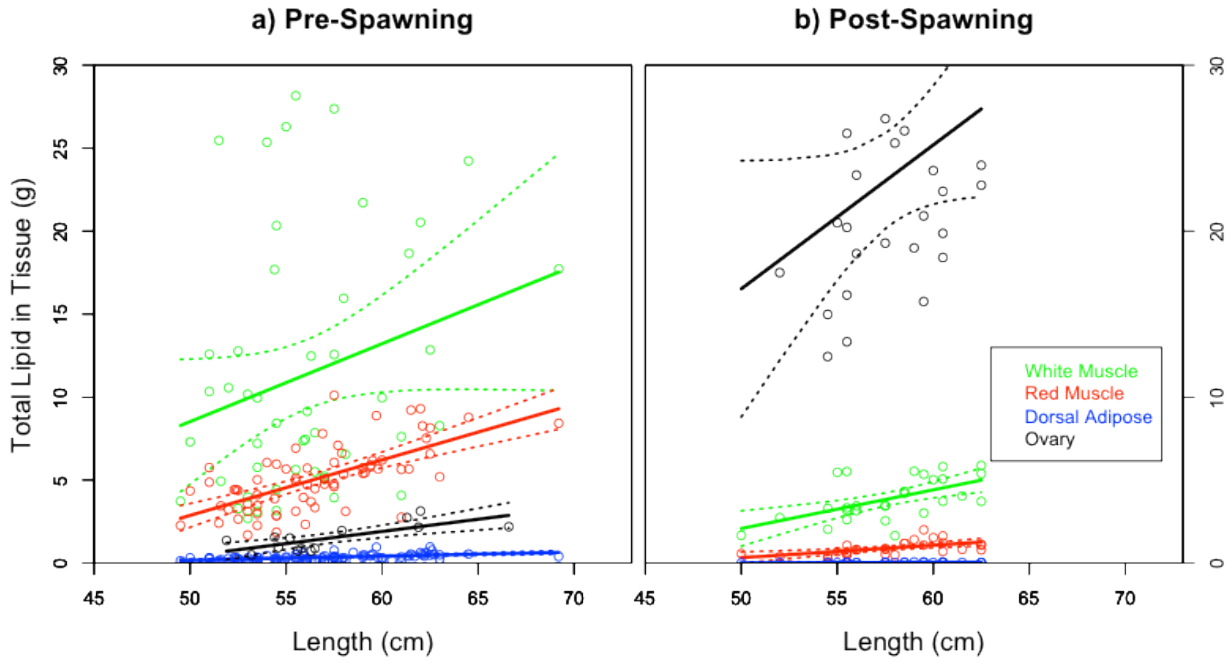

Figure A.3: Length of pre- (a) and post- (b) spawning fish plotted against weight of total lipid in the tissue (g). The GAM model prediction drawn (solid lines), 95% confidence intervals (dotted lines), demonstrate the relationship in pre- and post-spawning fish. Individual data points are shown coloured by tissue, as indicated in the legend.

There were very low levels of TAG measured in the eggs of the post-spawning fish and this did not vary significantly with fish length (Fig. A.4B, Table A.1). This is important because it indicates the high levels of lipid found in the eggs post-spawning are not comprised of triacylglycerides.

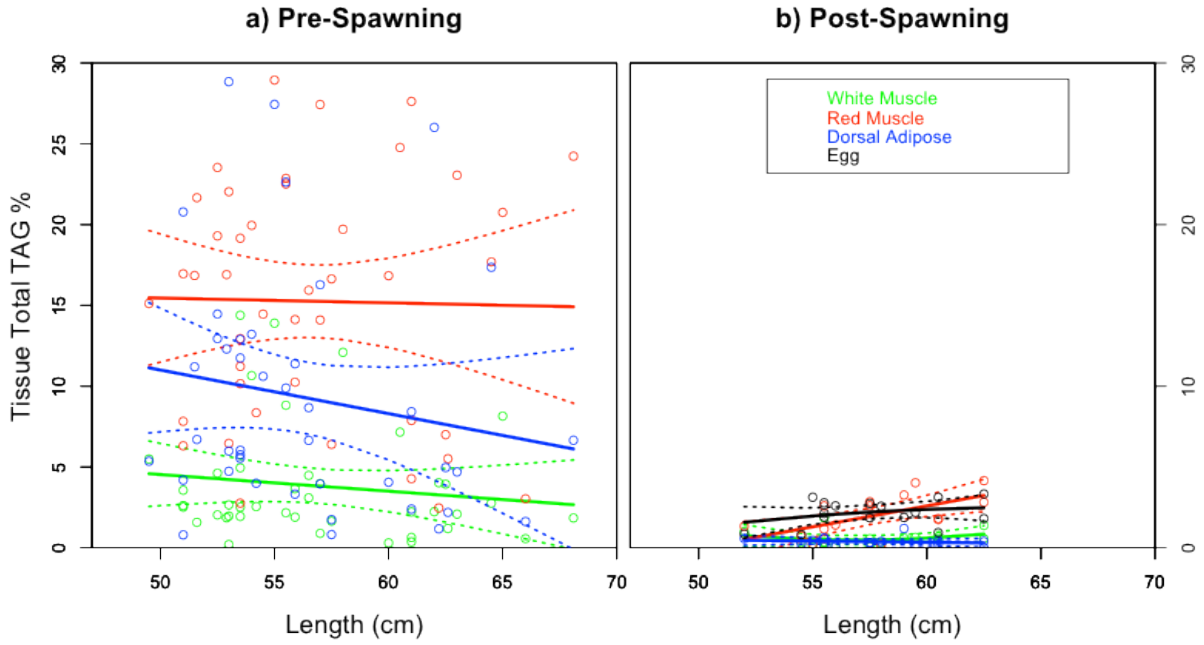

Figure A.4: Length of pre- (a) and post- (b) spawning fish plotted against weight of total tissue TAG concentration. The GAM model prediction drawn (solid lines), 95% confidence intervals (dotted lines), demonstrate the relationship in pre- and post-spawning fish. Individual data points are shown coloured by tissue, as indicated in the legend.

Discussion

Previous work considering the relationship between characteristics of egg and maternal condition has found that fecundity (number of eggs) decreases with maternal condition (Fleming *et al.* 1997; Berg *et al.* 2001; Burton *et al.* 2012). This Chapter clearly demonstrates that after spawning, some of the lipid lost from the body tissue of Atlantic salmon can be found in the eggs produced, showing that female salmon provision their eggs with body lipid. It can be shown here that:

1. Atlantic salmon eggs hold significantly more lipid after spawning maturation
2. Post-spawning there is a higher lipid concentration in the eggs than body tissues
3. Atlantic salmon eggs contain very low concentrations of TAGs
4. Longer salmon have higher lipid concentrations in their eggs

Maternal investment

Previous work on Atlantic salmon has consistently found an effect of fish size on the size of eggs produced by the female fish (Jonsson & Jonsson 1999; Berg *et al.* 2001). Such a pattern extends also to the close relative of the Atlantic salmon, the brown trout (*Salmo trutta*); an effect of maternal condition on the size, number and quality of eggs ultimately produced at spawning is, perhaps, to be expected – and has been reported (Hutchings 1991; Einum & Fleming 2000b; Berg *et al.* 2001) – because this is the only maternal care provided to salmonid offspring. The chapter shows that provisioning of the eggs occurs during the spawning migration, however, it does not find an effect on egg size.

Much of the previous work investigating maternal effect relies upon fecundity to quantify maternal provisioning. This demonstrates that fish size (a proxy for condition) has no effect either on fecundity or lipid provisioning (Forrest 2010). Eggs are consistently provisioned with statistically similar concentrations of lipids (and TAGs). However, when considering the total lipid contained within all of the eggs produced, there is an effect on body size, as reported in other studies (Jonsson & Jonsson 1999; Einum & Fleming 2000a; Berg *et al.* 2001). This chapter shows that longer females have a greater mass of lipid in their ovaries.

Very low levels of triacylglycerides have been identified in the eggs of salmon after spawning. This indicates that eggs are provisioned with other lipid classes in preference to TAG, which probably has been metabolically consumed to support the upstream spawning migration (Chapter 4). Previous work has suggested that TAGs are important in influencing juvenile survival (Næsje *et al.* 2006) and growth (Litvin *et al.* 2011), implying young fish are reliant on their diet for this source of nutrients.

Potential for iteroparity

Work on sockeye salmon (*Oncorhynchus nerka*) (Tierney *et al.* 2009) indicates that maternal investment is affected by iteroparity, in that there is a trade-off in producing more or better quality eggs, but found that larger sockeye salmon produce more offspring. Tierney et al. (2009) found that the migration process can be so costly that not all fish are able to spawn at its completion. Because Atlantic salmon also potentially are iteroparous, a similar trade-off may be applicable also to this species.

Armstrong and Nislow (2006) assumed that the female Atlantic salmon benefit by providing a large lipid supply to its offspring, but females also can improve their reproductive success relative to other females by producing more offspring. There is a trade-off between the two (Armstrong & Nislow 2006). In brown

trout it has been suggested that environmental factors may drive the number and quality of eggs produced (Acolas *et al.* 2008), environmental variation can be costly.

It has been suggested that female Atlantic salmon of poor body condition may respond by adjusting the size and number of eggs they produce (Jonsson *et al.* 1996; Burton *et al.* 2012). The egg counts and lipid concentrations recorded in this chapter seem to support this hypothesis, rather than early life conditions driving the number of eggs produced as has been suggested for cichlids (Taborsky *et al.* 2007). This is supported also by evidence from herring (*Clupea harengus*), which indicates that female fish subjected to nutritional stress are able to reabsorb their oocytes and effectively retrieve some of the resources invested in their development oocytes (van Damme *et al.* 2009; Kennedy *et al.* 2010).

The results of this chapter indicate that the Atlantic salmon studied are producing the maximum number of eggs possible. Juvenile success of salmon has been shown to be driven by egg size (and thus lipid concentration) (Hutchings 1991; Einum & Fleming 2000a) (Hutchings 1991; Einum & Fleming 2000b), and if these eggs are being provided only with the available lipids, not only are fewer offspring likely to be produced, but the juvenile alevins hatching will start river life in a poor condition. Large eggs have a better chance of survival (Berg *et al.* 2001).

Conclusions

This work demonstrate that lipid content and thus condition of female Atlantic salmon is important in driving offspring provisioning in terms of egg lipid concentration. Work collected in support of this indicates that there is a dramatic decline in egg numbers of Atlantic salmon during the spawning migration

(Forrest 2010; Burton *et al.* 2012), but that the eggs are also provided with low levels of lipid.

To draw any further conclusions from this a study would need to be performed on a range of the full life cycle of an Atlantic salmon population, to reduce any population bias from the results. However, this study clearly indicates that environmental experiences of salmon during their spawning migration affect fecundity. Maternal condition will influence reproductive success.

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