Environmental Influences on Muscle Growth and Flesh Quality in Farmed Atlantic Salmon (*Salmo salar* L.)

Submitted for the degree of Doctor of Philosophy to the University of St. Andrews by,

Christopher Beattie

School of Environmental and Evolutionary Biology

Gatty Marine Laboratory

University of St. Andrews

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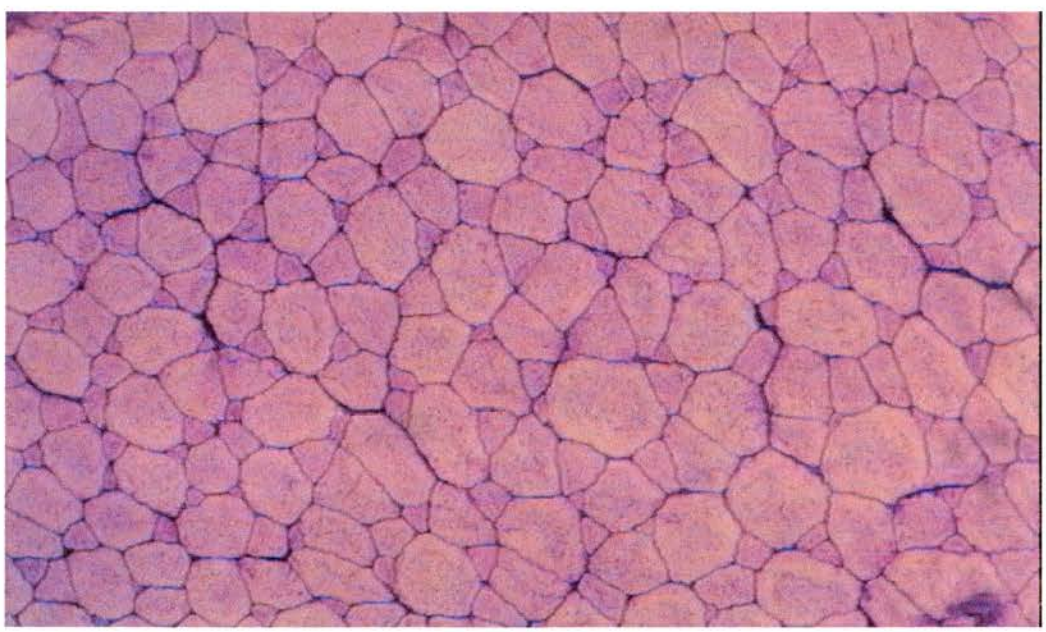
'The capacity of an organ to grow to meet it's physiological responsibilities depends mostly on it's ability to multiply the functional units of what it is composed, regardless of whether they are represented by organelles, by cells or by tissues' (Goss 1966).



Muscle section taken from a pre-smolt Atlantic salmon (*Salmo salar* L.)

Declaration

I hereby declare that the research represented in this thesis was carried out by me and that this thesis is my own composition. No part of this work has previously been submitted for a higher degree. The research was conducted at the Gatty Marine Laboratory, School of Biological Sciences, University of St. Andrews, under the supervision of Professor I.A. Johnston and at Matre Aquaculture Research Station, N-5198 Matredal, Norway under the supervision of Tom Hansen

Signed

Date 15/5/00

Certificate

I hereby certify that Christopher Beattie has spent eleven terms engaged in research under my direction and that he has fulfilled the conditions of General Ordinance No. 12 (Resolution of the University Courts No. 1, 1967), and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

Signed

Date 15/5/00

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SUMMARY

Chapter 1

General Introduction

The general introduction sets out the rationale for the study and it's applicability to aquaculture. A description of the life history of the Atlantic salmon (*Salmo salar* L.) is given, together with a description of the origin and current status of fish farming. The structure and function of fish muscle is explained in addition to its growth, development and inherent plasticity. Finally, the environmental influences on muscle growth in fish are discussed and this is followed by the aims of the thesis.

Chapter 2

Egg incubation temperature influences muscle fibre recruitment during seawater stages of Atlantic salmon (Salmo salar L.)

Eggs of Atlantic salmon (*Salmo salar* L.) were incubated in either heated or ambient water temperatures. The comparative whole animal and muscle growth performance of the two groups was monitored for 26 months. The number of white muscle fibres per myotome was significantly higher at hatch in the ambient group, resulting in a greater muscle cross sectional area. However, muscle fibre number was almost four-fold higher in the heated group at the S1 parr stage relative to the ambient group. Six months following seawater transfer the number of fast white muscle fibres was not significantly different between groups. The 5^{th} and 10^{th} percentile fibre diameters decreased significantly in both groups during winter and spring, indicating a relative

increase in the contribution of new fibre recruitment which was correlated with seasonal lows in water temperature and day-length. The results indicate that egg incubation temperature has persistent effects on muscle cellularity throughout the freshwater and early seawater stages of Atlantic salmon.

Chapter 3

Advanced photoperiod treatment affects smoltification and muscle growth in Atlantic salmon (Salmo salar L.)

Juvenile Atlantic salmon (*Salmo salar* L.) were reared under three different photoperiod regimes: constant light (CL), simulated short winter (SW) and simulated long winter (LW) from July to December 1997. Muscle fibre frequency analysis suggested increased contribution of muscle hypertrophy towards somatic growth in all groups during summer and autumn while increased fibre recruitment was found in the CL and SW groups during winter. The number of white muscle fibres per myotome was linearly related to total white muscle cross-sectional area with no significant differences between groups. This indicates that the SW treatment increased muscle growth but that the relative contribution of hypertrophy to fibre recruitment was not affected by photoperiod treatment at this stage in the life cycle.

Chapter 4

Cage systems and light manipulation influence muscle fibre cellularity and harvest quality of underyearling (0+) Atlantic salmon in seawater

Underyearling (0+) Atlantic salmon (*Salmo salar* L.) were reared in conventional sea cages and closed pens systems under natural light and additional continuous light for 14

months up to harvest. The comparative muscle cellularity of all groups was assessed in November 1995, together with harvest quality parameters including texture (hardness), percentage lipid and fillet colour. Muscle fibre frequency analysis revealed increased variation in fibre frequency in fish reared under constant light regimes. Two-way analysis of variance showed no significant differences in the 5^{th}, 10^{th} or 50^{th} percentiles between treatment, however a significant effect of light treatment was found on the 95^{th} percentile diameter value. Muscle fibre density (fibres/mm^2) was not significantly different between treatments although a within-fish comparison of the steak-section revealed significantly higher fibre densities in the dorsal compared to the lateral epaxial area. Lipid was positively correlated to body mass and regression analysis revealed no significant relationship between muscle fibre density and fillet hardness as measured by texture profile analysis or fillet colour measured by Roche colour card.

Chapter 5

Instrumental measurements of texture and it's relation to muscle fibre density in Atlantic salmon (Salmo salar L.)

Atlantic salmon (*Salmo salar* L.), 1-5kg (pre-gutted body mass) were subjected to a series of standard flesh quality tests along the length of the fillet and the results related to muscle fibre density. The NQC cut had a firmer texture than the Mowi. The NQC cut was also found to be significantly leaner with a lower % lipid than the Mowi region. Significant differences in Hunterlab® colour score were also found with the NQC region having significantly higher readings than those in the Mowi region. Muscle fibre density (MFD) (no. muscle fibres/mm^2 muscle cross-sectional area) was found to be significantly

higher in the NQC region than the MOWI region. A weak correlation was found between body mass and MFD in the Mowi but not in the NQC region. Muscle fibre density did however, show a positive correlation with maximum shear force. The results indicate that muscle fibre density influences texture in Atlantic salmon. The potential for the manipulation of muscle cellularity and hence flesh quality are briefly discussed.

Chapter 6

General Discussion

The major findings of the thesis are discussed in relation to previous work in addition to possible implications for salmon aquaculture and the scope for future study is evaluated.

Chapter 1

GENERAL INTRODUCTION

The vast majority of farmed Atlantic salmon (*Salmo salar* L.) are reared in fresh water tanks and ongrown in net cages in sea water. In these tanks and cages, salmon are exposed to a complex array of natural and artificial stimuli, which are not normally experienced in the wild (Juell 1995). In order to maximise production, fish are subject to manipulations in water temperature, photoperiod, salinity, oxygen levels and feed intake in addition to being reared in a restricted environment depriving them of the opportunity to carry out both feeding and spawning migrations.

Salmon farming has been in existence for a little over 30 years (Monahan, 1997) and salmon should still be regarded substantially as an undomesticated species, whose physiological and behavioural traits have evolved to maximise lifetime reproductive success in a natural environment (Adams and Thorpe, 1989; Huntingford and Thorpe, 1992). The imposition of widely varying environmental conditions on the salmon may have profound biological, ethical and economical implications for this relatively young industry. Indeed, Vøllestad and Hindar (1997) have described the salmon hatchery environment as developmentally 'hostile', inducing greater fluctuating morphological asymmetry than that found in wild fish. This would seem to suggest the presence of environmental or genetic stress during ontogeny, which reduces the efficiency of normal developmental processes (Clarke, 1992).

Many previous studies have focused on the impact of salmon farming practice on whole body growth of Atlantic salmon (for review see Shearer 1994), few have, however, delved further into the development of the main body tissue, the white muscle after the initial hatch stage (Totland *et al.,* 1987; Johnston *et al.,* 1999, 2000a,b). The main edible part of the salmon is composed of fast twitch muscle fibres that power high speed swimming using anaerobic pathways (for review see Bone, 1978; Johnston, 1981, 1994; Weatherley and Gill, 1987). White muscle in fish is extremely plastic in its development (Johnston, 1993) and can be influenced by a number of environmental variables commonly used in salmon farming to manipulate growth and development. Environmental influences have been shown to have a profound impact on the growth and development of fishes (Brett, 1979). This introduction will focus on muscle development in fish, taken into context with the Atlantic salmon's life cycle, the influence of salmon farming practice and is followed by a discussion on the manipulation of final product quality.

1.1 *Teleost fish*

1.1.1 *An introduction*

Fish are the most abundant and yet least known class of vertebrates on earth. Around 20,000 species are recognised at present (Bone and Marshall, 1982), and it is likely that the final total will show three in every five vertebrates to be a fish. The majority of fish species are teleosts and the remainder comprises some 50 species of jawless fish and about 800 species of cartilagenous fishes.

1.1.2 Geographical distribution

This huge array of species is irregularly distributed – no less than four out of ten fish species live in freshwater, although fresh waters make up only 0.0093% of the total water on earth. Cohen (1970) has estimated that 33% of all fishes belong to primary freshwater species, mainly carps, characins and catfishes. Half the marine species known are from the warm waters fringing the land, particularly where corals are most productive. Fishes have, however, colonised all marine environments and developed remarkable adaptations to enable them to make a living at the bottom of the deepest oceans as well as around the shores. All familiar teleosts, except herring and eels, belong to the five euteleost super-orders (see Fig 1.1) The first Protacanthopterygii, contains salmon, trout and a few deep sea forms

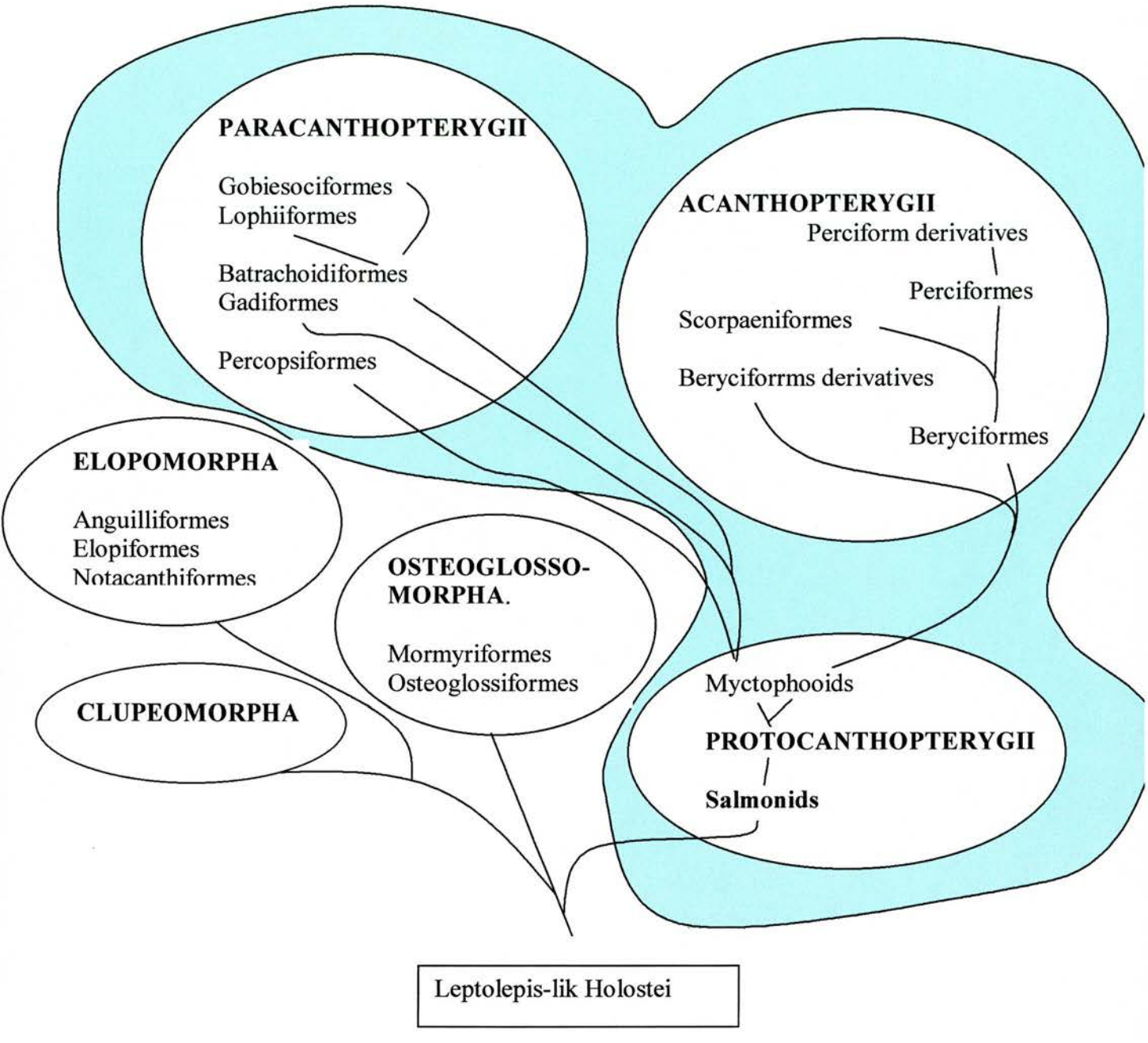


Figure 1.1 Relationships of euteleost groups (within shaded outline) after Bone and Marshall (1982) The position of salmonids are shown within the protocanthopterygii.

1.2 The Atlantic salmon (*Salmo salar* L.)

1.2.1 Classification

The Atlantic salmon (*Salmo salar* L.) (Fig. 1.2) is a member of one of the most primitive super-orders of the teleosts or bony fish, namely the Protacanthopterygii, which includes the salmonids and a few genera of deep-sea fish. The family salmonidae includes the Atlantic and Pacific salmon, the trout and the charr (also classified as the salmonidae), the grayling (Thymallinae) and whitefishes (Coregoninae).

Fig.1.2 The Atlantic salmon (*Salmo salar* L.)

1.2.2 Life cycle

Descriptions of the life cycle of the Atlantic salmon have been made for over two millennia; Pliny the Elder writes of salmon in the Historia Naturalis in the first century A.D. In 1527, Hector Boece gave a correct outline of the life history of the Atlantic salmon, while a sixteenth century priest, Peder Clauscon Friis, surmised that salmon bred

in fresh water, that the young spent a period in the river before migrating to sea and that, once there, they grow rapidly and carried out extensive migrations.

1.2.3 Migratory habits

The Atlantic salmon is an anadromous fish and, with rare exceptions, depends on two distinct environments for the successful fulfillment of its life history. A freshwater environment in which the reproductive and nursery phase of its life cycle can occur and a marine environment for its main feeding phase, during which rapid growth is achieved. Wild Atlantic salmon enter sea water to increase their feeding opportunities and return to fresh water to spawn. Knowledge of their behaviour during these long migrations is sparse and is largely based on indicators from mark-recapture trials, catch statistics and stomach contents analysis. It is assumed that movement at sea is random and influenced by water currents and prey distribution until sexual maturation motivates homing to the natal stream (Thorpe, 1988). Tracking of Atlantic salmon with ultrasonic transmitters have shown that it is a pelagic species, typically swimming in the upper 15m of the water column (Holm *et al.;* 1982, Westerberg, 1982a).

1.2.4 Smoltification

Prior to seaward migration, the stream-dwelling parr undergo a remarkable metamorphosis, the parr-smolt transformation when fresh water juveniles become behaviourally, morphologically, biochemically and physiologically prepared for ocean migration and a marine existence (Hoar, 1988, Wedemeyer *et al.,* 1980). The transformation may be triggered by one or more environmental factors but an increase in

daylength is thought be the most important element controlling the timing of smoltification (Villareal *et al.*, 1988).

1.2.5 Reproduction

Salmon re-enter rivers at all times of the year and spend between two, three or four years at sea before returning to fresh water to spawn. If the salmon has spent only a little over one year at sea before returning to it's natal river, it is termed a 'grilse'. Once in freshwater, the salmon will migrate upstream at varying speeds depending on the time of year, water temperature and speed of flow (Thorpe, 1989; Mills, 1992). By the time spawning commences, salmon will have occupied suitable spawning grounds which consist of silt free gravel in areas extending from the upper reaches of the watershed down to tidal level. On approaching fresh water, the salmon stops feeding and metabolic costs are met by breaking down sequentially fat and then protein tissues (Kiessling *et al.*, 1995).

After spawning, most of the males die but a small proportion of the females return to the sea as spent fish or 'kelt' and may spawn a second time. Following spawning, the salmon uses its tail to pile up gravel over the eggs, creating an underwater nest or 'redd'. In the wild, the eggs hatch in late March or early April and the newly emerged fish or 'alevin' feeds endogenously from an attached yolk sac. When the yolk sac has been fully absorbed, alevins make their way up through the gravel to emerge as exogenously feeding 'fry' four or five weeks later. At the end of their first year of life, the fry are

brownish-green in colour with distinctive finger marking on their flanks and are known as 'parr'.

1.2.6 Plasticity in life cycle

There is considerable plasticity in the life cycle of salmonids (Thorpe, 1989;1997) and the fish may remain as parr until the spring of their second, third or even fourth year or more depending on stream conditions. The progeny of one fish do not necessarily all go to sea in the same year or return at the same time and this phenomenon gives obvious survival advantages to the species. Some fish may remain resident and mature in the natal stream, and are referred to as 'precocious parr' (Thorpe, 1989).

1.3 Salmon Aquaculture

1.3.1 Economic importance of aquaculture

The farming of Atlantic salmon has become an increasingly important area of fish farming or 'Aquaculture'. Aquaculture refers to the rearing of aquatic species under controlled and supervised conditions from the egg to the market (Monahan, 1997). In 1997, aquaculture represented a $48.6 billion industry producing 28.8 million tonnes of fish worldwide and it is estimated that by the end of the millenium, approximately 90% of fish used for human consumption will be reared on dry commercial feed in an aquaculture environment (Åsgard *et al.*, 1995).

1.3.2 Origins of aquaculture

Aquaculture originated in eastern Asia some four thousand year ago. One of the several members of the carp family is the most likely candidate for the first cultivation of fish, which probably started from fishermen storing their surplus catch in baskets or small enclosures in ponds. Records of Chinese aquaculture, the probable source region for the practice, reach back to 1500 BC (Ling, 1977) and it has been suggested that the desire to please royalty may have been a force in the initial domestication of wild fish species.

Salmon farming today is the outgrowth of the trout culture, which developed in Denmark around 1890 and was introduced into southern Norway in 1912. Aquaculture progress in Europe was slow until the expansion of trout farming in seawater which occurred in the 1960s and 1970s. In 1965 A/S Mowi of Norway, started the rearing of salmon in a closed off part of the sea which led to the development of floating pen culture as we know it today.

1.3.3 Environmental requirements of salmon farming

Salmon require high levels of dissolved oxygen in the water (4-11mg/l) (Shepherd and Bromage, 1988) and the flow of water through densely stocked pens must be good to maintain adequate oxygen supplies. Water temperatures above 22°C and below -2 °C are usually lethal for salmon; an optimum range is generally regarded to be 4-15°C. To meet these environmental requirements, salmon farming is limited to locations between the latitudes 40° and 70° in both the northern and southern hemispheres. The distribution of salmon farming as far north as 70° in Europe is due to the presence of the warm Gulf

Stream. The absence of salmon farming in the more northerly coastal areas of eastern North America and eastern Asia is due to the cold Labrador and Kamchatka currents.

1.3.4 Biology of salmon farming

The biological aspects of salmon farming are subdivided into separate stages of the production process. In Fig 1.3, the first four boxes show the biological system including the broodstock, which is the source of eggs, incubation and hatching of the eggs, and the rearing through the smolt stage. Smoltification, the physiological change in the salmon moving from fresh water to salt water is normally carried out within the hatchery. Careful control of the water environment is particularly important during smoltification (Hansen 1998) and salinity is closely monitored. Smolts are transferred to the farms for grow out stage. Here fish are carefully monitored for feed uptake, disease and general well being. Harvest is carried out before sexual maturity, prior to the partitioning of protein from muscle to gonadal development.

1.3.5 The biological justification of aquaculture

Fish farming is a rational and effective form of meat production. The edible portion of the salmon for example contains about 30% of the protein provided in the feed. This compares to approximately 18% for chicken, 10% for pigs and 2% for sheep (Table 1.1). Thus, fish farming is an efficient way of producing high quality, high value protein for human consumption (Åsgard *et al.,* 1995). Farmed salmon is also more efficient than wild cod, from 10kg of capelin, we can obtain 4.6kg of salmon or 2.8kg of salmon fillet.

The same amount of capelin will at best produce 2.0kg of wild cod or 0.7kg of cod fillet (see Fig. 1.4)

Animal	**Salmon**	**Chicken**	**Pig**	**Sheep**
Feed consumption (kg/kg)	1.0	2.5	3.9	33
Gross Energy (%) found in product	27	12	16	0.8
Gross protein (%) found in product	30	18	13	2.1

Table 1.1 Exploitation of protein and energy from feed in the edible portion of salmon, chickens, pigs and sheep. After Åsgard *et al.,* 1995.

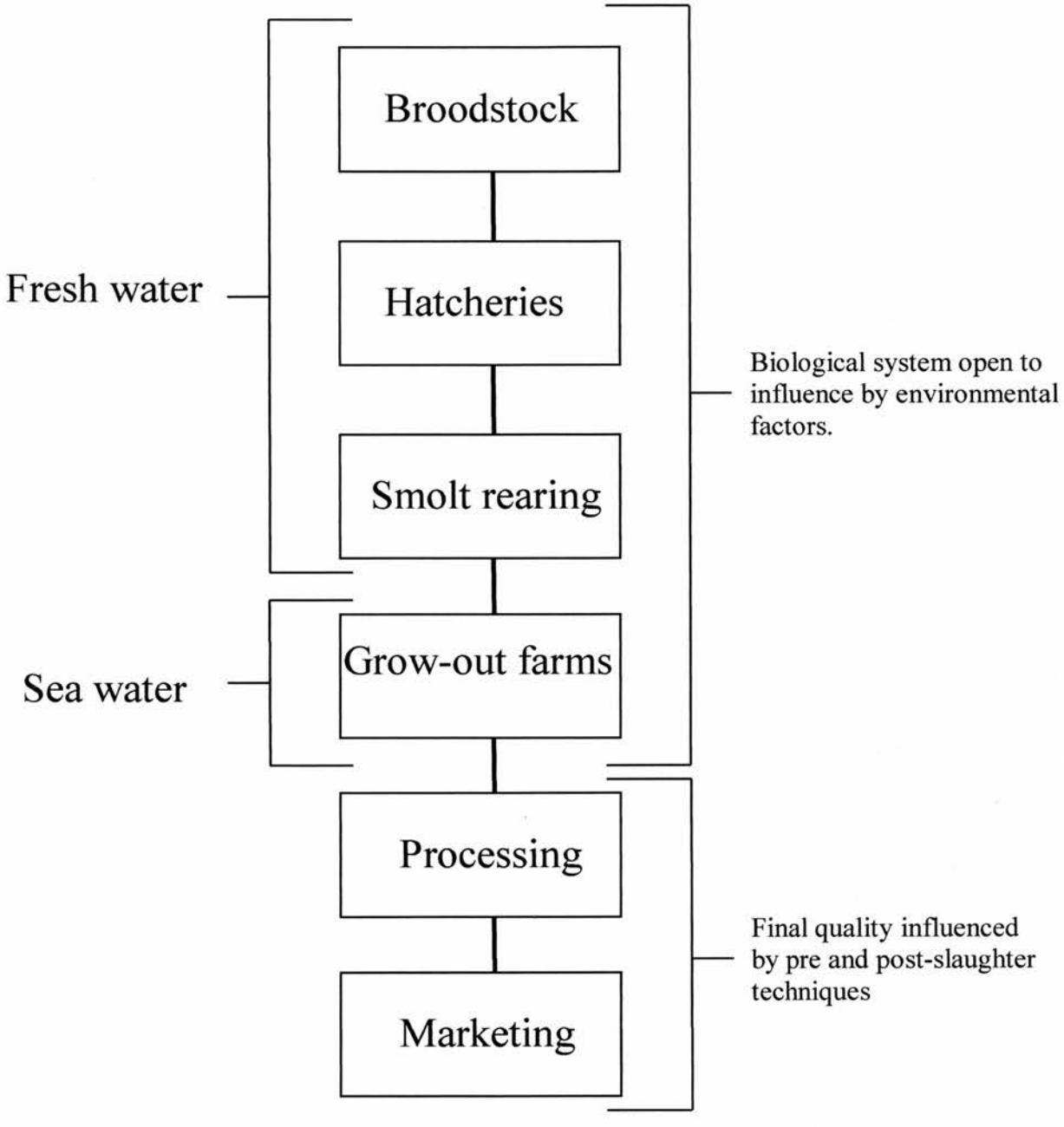


Fig. 1.3 Schematic outline of the salmon farming process showing the biological system open to influence by environmental factors. The current study will examine the influence of environmental factors in the hatchery, smolt-rearing and grow-out farm environments

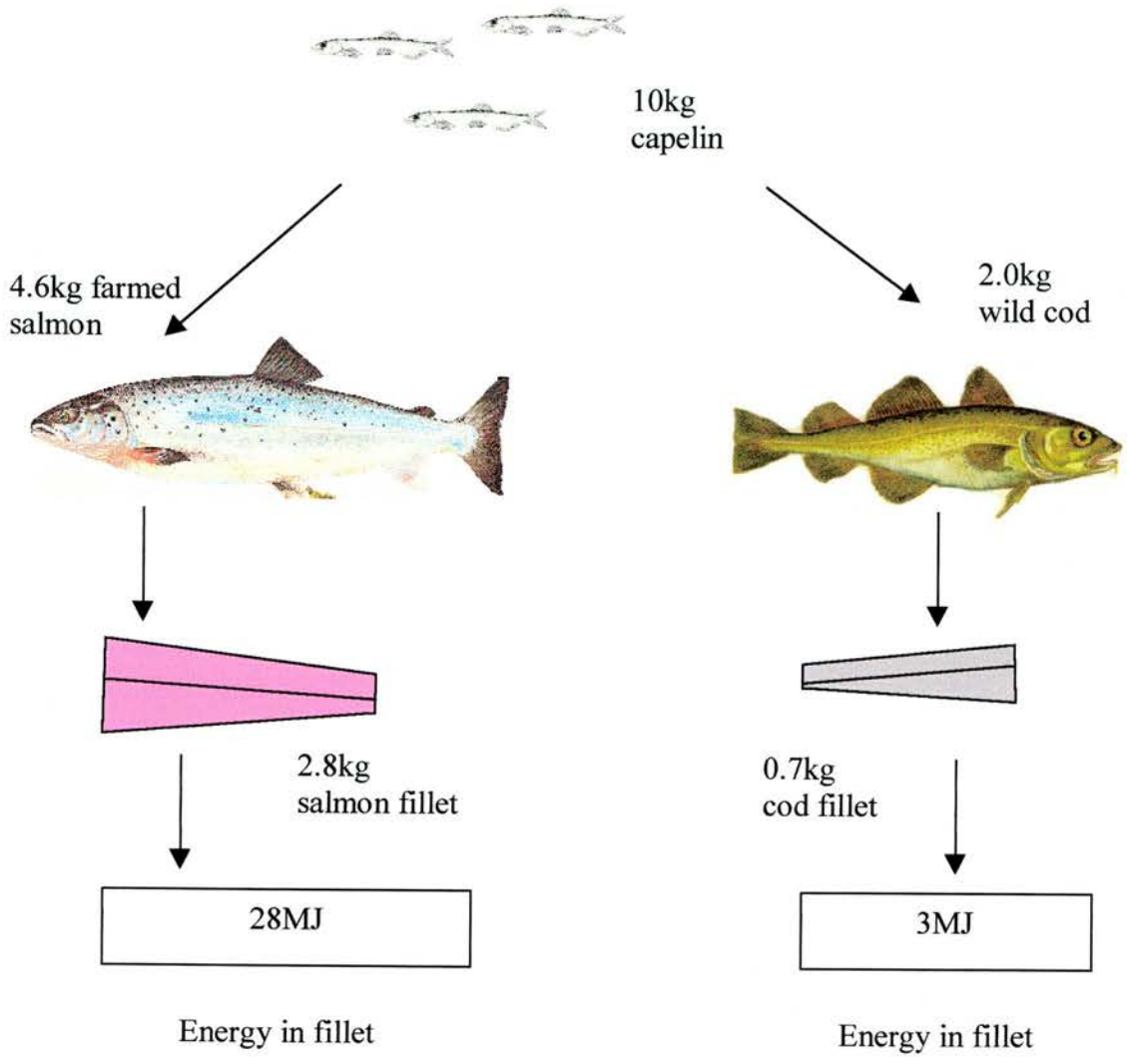


Fig. 1.4 Comparison of dietary resource exploitation in farmed Atlantic salmon (*Salmo salar* L.) and the Atlantic cod (*Gadus morhua*) after Åsgard *et al.*. 1995.

1.4 Aquaculture: the growth of muscle

White muscle tissue accounts for up to 65% of the body weight off the Atlantic salmon and over 90% of the final filleted product (Weatherley *et al.*, 1979). Aquaculture essentially involves the growing of muscle tissue as quickly and efficiently as possible to produce the highest quality product with the greatest profit. The growth mechanisms and plasticity of this tissue are, therefore, of great importance.

1.4.1 Structure of muscle

Fish myotomal muscle shows a number of structural and functional features common to striated vertebrate muscle in general. Striated muscle tissue consists of large numbers of multi-nucleated muscle cells or fibres ranging in diameter from 1 to 300μm, containing myofibrils composed of the thick and thin protein filaments myosin and actin (Huxley, 1953; Hanson and Huxley, 1953; Ebashi and Kodama, 1966a,b). Each myofibril consists of a series of repeating units or 'sarcomeres' (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954). The inter-digitating actin and myosin filaments gives rise to the dark 'A' band and a lighter 'I' band, imparting a striated appearance on the muscle (Keynes and Aidley, 1981; Luther *et al.,* 1996). The thin filaments are attached to the 'Z'-disc, and extend through the 'A' bands. The less dense region between the ends of two sets of thin filaments are termed the 'H' zone and is bisected by the 'm'-band (Fig 1.5). (For review see Ishikawa, 1983; Muntz, 1990).

Associated with the thin actin filaments are two accessory proteins, tropomyosin and troponin, which are involved in mediating calcium regulation and contraction. The

tropomyosin filament lies in the groove of the actin filament helix and is involved in the calcium regulatory system controlling actin and myosin interactions (Schiaffino and Reggiani, 1996). The other major regulatory protein (troponin) is a complex of three polypeptides; troponin-C (Tn-C), troponin-T (Tn-T) and troponin I (Tn-I) (Greaser and Gergely, 1971; Zot and Potter, 1987).

The myofibrils are surrounded by tubules of sarcoplasmic reticulum which make close contact with the in pushings of the sarcolemma. As development proceeds and more myofibrils are synthesized and assembled, they come to occupy the bulk of the cell Individual myofibres are surrounded by a delicate connective tissue sheath, the endomysium and grouped together in fasicles which make up the muscle. The endomysium contains types I, III and IV collagen as well as fibro-nectin, while the basal lamina contains type IV collagen, laminin and heparin sulphide proteoglycan (Kuhl *et al.,* 1986). The fascicles are surrounded by further connective tissue of the perimysium and the epimysium. (Muntz, 1990).

1.4.2 Organisation of muscle

Muscle fibres are packed into blocks or myotomes, which are held between collagen sheets known as myosepts. Salmonids have a selachian muscle arrangement where the pattern of fibres is directed to the top of the myotomal cone (Johnston, 1981) The myotomes are cone shaped and stacked in a metamerical 'w'arrangement on both sides of the medial septa which separates the dorsal epaxial and ventral hypaxial musculature. Within each myotome the muscle fibres run approximately parallel to the

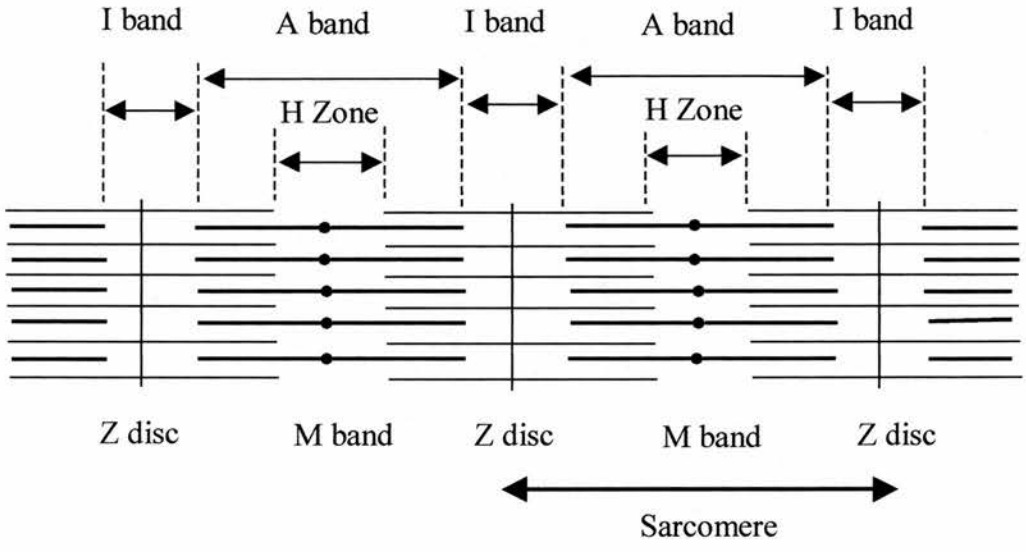


Figure 1.5 Schematic representation of striated muscle (After Huxley, 1953)

long axis of the fish body, although some may deviate by as much a 35° from normal (Alexander, 1969). Most muscle fibres do not attach to skeletal parts but instead to tough sheets of connective tissue, the transverse septa, which separate adjacent myomeres.

1.4.3 Muscle fibre types

The fish muscle system contains two fundamentally different fibres types designated red and white because of their colour (Arloing and Avocat, 1875; Patterson and Goldspink, 1976; Higgins, 1990). This situation is slightly confused in the salmon since the white muscle is coloured pink by carotenoid pigments (Torrissen and Nævdal, 1988). Red muscle is superficial, lying in a relatively thin strip along each flank with it's greatest thickness forming a wedge shape along the outer margin of the major horizontal septum which divides the muscle into dorsal and ventral bands. The red and white fibres differ not only in their location but also in their diameter, ultrastructure and histochemistry (Nag and Nursall 1972; Higgins, 1985). Red muscle is highly vascularised, rich in mitochondria and possesses an enzyme apparatus geared towards aerobic metabolism of primarily, fatty acids (Satchell, 1991). Red muscle utilises aerobic metabolism as an energy source and has high concentrations of the oxygen transport pigment myoglobin. It has low contractile power and is used for prolonged swimming activity using aerobic metabolism (Johnston and Goldspink, 1973a). Fish whose lifestyle demands long periods of continuous swimming, such as mackerel (*Scombrus scombrus*), have relatively higher proportions of red muscle than those who rely more on burst activity such as the cod (*Gadus morhua*) (Greer-Walker and Pull, 1975).

The bulk of salmon muscle consists of fast white fibres; the pale colour is caused by poor vascularisation and by a low concentration of the respiratory pigment myoglobin (Satchell 1991). White muscle is faster, more powerful and capable of anaerobic burst activity (Johnston, 1981; Altringham and Johnston, 1988; Rome *et al.,* 1988). White muscle can produce higher swimming speeds but the exhaustion of metabolic fuels and the build up of waste products from anaerobic metabolism means that this activity can only be maintained for short periods of time. Many species of fish possess a third type of muscle, the intermediate or 'pink' muscle, which lies peripheral to the white muscle (Bone, 1966). Pink fibres are intermediate in their average diameter, myoglobin content and have a unique myosin heavy-chain (Rowlerson *et al.,* 1985).

1.4.4 Innervation

Fish muscle is innervated by anatomically discrete groupings of motorneurons or motorneuron pools, which occur in characteristic locations within the spinal cord (Westerfield *et al.*, 1986). In teleost fish, red muscle fibres are multiply innervated by motor axons that terminate in *en grappe* endings (Bone, 1964; 1966; 1970; Best and Bone, 1970). The innervation of fast fibre types is however of more interest than that of slow fibres due to large differences between fish groups such that the pattern of innervation may serve as a taxonomic character (Bone, 1970; Best and Bone 1973). Multiple innervation is found in the white muscle fibres of higher teleosts including the salmon, where several nerve terminals are distributed along the length of the muscle fibre (Bone, 1970).

1.4.5 Muscle contraction

Contraction is initiated when a nerve impulse arrives at a motor-end plate. A transmitter substance, usually acetyl-choline is released and causes the muscle fibre membrane to depolarise (Kuffler, 1946). This action potential is propagated along the fibre and into the T-tubule system, which infiltrates each individual myofibril. T-tubules usually lie at the level of the Z-band adjacent to the terminal cisternae of the sarcoplasmic reticulum, which in the relaxed state, contain a high concentration of Ca^{2+} ions. During contraction, the propagated action potential triggers the release of calcium ions (Heilbrunn and Wiercinski, 1947) which bind to TnC , releases the inhibitory effect of TnI on ATP-ase activity and stimulates the contraction of the sarcomeres. A conformational change takes place in the tropomyosin complex leading to the attachment, detachment and subsequent re-attachment of the myosin heads to the actin filament. The filaments themselves do not shorten but slide past each other, causing the muscle to shorten, the so-called 'sliding filament hypothesis' (Huxley and Niedergerke, 1954; Zot and Potter, 1987). The force developed by the muscle is related to the amount of overlap of the filaments (Gordon *et al.,* 1966a;b).

1.4.6 Phases of muscle development in fish

Three distinct phases of muscle growth can be distinguished in fish:

1.4.6a Embryonic phase

The 'embryonic' phase leads to the formation of embryonic muscle fibres, together with a population of undifferentiated myoblasts that are the source of subsequent growth (Devoto *et al.*. 1996; Johnston 1999a). As in other vertebrate embryos, the newly

formed somites of fish consist of radially arranged epithelial balls of mesoderm (epithelial somites). The epithelial somites undergo a series of morphological changes that eventually result in their dissolution and differentiation into bone cartilage, dermis and muscle (Keynes and Stern, 1988). Muscle formation begins before all the somites of the trunk have been formed and proceeds in a rostral-caudal direction (Waterman 1969, Johnston and Vieira 1996). Muscle fibres develop from myotubes and two classes can be distinguished in fish embryos. The first to form are the muscle pioneer fibres at the horizontal septum, formed by a single myoblast elongating to span the entire somite (van Raamsdonk *et al.,* 1974; Hanneman, 1992; Johnston *et al.,* 1995). The muscle pioneers express members of the homebox gene family *engrailed* (Hatta *et al.,* 1991) and are thought to have a role in patterning the characteristic chevron shape of the myotome and in guiding the growth cones of the pioneer neurones that first innervate the somites (Kimmel *et al.,* 1991).

Synthesis of myosin and actin filaments follows at the periphery of the myotubes in association with electron dense material of the presumptive z-line (Johnston and Vieira 1996). Initially, the nuclei are centrally placed and displace the myofibrils peripherally giving a tube-like appearance. Secondary myotubes are then formed as 3-6 myoblasts align in orderly rows between adjacent myosepta forming elongated, cylindrical, multi-nucleated secondary myotubes (Fischman, 1967; Yaffe, 1969). As development proceeds and more myofibrils are synthesised and assembled, they come to occupy the bulk of the cell and push the myonuclei into their characteristic peripheral position, just inside the sarcolemma (Ishikawa, 1983). By the end of embryonic development in fish, superficial

(presumptive red) muscle fibres are usually present in a single layer, while the remainder of the myotome contains inner (presumptive white) muscle fibres (Batty, 1984). Both larval fibre types are, however, relatively aerobic in character compared with adult muscle fibre types (El Fiky *et al.,* 1987) as most of the oxygen required for metabolism is supplied by diffusion across the skin and intervening tissues (Batty, 1984). As body size and therefore diffusion distances increase, the capacity for aerobic metabolism decreases and anaerobic pathways become more important (Johnston, 1994).

1.4.6b Germinal Phase

When all of the myogenic cells in the main mass of the somite have entered the phase of post-mitotic differentiation, further muscle growth takes place by myoblast proliferation in germinal zones at the dorsal and ventral apices of the myotome (Stickland *et al.,* 1988; Vegetti *et al.,* 1990; Brooks and Johnston, 1993; Rowlerson *et al.,* 1995). The majority of both red and white muscle fibres result from the fusion of myoblasts, forming multi-nucleated myotubes (Waterman, 1969). This embryonic pattern of fibre recruitment results in a decrease in mean fibre size from medial to superficial positions, which can persist into the adult stage (Kiessling *et al.,* 1991). Additional red muscle fibres are added solely at the dorsal and ventral extremities of the superficial red layer, which in embryos is only one fibre in thickness (Brooks and Johnston 1993). Finally, as these germinal zones become exhausted, the fish enter the 'satellite cell' phase and myoblasts on the surface of embryonic muscle fibres are activated, a process which can continue throughout much of adult life. Koumans *et al.,* 1994; Johnston *et al.,* 1995, Rowlerson *et al.,* 1995).

1.4.6c Myosatellite phase

In mammals, most muscle growth is by hypertrophy, an increase in the length and girth of muscle fibres with hyperplasia restricted largely to the pre and peri-natal period (Goldspink, 1972; 1974; Schultz, 1974; Stickland, 1981; Muntz and Boudjelida, 1986). By contrast, hyperplasia continues in teleosts together with hypertrophic growth, into the adult stage (Fig.1.6) (Greer-Walker, 1970; Stickalnd, 1983; Weatherley *et al.,* 1988; Weatherley, 1990) a process requiring the synthesis of myonuclei (Schultz and McCormick, 1994). Fibre enlargement or hypertrophy also requires additional myonuclei as fibres expand in order to maintain a relatively constant nuclear to cytoplasmic ratio (Koumans *et al.,* 1994; Cardasis and Cooper, 1975; Enesco and Puddy, 1964). Fish muscle is a post-mitotic tissue, however, incapable of undergoing mitosis (Campion, 1984) and in the absence of embryonic myoblasts, another source of myonuclei is required (Cardasis and Cooper, 1975; Enesco and Puddy, 1964). The additional source of nuclei is thought to originate from undifferentiated myoblasts, which become enclosed within the basal lamina of existing muscle fibres during the larval stage and are collectively referred to as satellite or myosatellite cells (Campion, 1984; Vegetti *et al.,* 1990; Devoto *et al.,* 1996).

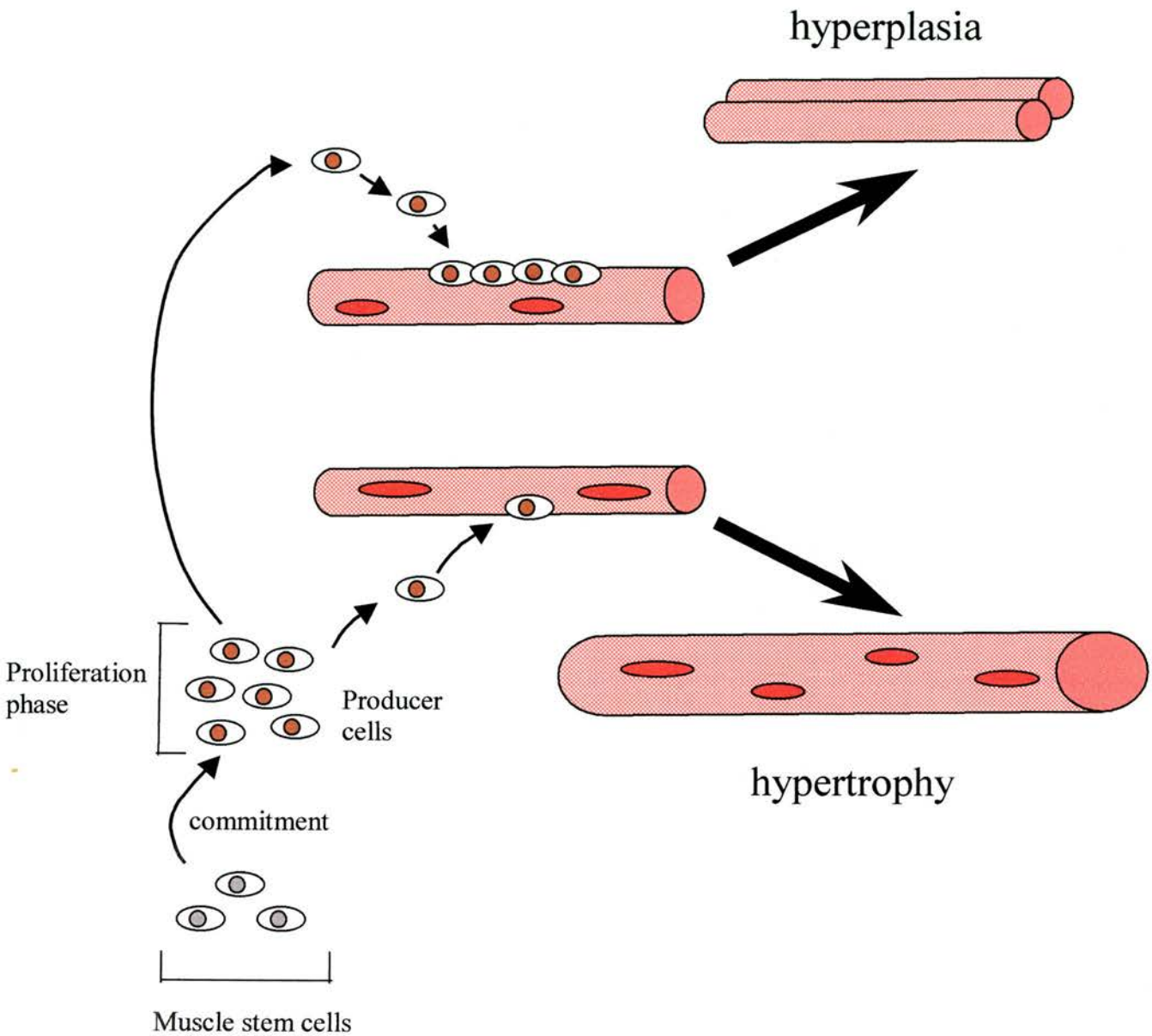


Figure 1.4 A model for the cellular basis of muscle growth (after Johnston, 1999a). Producer cells originate from the muscle stem cells and undergo a limited number of further divisions controlled by a balance between proliferation and differentiation signals. These myoblasts are either absorbed within the existing muscle fibres to maintain the nuclear to cytoplasm ratio in hypertrophying cells or fuse together on the surface of existing fibres forming a myotube which matures into a new muscle fibre (hyperplasia).

1.4.7 Myosatellite Cells

Myosatellite cells are small, spindle shape cells which contain a heterochromatic nucleus and, with the exception of free ribosomes and polysomes, their small amount of cytoplasm contains only a small number of other organelles (Schultz and McCormick, 1994). They are situated between the sarcolemma and the basal lamina of fully differentiated skeletal muscle fibres and retain their proliferative capacity following the initial embryonic phase. (Mauro, 1961; Campion, 1984). On the basis of ultra-structural observations on several cyprinid species and rainbow trout, it was suggested that undifferentiated myoblasts arise from, and proliferate within, the adjacent mesenchymal lining and enter the muscle via the myosepta (Stoiber and Sanger, 1996).

Proliferating cells can be readily labelled *in vivo* using either [^{3}H] thymidine or 5-bromo-2'-deoxyuridine (BrdU) a thymidine analogue as both labels are incorporated into replicating DNA and can be detected by autoradiography or immunohistochemistry. Moss and Leblond (1970; 1971) demonstrated that a pulse of ^{3}H-thymidine labelled proliferating myosatellite nuclei but did appear in post-mitotic nuclei. As the percentage of labelled myosatellites decreased, labelled nuclei also appeared in muscle fibres indicating that the myosatellite cells were the origin of post-embryonic nuclear material responsible for restoring the nuclear to cytoplasm ratio.

Myosatellite cells observed at an ultrastructural level are thought to constitute a mixture of muscle stem cells and a population of proliferating myoblasts that are committed to differentiation (Mauro, 1961; Johnston, 1998). Additionally, *in vitro* studies

on common carp, *Cyprinus carpio* L., suggest that hypertrophy and hyperplasia of fish muscle might depend on myosatellite cells, which have entered different pathways. (Koumans *et al.,* 1993; Devoto *et al.,* 1996). Koumans *et al.* (1993) postulated that hypertrophy of fish muscle is particularly dependent on myosatellites which have entered proliferation pathways and which divide to produce the additional nuclei required by fibres that are increasing in size. Hyperplasia on the other hand is believed to rely more on myosatellites which have entered differentiation pathways, and which fuse to produce new myotubes/immature muscle fibres. However, *in vivo* experiments in herring (*Clupea harengus*) by Johnston *et al.* (1998) showed that all of the nuclei in newly formed myotubes were BrdU positive, indicating that they had undergone recent divisions. Johnston *et al.* (1998) concluded that the possibility of separate myosatellite populations for hypertrophy and hyperplasia existed but that it is just as likely that local signals from the muscle fibres themselves determine the fate of the producer cell nuclei.

1.4.8 Control of proliferation and differentiation

The control of proliferation and differentiation of myosatellite cells are incompletely understood in mammals and hardly studied at all in fish. In mammals, members of the MyoD gene family are thought to activate the muscle differentiation programme and inhibit proliferation of the myoblast producer cells. The MyoD family are components of a highly redundant and poorly understood regulatory system modulating muscle growth which includes numerous growth factors including insulin-like growth factor-1 (IGF-1) (Olson, 1992). IGF-1 has a role in promoting differentiation and mutations results in mice with poorly developed muscles (Florini *et al.,* 1991).

1.4.9 Muscle growth as related to somatic growth

The capacity of an organ to grow depends mostly on it's ability to multiply the functional units of which it is composed (Goss, 1966; Bulow 1970; Mustafa *et al.,* 1991). Since white muscle accounts for up to 65% of the total body mass of a salmon, the formation of new muscle cells or myogenesis dominates this multiplication process. It has been hypothesized that this relationship between muscle fibre frequency and body size ensures muscle tissues meet the functional and structural requirements imposed by a particular body size (Gill *et al.,* 1989). This imparts a high degree of growth control in fishes, which enables them to conserve the size of organs and tissues relative to the whole body size (Weatherley, 1990).

Stickland (1983) concluded that any increase in the observed number of fibres per cross section in rainbow trout (*Oncorhyncus mykiss*) must be partly due to the formation of new fibres. The smallest fibres being those most recently formed which subsequently increase in size with overall body growth. Within the large mass of white muscle there are small diameter fibres which give the muscle a mosaic appearance Weatherley *et al.,*1980a; 1980b). These fibres were shown by Johnston *et al.* (1975) to have the same biochemical properties as the larger white fibres, suggesting that they are a stage in the development of larger fibres.

Weatherley and Gill (1987), Stickland 1988 and Vegetti *et al.* (1990) report than in growing fish, the role of hyperplasia decreases while that of hypertrophy increases with increasing length until in large fish, hypertrophy seems to be the sole process of muscle growth. A large ultimate body mass and fast growth rate is positively correlated

with the ability to produce new muscle fibres for a longer fraction of the lifespan (Weatherley *et al.,* 1979; 1980a; 1988). Suresh and Sheehan (1998) found that in rainbow trout, both triploids and diploids, the proportion of small fibres decreased with body size indicating the declining contribution of hyperplasia to muscle growth as body size increased. Weatherley *et al.* (1988) found that the largest and fastest growing fish species including whitefish (*Coregonus lavaretus*), rainbow trout (*Oncorhyncus mykiss*) and muskellunge (*Esox masquinongy*) show evidence of sustained recruitment of muscle fibres to a large size in contrast to the slower growing, bluntnose minnow (*Pimephales notatus*) and longnose dace (*Rhinicthys cataractae*). Willemse (1976) found a good correlation between muscle fibre growth and overall growth in glass eels. (*Anguilla anguilla*) while Villareal (1983) also found that the proportion of small diameter fibres was greatest in the fastest growing juvenile salmon.

This positive correlation between hyperplasia of muscle tissue and growth rate has been described in rainbow trout (Luquet and Durand, 1970), carp (*Cyprinus carpio*) (Koumans *et al.,* 1993; Alami-Durante *et al.*, 1997) and the European sea bass (*Dicentrarchus labrax* L.) (Nathanailides *et al.,* 1996) and has been confirmed on the basis of biochemical tests of muscle in the trout (Stickland, 1983; Weatherley and Gill, 1984; Weatherley, 1990), and carp (Koumans *et al.,* 1993). Valente *et al.* (1998) found a higher concentration of muscle DNA in a fast growing strain of rainbow trout suggesting a smaller cell size and larger number of cells per unit weight of tissue. A faster decrease of DNA concentration, with increasing body weight in a slower growing strain of rainbow trout reflected a lower percentage of hyperplasia and consequently a lower capacity for growth.

1.4.10 The relationship between hypertrophy and hyperplasia

Several investigations have established the relative importance of hypertrophy and hyperplasia by correlating muscle development with fish length. In the cod (*Gadus morhua*), early growth up to 35cm was predominantly by hyperplasia, followed by a phase (35-75cm) when hypertrophy also contributed substantially (Greer-Walker, 1970). In the largest fish (75-120cm) hyperplasia predominated again. Similar growth phases have been recorded for rainbow trout (*Oncorhyncus mykiss*) (Weatherley *et al.,* 1980b; Stickland 1983) where hyperplasia is again the main growth process up to 20cm, with hypertrophy assuming increasing importance until at around 60cm, when it becomes the sole growth process.

1.4.11 Muscle growth in Atlantic salmon

In Atlantic salmon, distinct germinal zones of myoblasts are present at the ventral and dorsal apices of the myotome during the yolk sac stages (Higgins and Thorpe, 1990; Johnston and McLay, 1997). Alevins hatch from the egg case with between 4000-6000 fibres per myotome (Stickland *et al.,* 1988; Johnston and McLay, 1997) rising to approximately 10,000 per myotome by first feed (Usher *et al.,* 1994). At this stage, myosatellite cells are activated throughout the myotome on the surface of existing fibres (Johnston and McLay, 1997). Over the following 12 months, upper-modal group fish will recruit up to 100,000 additional muscle fibres, the rate of recruitment increasing immediately prior to seawater transfer (Higgins and Thorpe, 1990). Discrete cycles of recruitment take place over the proceeding two sea-winters, generating distinct bi-modal frequencies in the muscle fibre frequency (Fig 1.7) and producing a 10 fold increase in

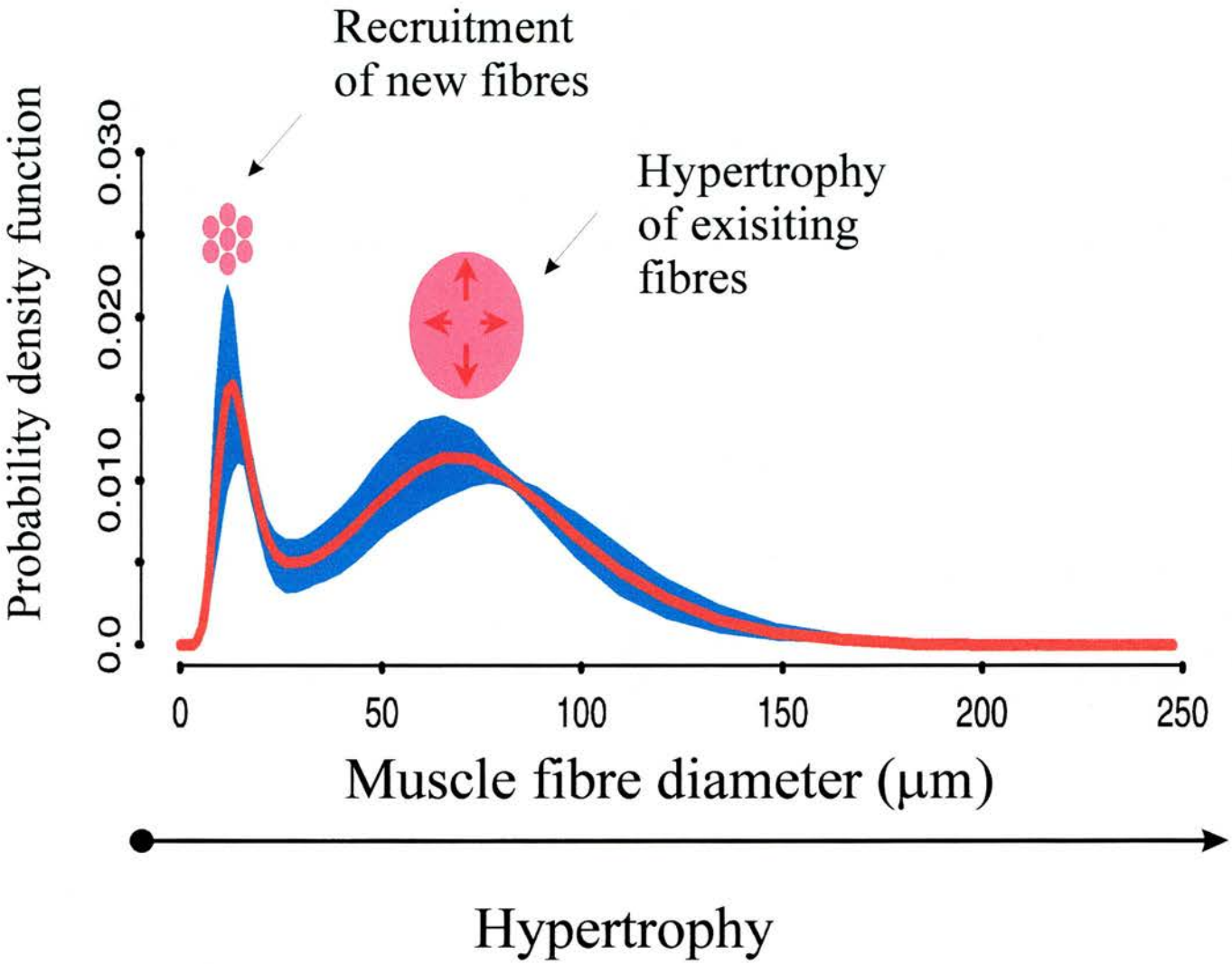


Fig. 1.7 Diagram representing the effect of hyperplasia (fibre recruitment) and hypertrophy (fibre enlargement) on muscle frequency distributions taken from 775d old Atlantic salmon showing a new modal peak of recruitment. The red line represents the mean probability density function while the blue shaded area corresponds to the variability band of the 100 bootstrap estimates, after Johnston *et al.*, (1999b).

fibre number (Johnston *et al.*, 1999b). Recent studies by Johnston *et al.* (1999b) and Johnston *et al.* (2000a) have indicated significant differences in muscle growth pattern in

Atlantic salmon linked to ploidy, growth rate and genetic strain. Diploid salmon were found to have a significantly higher muscle fibre recruitment rate than triploid fish at an equivalent stage and had significantly more muscle fibres per myotome. A study of fast and slow growing strains of Atlantic salmon by Johnston *et al.* (2000a) revealed that the slower growing strain recruited more muscle fibres for a given cross-sectional area and had had a higher muscle fibre density than the fast growing strain. Together with the temperature incubation studies of Stickland *et al.,* (1988); Usher *et al.,* (1994); Johnston and McLay (1997), this suggests that there is a wide scope of environmental and genetic influences which may be utilised to alter muscle cellularity in Atlantic salmon.

1.5 Plasticity of muscle growth and the influence of environmental variables

The ability to recruit new muscle fibres throughout the life cycle distinguishes fish from higher vertebrates and imparts considerable plasticity of growth, which is absent from mammals and birds (Weatherley, 1972; Weatherley and Rogers, 1978; Carpené and Vegetti, 1981; Johnston, 1993). Scheiner (1993) defined phenotypic plasticity as a change in the expressed genotype as a function of the environment, which plays two main roles in the evolutionary process. First the environment establishes the relationship between the phenotype of an individual and fitness, the so called 'fitness function'. Secondly, the environment interacts with the development process and plays a role in determining the phenotype. Developmental plasticity can arise through thermal activation of switches in the developmental programme leading either to the initiation of developmental arrest or to the production of alternative phenotypes differing in morphology, physiology and life history characters (Scheiner, 1993). A striking example

of this type of plasticity is the determination of the sex of some fish, such as the pejerrey (*Odontesthes bonariensis*) (Strussman *et al.,* 1996) and the Atlantic silverside (*Menidia menidia*) (Lagomarsino and Conover, 1993), by temperature during a critical period in development.

1.5.1 Temperature

In general, there is a positive correlation between rearing temperature, the growth rate of fish and therefore muscle (Weatherley and Gill, 1987; Austreng 1987). Muscle cellularity shows considerable plasticity with respect to exercise, feeding and environmental factors (Totland *et al.,* 1987; Kiessling *et al.,* 1991; Stickland *et al.,* 1988; Johnston *et al.,* 1993; Matschak *et al.,* 1995) although the responses observed vary with developmental stage and species. Temperature is one of the most pervasive abiotic environmental factors influencing living organisms because of its effect on the rate of chemical reactions (Schmidt Neilsen, 1975). While mammals and birds evade the effects of temperature changes by maintaining a constant core body temperature, endothermy is not found in most fishes (the exceptions being Tuna and their relatives) (Stevens and Neil, 1978). Early life stages are much more sensitive to temperature change, with temperature tolerances of embryos and larvae being more restricted than those for adults (Blaxter, 1992). The thermal environment experienced by fish can change dramatically due to seasonal, diurnal warming or cooling as the embryos develop and, therefore, temperature variation may result in a degree of plasticity in larval fish development, which persists into adult life (Brett, 1979).

Temperature incubation during early development can affect the number and size distributions of embryonic white muscle fibres at hatching in the Atlantic herring (Vieira and Johnston, 1992; Johnston *et al.,* 1998) the plaice (*Pleuronectes platessa*) (Brooks and Johnston, 1993) whitefish (*Coregonus lavaretus*) (Hanel *et al.,* 1996) and Atlantic salmon (Stickland *et al.,* 1988; Usher *et al.,* 1994; Nathanailides *et al.,* 1995; Johnston and McLay, 1997). Stickland *et al.* (1988) found that salmon eggs reared at 10°C produced fish with 33% fewer white muscle fibres of larger average cross-sectional area than those reared at lower ambient temperatures. Even larger differences in muscle cellularity were reported in a subsequent study with embryos reared at 5°C having almost 2 times as many white fibres as those reared at 11°C (Usher *et al.,* 1994). Using different stocks of wild salmon, Johnston and McLay (1997) found that, on average, there were 16% fewer embryonic white muscle fibres with a lower mean cross-sectional area in 5 families reared at 8°C than at variable ambient temperatures averaging 4.3°C. In contrast, Johnston (1993) found that in herring, the number of inner white muscle fibres were significantly higher in larvae reared at 15°C than at either 10°C or 5°C. The average diameter of muscle fibres was inversely correlated with temperature.

A great deal of debate surrounds the exact mechanism through which temperature alters muscle cellularity. Higgins and Thorpe (1990) suggest that in salmon embryos, there is survival value in reaching the largest possible size and developmental stage before the yolk sac is depleted. At high water temperature, higher metabolic costs would mean that protein synthesis, fibre hypertrophy, would be a more energy efficient method of increasing body size than the nuclear division involved in hyperplasia. When the

limiting factor of food is removed at first feeding, the fish can fuel further growth through fibre recruitment. Matschak *et al.* (1998) suggested that the observed temperature effect linked to muscle cellularity is due to physiological hypoxia at higher temperatures. A direct effect of temperature on myosatellite cells has also been demonstrated with higher temperatures leading to an increased differentiation rate but not proliferation (Matschak and Stickland 1995).

1.5.2 Light

Salmonids have a seasonally changing physiology and experience cycles of growth, precisely timed migrations and seasons of reproduction (Hoar, 1965). In addition to temperature, photoperiod is an important factor in synchronising the seasonally changing physiological processes of maturation, spawning, growth, smoltification and migration (Wedemeyer *et al.,* 1980; Adams and Thrope, 1989; Thorpe, 1987; Thorpe *et al.,* 1989; Villareal *et al.,* 1988). Extended daylength has a growth promoting effect on juvenile Atlantic salmon in fresh water (Berge *et al.,* 1995; Saunders *et al.,* 1985; McCormick and Saunders, 1987) and in sea water (Endal *et al.*, 1991; Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Taranger *et al.*, 1995; Oppedal *et al*, 1997; Duncan *et al.*, 1999) and has been utilised in aquaculture as a means of increasing growth rates. Photoperiod manipulation is now widely used in aquaculture to stimulate early smoltification and growth to produce 0+ smolts in autumn of the first year of life (Clarke, 1989; Duston and Saunders, 1990; Gaignon and Quemener, 1992).

Little is known of the physiological changes that take place in light manipulated salmon. McCormick *et al.* (1987) found that an abrupt increase in day length altered the timing and relative amounts of plasma growth hormone in salmon while Bjornsson *et al.* (1995) found high levels of insulin-like growth factor I (IGF-1) in salmon undergoing the parr-smolt transformation. Plasma growth hormone levels are prone to seasonal variation in salmonids and usually increase during parr-smolt transformation (Bjornsson *et al.*, 1989). Data from salmon reared on natural or simulated natural photoperiod indicate that growth hormone levels increase steadily from January to June irrespective of latitude (Young *et al.*, 1989a; Prunet *et al.*, 1989). Fauconneau *et al.* (1997) found that growth hormone supplementation enhanced the percentage of small diameter fibres in the rainbow trout, indicating a role of this hormone in hyperplastic growth. Musarò *et al.* (1999) found that virally delivered IGF-1 genes induced muscle hypertrophy and regeneration in mice.

1.5.3 Exercise

Many studies have investigated the effect of exercise on the growth and physiology of fish (for reviews see Hoar and Randall, 1978; Blake, 1983; Davison, 1989 and Davison, 1997). In salmonids, exercise promotes growth, with maximum growth occurring with continuous exercise at 1-1.5bl/sec (body lengths per second) depending on the size of the fish (Davison and Goldspink, 1977; Greer-Walker and Emerson, 1978; Nahhas *et al.*, 1982b).

In general, training at sustainable swimming speeds leads to increased proportions of red muscle associated with increased numbers of cells and cell diameters. White muscle also appears to be affected, leading to increased cell size even at low speeds where contraction of the white muscle would be passive (Johnston and Moon, 1980). In recent studies where muscle histology was analysed, white muscle has either not changed (Sänger, 1992; Hinterleitner *et al.,* 1992) or shown increased fibre diameter (Davison, 1994; Hinterleitner *et al.,* 1992; Totland *et al.,* 1987) depending on the species. For many salmon species, exercise has a marked positive effect on feed conversion efficiency (Christiansen and Jobling, 1989; Greer-Walker and Emerson, 1978 and Leon, 1986).

1.6 Muscle growth and flesh quality

White muscle accounts for over 95% of the tissue that is the final product of aquaculture and its growth and development is ,therefore, of great economic importance. In Scotland, the production of farmed Atlantic salmon (*Salmo salar* L.) has increased dramatically in the last 5 years from 64,064 tonnes in 1994 to 120,000 tonnes in 1999 (Data source: Federation of European Aquaculture producers). As overall production rises and prices fall, there is an increasing focus on the quality of salmon. Flesh quality is a highly subjective and broad term encompassing numerous physico-chemical properties (for reviews see Dunajski, 1979; Love, 1980; Johnston, 1999a). Haard (1992) defined the important attributes of food as being safety, nutrition, texture, flavour, colour appearance and the suitability of the product for processing and preservation. Research on mammalian species has shown that both the connective tissue matrix and the muscle fibres themselves contribute to the textural qualities of meat (Tornberg, 1996, Offer *et al.,*

1989). Collagen in fish muscle is significantly less cross-linked than that of birds and mammals (Hallet and Bremner, 1988) but still plays a role in the determining the textural properties of the tissue (Eckhoff *et al.,* 1998). Textural properties of Atlantic salmon have been related to pre and post-slaughter treatment (Wathne, 1995), time spent in rigor (Montero and Mackie, 1992) and the degree of collagen break down in the muscle (Ando *et al.,* 1991a,b; Hallet and Bremner, 1988).

Recent work on flesh quality in fish has related muscle cellularity to different flesh quality traits. Hurling *et al.* (1996) showed that muscle fibre cross-sectional area was related to sensory firmness in a number of different species including the flying fish (*Exocoetidae*), Tuna (*Katsuwomas pelamis*), swordfish (*Xiphiidae*), monkfish (*Lophius piscatorius*), cod (*Gadus morhua*), skate (*Raja batis*) and dab (*Limanda limanda*). Hatae *et al.,* (1990) proposed a 'lattice' theory of sensory firmness in which he related the textural properties of the fish to the basic structural element of the muscle fibre. Smaller, more closely packed muscle fibres provide a denser, firmer structure than larger, more loosely packed fibres which give a softer texture.

Colour is a particularly important quality indicator in salmonids (Koteng, 1992) with a uniform red colour being preferred by the industrial (smokehouse) and individual consumer. The pigmentation of wild salmon is derived from the absorption and deposition of oxygenated carotenoids from the diet (Christiansen *et al.*, 1995). The main pigment, astaxanthin is thought to bind non-specifically to hydrophobic sites on the actomyosin complex (Hemni *et al.,* 1990) and is partly genetic but also varies with age,

growth rate and maturation (Torrissen and Nævdal, 1984; 1988; Choubert *et al.,* 1997). Johnston *et al.* (2000b) found a relationship between visual colour score and fibre density in Atlantic salmon suggesting that higher densities gave a higher visual score reading.

The quality of flesh is known to be influenced by external factors such as feeding regime, diet composition and environment which impact upon the growth of the fish and the metabolic characteristics of the muscle tissue (Dunajski, 1979; Fægermand *et al.,* 1995; Fauconneau *et al.,* 1995 and Gjedrem, 1997). Totland *et al.,* (1987) suggested that the firmness was increased by exercising the fish in a raceway and attributed this to an increase in the diameter of muscle fibres. The relationship between flesh quality, muscle structure and the inherent plasticty within the tissue suggests that it may be manipulated to improve salmon quality (Johnston, 1999).

1.6 Aim

The primary aims of this thesis were to investigate the influences of salmon farming practice on muscle growth and flesh quality in Atlantic salmon. A particular focus was the effect of temperature incubation on the long term development of white muscle which, to this point has not been described. The studies were carried out in commercial scale conditions using hatchery stock Atlantic salmon (*Salmo salar* L.) at Matre Aquaculture Research Station, Norway.

A number of questions central to the operation of aquaculture were addressed. Does high temperature incubation affect long term muscle growth? Does light manipulation of juvenile salmon influence normal fibre recruitment and potential for future growth? Do cage rearing types and light manipulation in seawater affect muscle growth and flesh quality? Finally, the direct effects of muscle fibre density on flesh quality were assessed and the possible effects of muscle growth plasticity evaluated.

Few longitudinal studies have investigated the long-term effects of environmental variables on muscle growth in salmon. Fewer still have adequately described the relationship between muscle fibre growth and flesh quality. This study required the development of methods in the sampling of muscle in large fish reared under commercial conditions and the extensive development of methods to quantify flesh quality by instrumental methods. Data resulting from the study also stimulated the development of a new and powerful statistical method for assessing changes in muscle fibre frequencies in growing fish devised by Dr. Marti McCracken and Professor Ian A. Johnston.

Chapter 2

EGG INCUBATION TEMPERATURE INFLUENCES MUSCLE FIBRE RECRUITMENT DURING SEA WATER STAGES OF ATLANTIC SALMON (*Salmo salar* L.)

2.1 Introduction

Water temperature is one of the most important abiotic factors influencing the development and survival of marine fish. Anadromous salmonids such as the Atlantic salmon (*Salmo salar* L.) spawn in fresh water, burying their eggs in the river gravel of upland streams where they incubate over winter to hatch the following spring (Mills, 1992). Salmon eggs may routinely experience widely fluctuating but predominantly low water temperatures in the wild. In commercial aquaculture however, heated water is commonly used to facilitate early hatching and rapid on-growing. The temperature regime experienced by a salmon reared in a commercial hatchery may therefore be vastly different from that which is may encounter in the wild (Vøllestad and Hindar (1997).

The effects of early thermal experience on muscle growth during the embryonic phase has been investigated in various fish species including herring (*Clupea harengus*) (Vierra and Johnston, 1992; Johnston *et al.*, 1993; 1995; 1997) turbot (*Scopthalmus maximus*) (Gibson and Johnston 1995) rainbow trout (*Oncorhyncus mykiss*) (Matschak *et al.*, 1998). Stickland *et al.*, (1988) found that in Atlantic salmon, temperature during early development affected the number and size distributions of embryonic white muscle fibres at hatching. Fish reared at 10°C had 33% fewer white muscle fibres of larger average cross-sectional area than those reared at lower ambient temperatures. Even larger differences in muscle cellularity

were reported in subsequent studies with embryos reared at 5°C having almost 2 times as many white fibres as those reared at 11°C (Stickland *et al.*, 1988; Usher *et al.* 1994; Nathanailides *et al.*, 1995; Johnston and McLay, 1997). Temperature has also been shown to affect the cellularity of embryonic muscle in whitefish (*Coregonus lavaretus*) (Hanel *et al* 1996) and wild plaice (*Pleuronectes platessa*) (Brooks and Johnston, 1993). Johnston and McLay (1997), found that on average there were 16% fewer embryonic white muscle fibres with a lower mean cross-sectional area in 5 families reared at 8°C than at variable ambient temperatures averaging 4.3°C. During the germinal phase, alevins reared at 8°C were more effective in translating yolk into muscle than those reared at ambient temperature, such that by first feeding there were no significant differences in fibre number or cross-sectional area.

Three distinct stages of myogenesis can be identified in Atlantic salmon. The embryonic phase of myogenesis is followed by a germinal zone phase in which hyperplastic growth proceeds from regions of the myotome containing myoblasts. These myoblasts become exhausted by first feeding and subsequent muscle growth involves the activation of a muscle stem cell population of myogenic precursors (satellite cell phase) which are distributed throughout the myotome (Higgins and Thorpe 1990, Johnston and McClay 1997). Recruitment of new muscle fibres (hyperplasia) gradually decreases after first feeding until the juvenile parr begin to smolt, almost 14 months after hatch. At this point the parr (now referred to as 1+ smolts) show an acceleration of muscle fibre recruitment and a period of rapid somatic growth ensues (Higgins and Thorpe, 1990). New fibres are formed on the surface of pre-existing muscle fibres, giving the muscle a mosaic appearance in transverse section (Higgins and Thorpe, 1990; Johnston and McLay, 1997). Atlantic

salmon have on average, 180,000 fibres by sea water transfer, 750,000 after the first sea winter and over 1,000,000 after two sea winters (Johnston 1999; Johnston *et al.*, 1999a).

No studies have followed the effect of early thermal experience past first feeding and analysed the long term effects of varying incubation temperature on muscle growth in fish during the satellite cell phase. This study sets out to investigate the long term changes in growth and muscle cellularity which result from the use of heated and ambient egg incubation temperature under commercial farming conditions.

2.2 Materials and Methods

2.2.1 Fish Husbandry

Approximately 10,000 Atlantic salmon eggs from the Namsen hatchery broodstock at Matre Aquaculture Research Station, Norway (61°N) were divided into 4 EWOS Californinan fibre glass hatching trays lined with astro-turf™ on December 3rd 1996. Two hatching trays, each containing ca. 2500 eggs were incubated in water heated to 4-10°C (mean 5.8°C) from December to February (heated group) while the remaining two trays were supplied with river water at ambient natural temperature at 2-6°C (mean 2.5°C) from December to May (ambient group) (Fig. 2.1). River water was used to incubate the ambient group eggs and was, therefore subject to normal environmental fluctuation. Heated water was drawn from the nearby Hydro-electric power station and supplemented by a heat pump. Hatch date was determined as the point at which 50% of the alevins had emerged from the egg cases. Eggs in the high temperature group started to hatch on 26th January 1997 and 50% hatch had been reached by 2nd February 1997 (382 degree-days post- fertilisation). The alevins were

retained in the hatchery until 28th February 1997 when yolk sac resorption was completed and the fry were able to feed exogenously ('first feed'). Fry from the high temperature group were then transferred to separate feeding

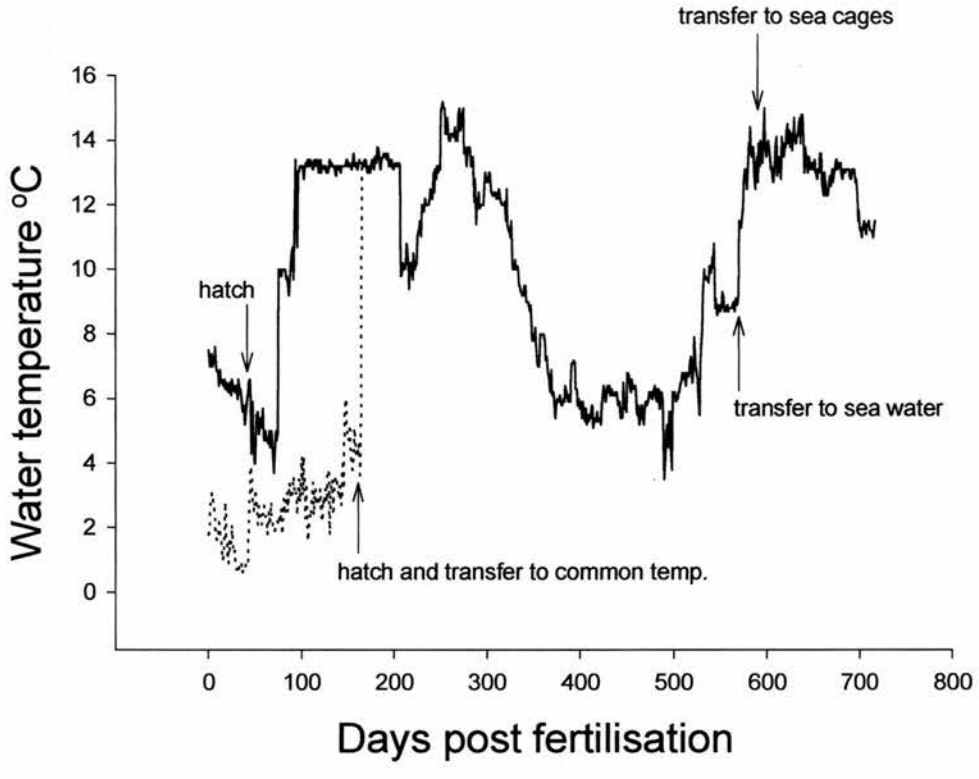


Fig. 2.1 Mean daily water temperatures for treatments groups reared in heated water (solid line) and ambient temperature (broken line) and following transfer to common temperature (mixed line)

units ($0.50m^2$) maintained at a constant high temp (ca. 8°C). Eggs incubated at ambient temperature began hatching on 11th May 1997 and reached 50% hatch by 15th May (441 degree-days post-fertilisation) and were transferred directly to $0.50m^2$ tanks maintained at ca 8°C. First feeding in the high and low temperature groups occurred at 576 and 908 degree-days respectively.

Following first feeding, all fish were transferred to four separate $1m^2$ square covered fibre-glass 350 L tanks with a water flow of 8-10L/min $^{-1}$. All fish were reared under a simulated ambient photoperiod (61°N) supplied by two 18W flourescent daylight tubes installed in the tank cover. All groups were fed commercial dry feed (T. Skretting Ltd., Stavanger, Norway) in excess from automatic feeders according to temperature and fish size (0.15-1.1g) 0.6mm (0.9-6g) 1mm (4-20g), 2mm (15-50g), 3mm (50-200g), 4mm (300-800g), 6mm (700-2000g) 9mm (2000g+) (Austreng 1987).

Heated and ambient incubation groups were vaccinated on 27th November 1997 (Norvax Protect, Intervet Norbio, Bergen, Norway) and transferred to four larger tanks (1.5x1.5m with a water flow of 30L-min $^{-1}$) but otherwise reared as described previously. Assessment of smolt status was carried out on 16th April 1998 to quantify the effect of temperature incubation on numbers of fish entering the upper modal group. Smolts were identified by their size, silvery appearance, marine pelagic shading and darkened fin margins. Lower modal fish were identified by their smaller size and greenish brown freshwater shading pattern. Precocious sexual maturity was assessed in all fish taken for muscle sampling by running a finger along the ventral surface of the fish and checking for the release of milt. All upper mode fish were fully

smolted in May 1998 and tank inlets were subsequently switched to provide sea water (salinity 28-30 °/∞).

On 26th June 1998 all remaining fish were judged to be osmoregulatory competent following sea water challenge tests and transferred to four sea cages (5.5mx5.5mx5.5m), reared under ambient light conditions and fed commercial dry feed (T. Skretting Ltd., Stavanger, Norway) in excess from automatic feeders according to temperature and fish size (Austreng 1987).

At hatch, first feeding and subsequently at monthly intervals 100 fish from each group were anaesthetised using metomidate hydrochloride (Wildlife Pharmaceuticals Co. USA) and measured for fork length (nearest 0.1cm) and body mass (nearest 0.1g). Following the development of length bimodality (October 1997), care was taken to sample fish from the upper modal group only, all fish <8cm fork length were discarded from the sample. Lower modal fish were however, retained in the tanks in order to assess the impact of ambient temperature incubation on bimodal recruitment. Fulton's condition factor (K) was calculated using the formula: $K= (W \times L^{-1})$, where W is the live body mass (g) and L is the fork length (cm) of each fish (Busacker *et al.*, 1990). Specific growth rate was calculated using the formula: $SGR =(e^{g}-1)100\%$ (Houde and Scheckter, 1981) where $g = (\ln(w_2)-\ln(w_1)(t_2-t_1)^{-1}$ (Bagenal and Tesch, 1978) and where w_2 and w_1 are the mean body mass at days t_2 and t_1 respectively. Condition factor (CF) was calculated as: $CF = BW \times L^{-3} \times 100$, where BW is body mass (g) and L is fork length (cm).

2.2.2 Histology

Muscle cellularity was quantified using Bouin's fixed material at hatch and first feeding and from frozen sections for subsequent age classes. Five newly hatched alevins were sampled from each heated water and ambient temperature group within 24h of 50% hatch. Further samples (n=5) were obtained from each group at first feeding. All fish sampled for muscle analysis were killed by anaesthetic overdose (benzocaine 1:2000(m/v)) and fixed in Bouin's fluid for 24h. The muscle tissue were dehydrated in a graded ethanol series and embedded in wax. Serial sections 7μm thick were cut transversely to the long body axis anterior to the adipose fin, stained with haematoxylin and eosin and mounted on gelatin coated slides.

Total muscle cross- sectional area was determined at a magnification of X40 using a drawing arm microscope attached to a Video-Plan image analysis system (Kontron Electronics, Basel). The outlines of individual muscle fibre areas were digitised and equivalent diameters calculated for the entire right side epaxial and hypaxial white muscle quadrants. Fibre counts from half sections were then multiplied by two to determine total fibre number, based on the assumption that the fish were bilaterally symmetrical (Vierra and Johnston, 1992).

Mean body mass from each monthly sample following first feeding was used to calculate a mean body mass range from which a representative sample of fish could be taken for muscle analysis. Fish sampled for muscle analysis were anaesthetised using metomidate hydrochloride (Wildlife Pharmaceuticals Co. USA) and killed by a sharp blow to the head. Whole steak sections were taken from a point immediately anterior to the adipose fin (Fig.2.2a) and photographed to determine total white

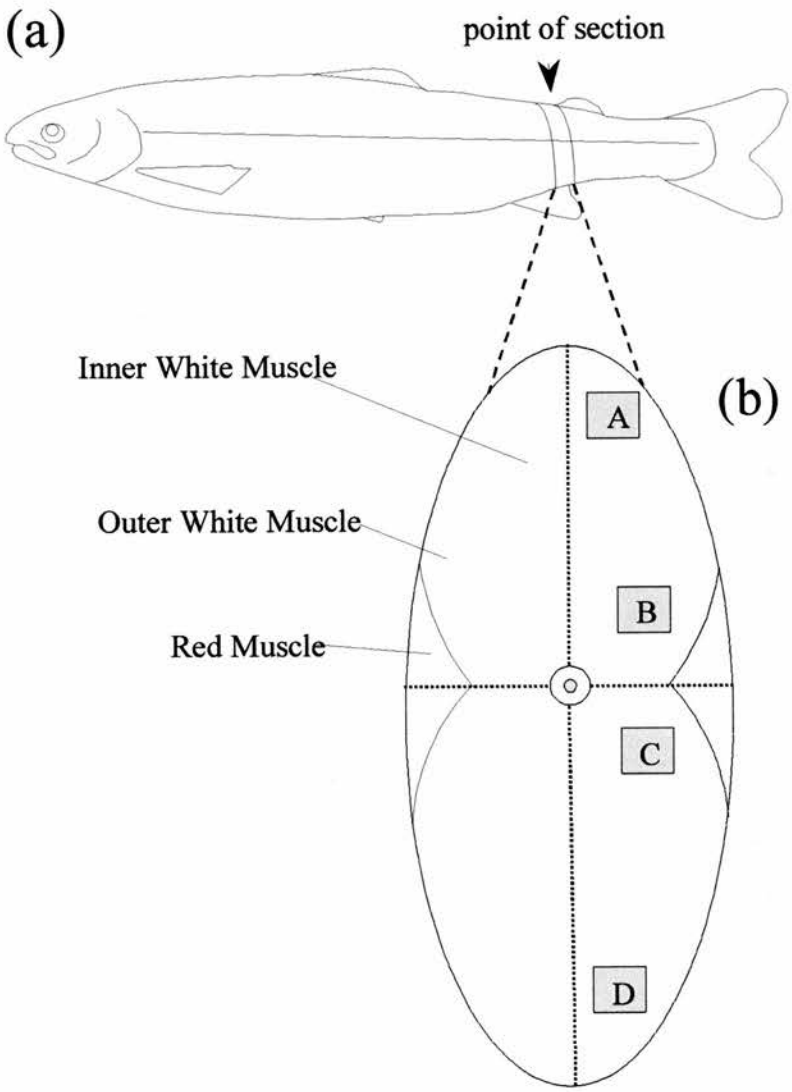


Fig. 2.2 Diagrams showing (a). sample position of the trunk muscle section and (b) standardised sample points used to estimate mean cross-sectional fibre areas.

muscle cross-sectional area. The right side epaxial and hypaxial quadrants were then isolated, immersed in cryo-protectant (Tissue-Tek, Shandon U.S.A.) and frozen in 2-Methylbutane (iso-pentane) cooled to it's melting point in liquid nitrogen (-196°C). Frozen muscle blocks were wrapped in aluminium foil to avoid desiccation, sealed in plastic bags and stored at -80°C prior to sectioning. Transverse sections were cut at 10-12μm on a Bright Starlet Microtome set to -20°C and mounted on poly-L-lysine coated slides (Sigma Chemicals, Poole, U.K.). Muscle sections were air dried and then stained with 1% Periodic acid-Schiff (PAS) solution (Sigma) before mounting in glycerol gelatin (Sigma, Poole, U.K.).

The cross-sectional areas of white muscle fibres were measured at four main sites on the section (Fig.2.2b) using an Image Pro 3.0 analysis system (Caltech. Co. USA) and equivalent diameter calculated. Approximately 400 fibres in total were measured per fish from each area of the myotome. Total white muscle cross sectional area was determined for each sample date while muscle fibre number was estimated by dividing the total white muscle cross sectional area by mean white muscle fibre cross-sectional area.

2.2.3 Statistics

Data were analysed using Statistica 5.1 software package (StatSoft Inc., Tulsa, USA). Data on length, body mass and condition factor were checked for normal distribution using normal distribution plots, and for homogeneity in variance by Levenes test (Sokal and Rohlf, 1995). The effects of temperature treatment on length, body mass and condition factor were tested using a nested one-way ANOVA (replicate groups nested within temperature treatment, Zar 1996). The effects on SGR

and length growth were tested with Kruskal-Wallis non-parametric ANOVA (Sokal and Rohlf, 1995. A significance level (α) of 0.05 was applied in all tests.

Non-parametric smoothing and bootstrap techniques were employed to investigate the changes in muscle fibre diameter during hypertrophic growth (Johnston *et al.*, 1999). The distribution of muscle fibre diameter was analysed by constructing smooth non-parametric estimates of the probability density function (pdf) using the kernel approach (Silverman, 1986) within the S-Plus computing environment utilising the **sm** library (Bowman and Azzalini, 1997). Pooled groups of 10 fish (5 fish from each of the heated and ambient groups) were compared for each sample date (400 fibres per fish). The average pdf for heated (f^{H}) and ambient (f^{A}) groups were then estimated using diameters pooled over groups using a common smoothing parameter (h) equal to the mean of the normal optimal smoothing parameters over fish within an age class. Variability within and between fish samples was accounted for by plotting a variability band around the pdf estimate. For each bootstrap sample, the average density was estimated and the area between this estimate and f^{H} or f^{A} was shaded. The final shaded area represents the maximal polygon created by the 100 bootstrap estimates of density and is referred to as the variability band. Structural characteristics within the polygon density plot were analysed visually for evidence of bimodality and changes in the overall fibre distribution. Finally, the values of specific percentiles of the estimated fibre densities for each fish were compared using the Wilcoxon two-sample nonparametric test. Median values of the 5^{th}, 10^{th}, 50^{th} and 95^{th} percentiles were compared for each group sampled.

2.3 Results

2.3.1 Somatic Growth - Hatch and First-Feeding

A summary of temperature during the egg incubation period and timing of hatch is given in Table 2.1. Mean body mass at hatch in the heated temperature groups were 0.145g ± 0.02 and 0.144g ± 0.02, compared with 0.139g ± 0.02 and 0.136g ± 0.02 in the ambient temperature groups (mean ± SE, n=5). Heated temperature fish were significantly heavier at hatch compared to those reared at ambient temperature ($F_{1,98}$=29.01, P<0.05, Fig. 2.4a), although fork length was similar ($F_{1,98}$ = 0.77, P>0.05, Fig. 2.3a). Body mass at first feeding was not significantly different between temperature groups ($F_{1,98}$ = 7.7, P>0.0.5, Fig. 2.4b) although heated group fish were significantly longer than ambient group fish ($F_{1,98}$=91.1, P<0.001, Fig. 2.3b).

2.3.2 Growth following the onset of exogenous feeding

Fork Length

Fork length remained significantly higher in the heated temperature group from hatch until the end of the experiment, (Fig.2.5a, Table 2.2). Fork length increase followed a seasonal pattern in both groups with higher growth rates between May and November in both years of the study, and depressed rates of growth in the winter period from November 1997 to May 1998. Following sea water transfer in June 1998, length growth rate increased markedly from less than 0.4 mm/day to around 1.2mm/day in both groups.

Body mass

Body mass increased in both treatment groups following a seasonal pattern with fastest mean body mass growth taking place from August 1997 to November 1997 (Fig. 2.5b) and from June 1998 to August 1998 following sea water transfer. The earlier hatching heated groups achieved a greater body mass in the summer period (June to October 1997) after which, body mass increase slowed markedly. Body mass at sea water transfer in both heated temperature groups was significantly higher than that in the ambient temperature tanks ($F_{1,198}$=9.99, P<0.01). Body mass in the heated temperature group was significantly greater than the ambient temperature group at all sample points following first feed (Fig. 2.5b, Table 2.2) apart from the final sample in February 1999. The body mass to fork length relationship in both treatments was not significantly different ($F_{1,5395}$ =0.802 P>0.05) indicating no effect of temperature on the gross morphology of the fish (Fig. 2.6).

Specific Growth Rate (SGR)

A sharp increase in SGR can be seen in both ambient temperature groups following transfer to higher temperature following first feeding. SGR in heated temperature and ambient temperature groups then followed a seasonal pattern with higher growth rates in spring/summer which subsequently decreased towards winter as temperatures and feed intake dropped (Fig. 2.7a). The ambient temperature groups exhibited a relatively higher SGR from August until January and subsequently remained indistinguishable from that of the heated temperature group with no consistent difference between groups. SGR increased in all groups following transfer to seawater and subsequently decreased as the fish entered their first winter in seawater.

Condition Factor

Condition factor varied considerably in both treatment groups with increased condition factors of 1.2 to 1.4 coinciding with increased body mass gain during late summer and autumn (Fig. 2.7b). There were no significant differences in condition factor throughout the freshwater stages. Condition factor in all groups began to decrease in mid April 1997 from around 1.4 to 1.1 in accordance with the onset of smoltification (Hoar *et al.* 1988). Condition factor continued to decrease following sea water transfer as a result of increased length growth and subsequently increased during late summer and autumn, with the heated temperature group having a significantly higher condition factor ($F_{1,198}$=3497.47 p $\leq$0.001) than the ambient temperature group in August(Fig. 2.7b, Table 2.2). No significant difference in condition factor between heated temperature and ambient temperature groups were found at the final sample in February 1999.

Development of bimodality and precocious maturity

Recruitment of fish to the upper modal group (UMG) in the heated temperature replicates was 98% and 98% while the ambient temperature replicates had markedly lower UMG recruitment of 75% and 73%. No evidence of precocious maturity was found in any of the groups sampled.

	Ambient group		Heated group	
	Hatch	First feed	Hatch	First feed
Degree days	441	908	382	576
Mean temp.	2.70	4.5	6.07	6.47
St. dev.	1.11	4.18	0.83	1.70
Max. temp.	6.0	13.8	7.6	10.2
Min. temp.	0.6	0.6	4.0	3.7

Table 2.1 Temperature records for ambient and heated temperature groups indicating degree days to hatch, first feed, mean (± S.D.) minimum and maximum temperatures during early life stages of the fish.

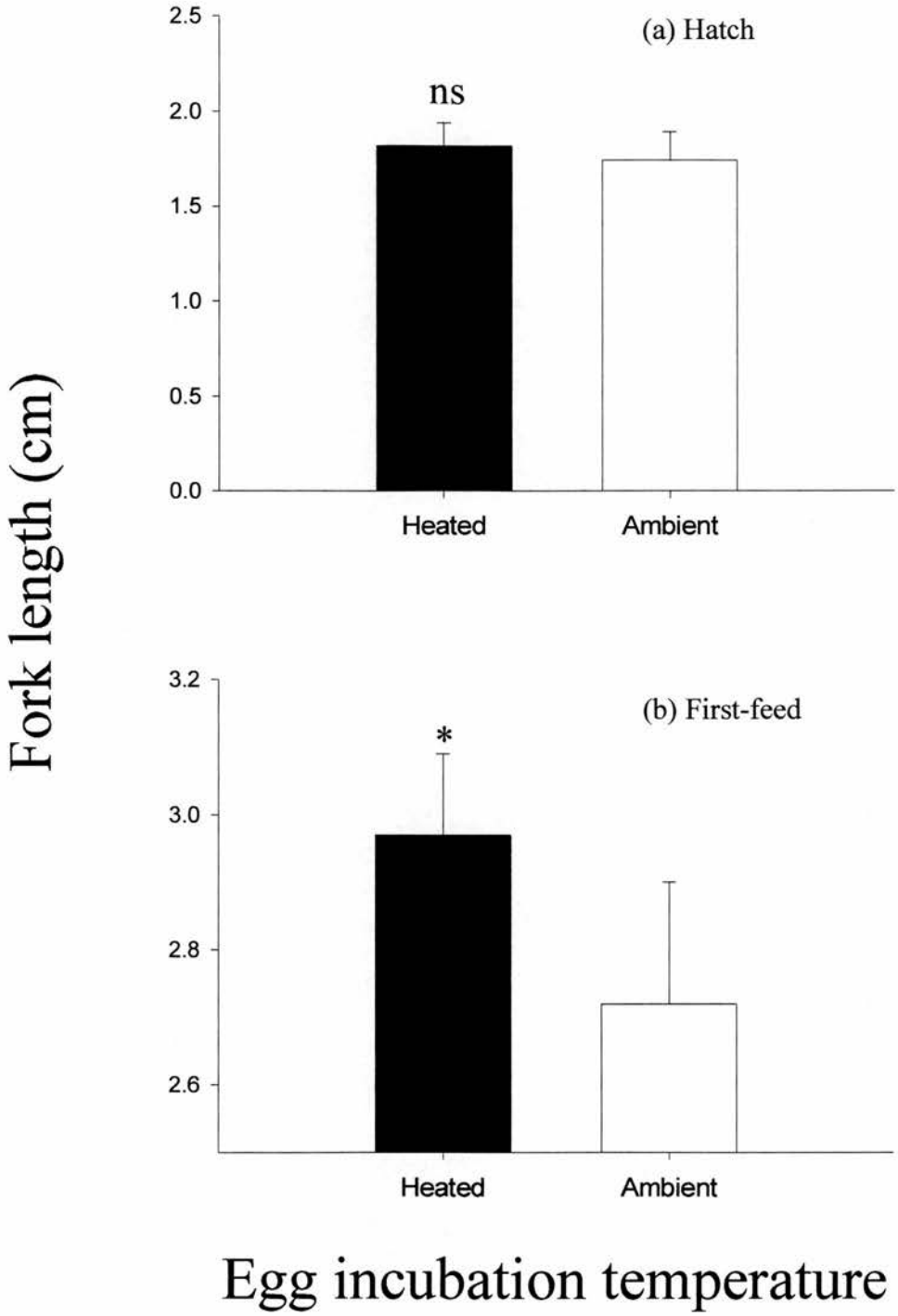


Fig. 2.3 Fork length of juvenile Atlantic salmon incubated at heated and ambient temperature at (a) hatch and (b) first feeding. Values are means +SE for 100 fish per group (pooled replicates). Asterisks indicate significant differences between treatment groups (ANOVA, $P<0.05$) ns = not significant (ANOVA, $P>0.05$)

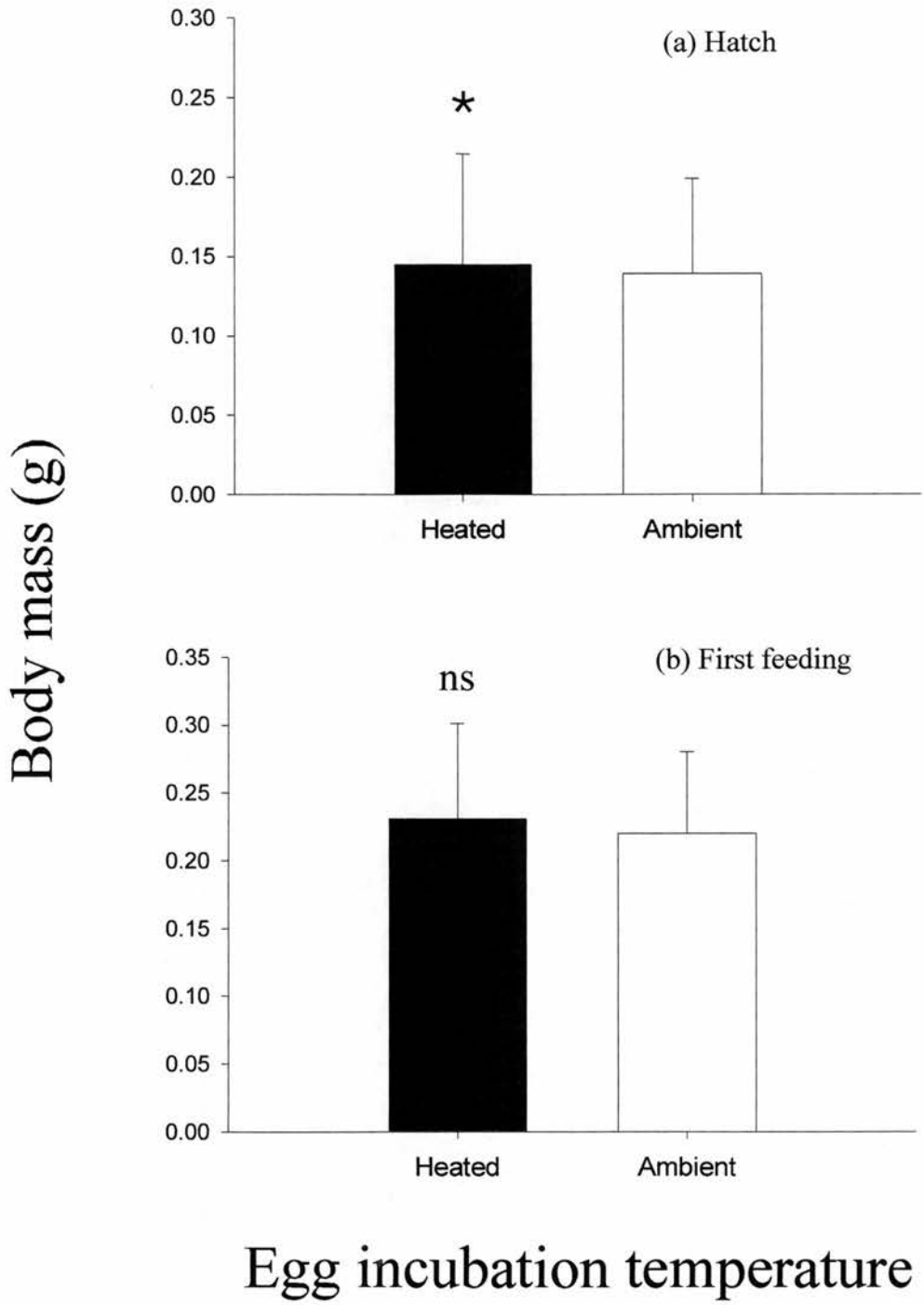


Fig. 2.4 Body mass of juvenile Atlantic salmon incubated at heated and ambient temperature at (a) hatch and (b) first feeding. Values are means +SE for 100 fish per group (pooled replicates). Asterisks indicate significant differences between treatment groups (ANOVA, $P<0.05$) ns = not significant (ANOVA, $P>0.05$)

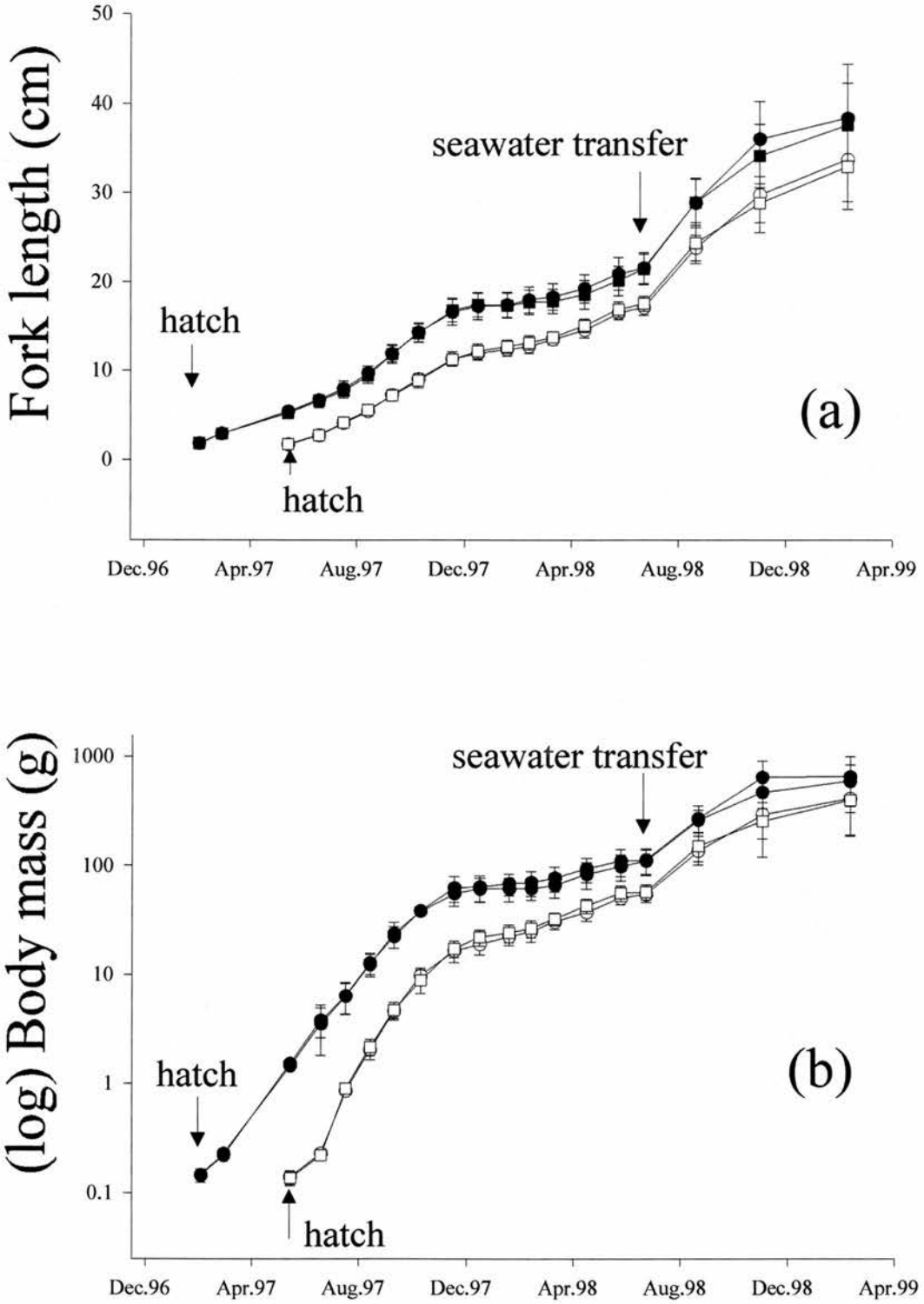


Fig.2.5 (a) Fork length and (b) body mass in Atlantic salmon incubated at heated (solid symbols) and ambient (open symbols) temperatures. Results of ANOVA are given in Table 2.2.

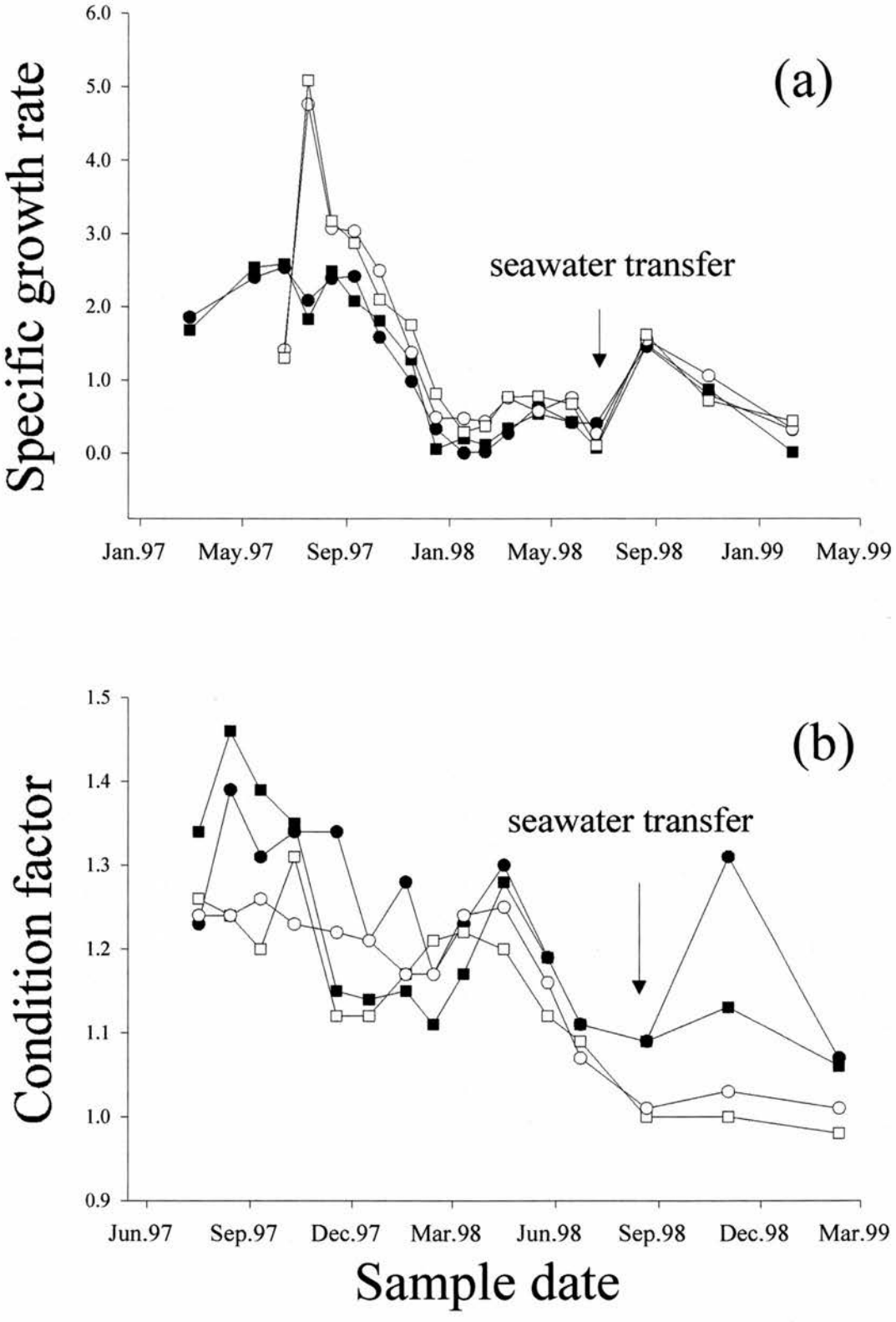


Fig.2.7 (a) Specific growth rate and (b) condition factor in Atlantic salmon incubated at heated (solid symbols) and ambient (open symbols) temperatures. Results of ANOVA are given in Table 2.2.

	Fork Length		Body mass		Condition Factor	
Date	$F_{1,198}$ n=100	P value	$F_{1,198}$ n=100	P value	$F_{1,198}$ n=100	P value
Hatch	7.78	0.107992	29.00	0.03278	_	_
First Feed	91.10	0.014789	0.77	0.47268	_	_
July 1997	768.56	0.0012	6155.17	0.00001	0.28	0.6455
Aug. 1997	661.81	0.0015	2045.97	0.0004	20.44	0.0455
Sept. 1997	1426.98	0.0008	711.76	0.0014	5.68	0.1398
Oct. 1997	3480.80	0.0002	3887.33	0.0002	4.35	0.1721
Nov. 1997	1646.64	0.0006	136.10	0.0072	0.47	0.5620
Dec. 1997	1056.68	0.0009	476.69	0.0020	0.04	0.8514
Jan. 1998	1289.74	0.0007	129.22	0.0076	0.44	0.5736
Feb. 1998	913.42	0.0010	104.60	0.0094	1.31	0.3698
Mar. 1998	302.56	0.0032	60.06	0.0164	1.16	0.3931
Apr. 1998	231.76	0.0043	68.60	0.0142	4.96	0.1557
May 1998	75.23	0.0130	60.10	0.0162	6.46	0.1260
June 1998	336.80	0.0029	893.74	0.0011	9.99	0.0871
Aug. 1998	271.19	0.0036	209.79	0.0047	3497.47	0.0002
Nov. 1998	35.36	0.0271	10.20	0.0855	3.45	0.2042

Table 2.2 Results of two-way nested ANOVA for fork length, body mass and condition factor in Atlantic salmon fry hatched from eggs incubated at heated temperature and ambient temperature (significance level $p<0.05$).

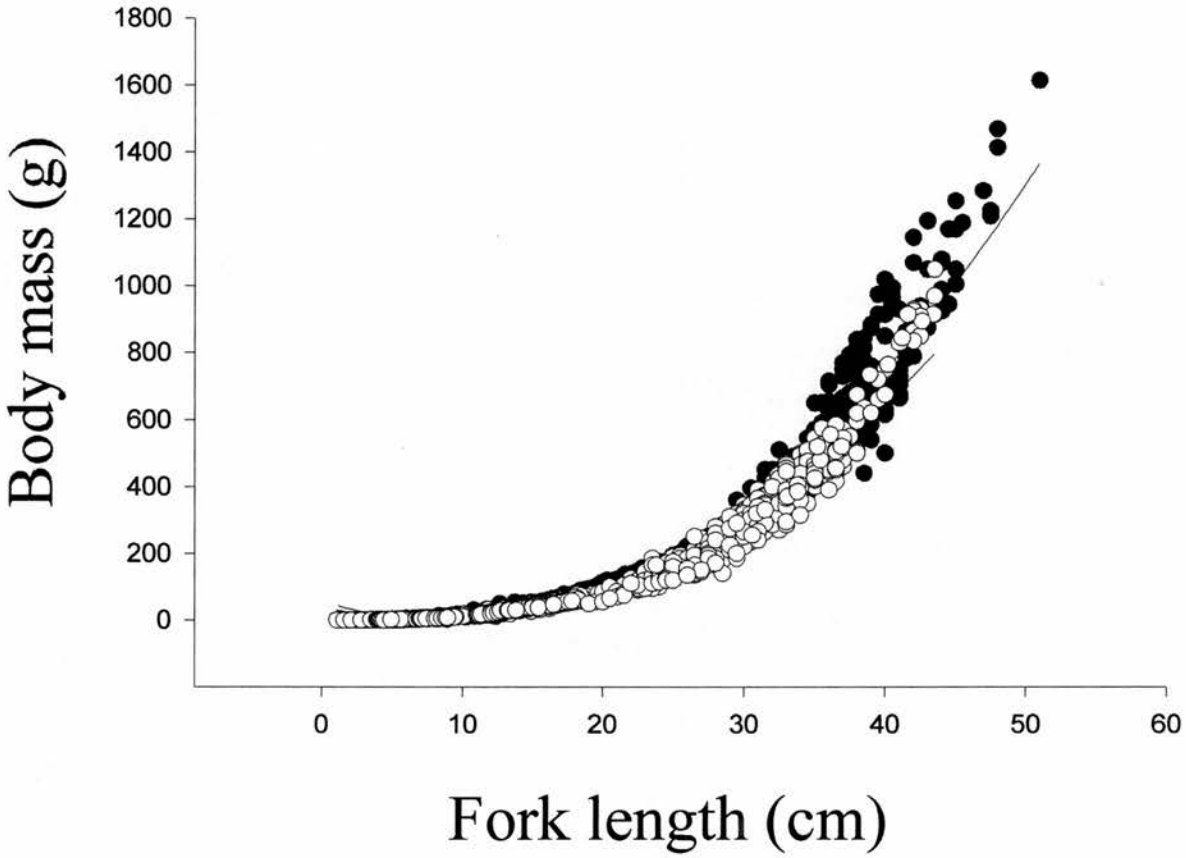


Fig.2.6 Relationship between fork length and body mass in Atlantic salmon incubated at heated (solid circles) and ambient temperature (open circles). The lines are fitted with an exponential regression

2.3.3 Changes in muscle cellularity during ontogeny - Hatch and First Feed

The total cross sectional area (TCA) of white muscle was significantly higher at hatch (Fig.2.8a) in the ambient compared to the heated temperature group ($F_{1,8}$=21.18 P<0.05) but this difference was not apparent at first feed (Fig. 2.8b). The mean diameter of white muscle fibres was not significantly different at hatch or first feed (Fig. 2.9a,b; Table 2.3). In contrast, white muscle fibre number was significantly higher in the ambient temperature fish at hatch than the heated temperature group ($F_{1,8}$=31.19 P<0.05) although by first feed, these differences were no longer statistically significant (Fig. 2.10a,b).

	Hatch		First Feed	
	Heated	Ambient	Heated	Ambient
TCA	0.51	0.62	0.96	1.22
(mm^2)	(0.06)	(0.10)	(0.13)	(0.04)
WMFD	121.80	140.80	300.03	338..42
(μm)	(49.52)	(20.41)	(41.62)	(14.20)
WMFN	4167	4443	6364	7113
	(164)	(221)	(611)	(308)

Table 2.3 Muscle growth parameters (total white muscle cross sectional area TCA, white muscle fibre area WMFA and white muscle fibre number WMFN, pooled replicates) at hatch and first feed in Atlantic salmon fry hatched from eggs incubated at heated temperature and ambient temperature.

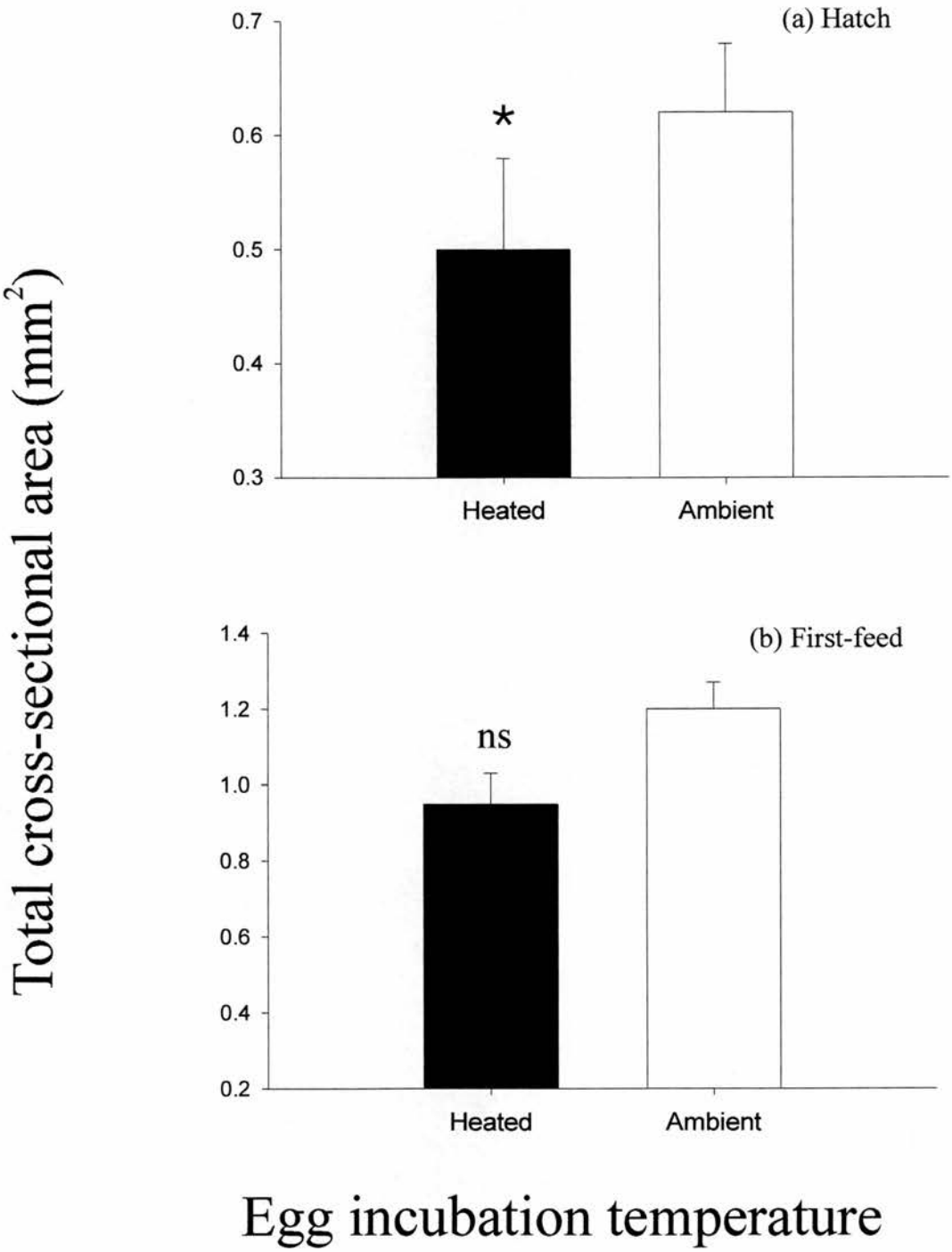


Fig. 2.8 Total white muscle cross-sectional area in juvenile Atlantic salmon incubated at heated and ambient temperature at (a) hatch and (b) first feeding. Values are means +SE for 100 fish per group (pooled replicates). Asterisks indicate significant differences between treatment groups (ANOVA, $P<0.05$) ns = not significant (ANOVA, $P>0.05$)

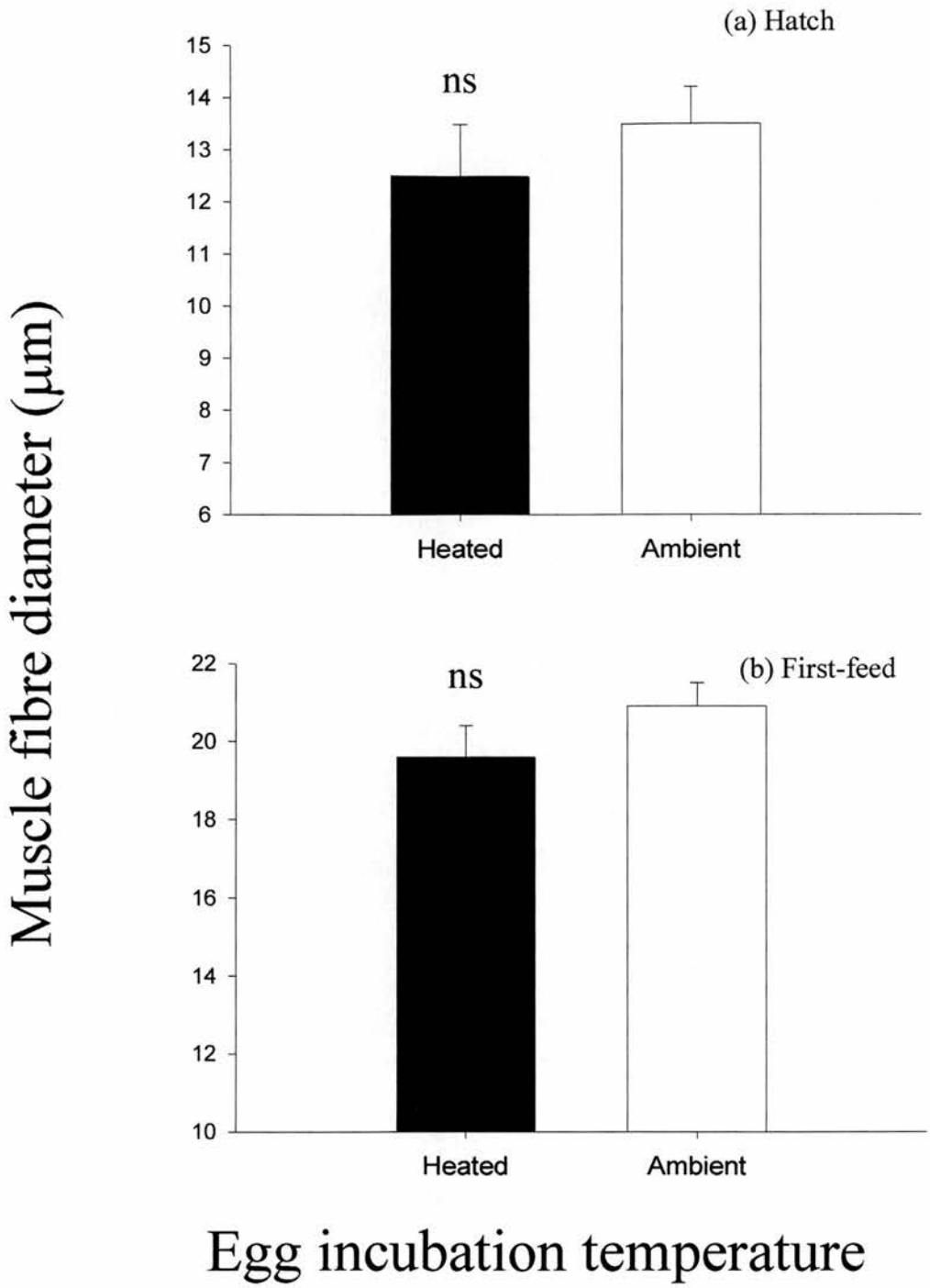


Fig. 2.9 White muscle fibre diameter in juvenile Atlantic salmon incubated at heated and ambient temperature at (a) hatch and (b) first feeding. Values are means +SE for 100 fish per group (pooled replicates). Asterisks indicate significant differences between treatment groups (ANOVA, $P<0.05$) ns = not significant (ANOVA, $P>0.05$)

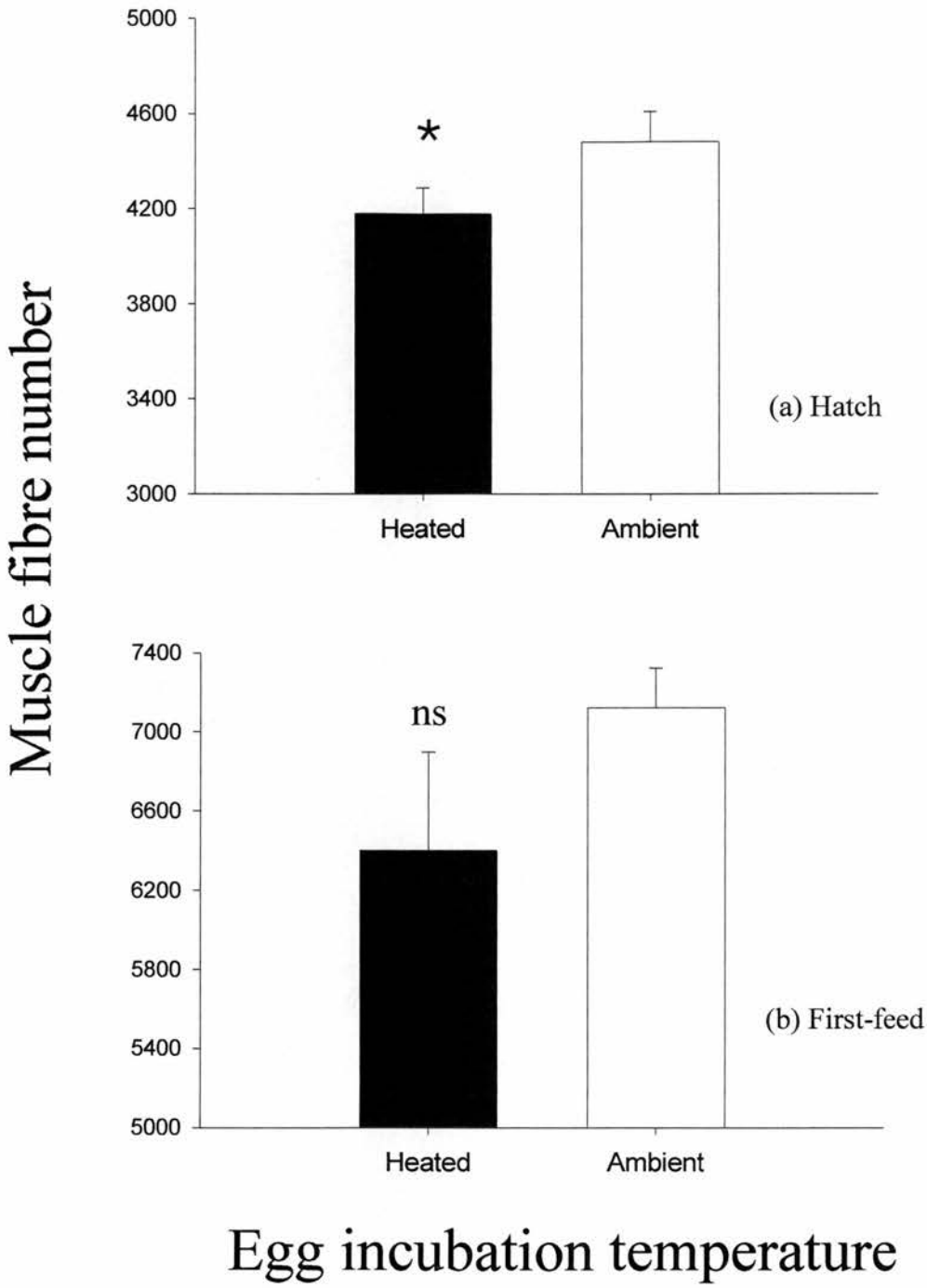


Fig. 2.10 White muscle fibre number in juvenile Atlantic salmon incubated at heated and ambient temperature at (a) hatch and (b) first feeding. Values are means +SE for 100 fish per group (pooled replicates). Asterisks indicate significant differences between treatment groups (ANOVA, $P<0.05$) ns = not significant (ANOVA, $P>0.05$)

2.3.4 Muscle growth following the onset of first feeding

Total cross-sectional area (TCA)

Following an earlier hatch date (February 2nd 1997 compared with 15th May 1997), total white muscle cross sectional area (TCA) remained significantly higher in both heated temperature groups up to sea water transfer (Fig.2.11a Table 2.4). TCA increased markedly in all groups following transfer to seawater with heated fish maintaining a significantly higher TCA 6 months after transfer. (Fig.2.11a). TCA was closely related to fork length in both ambient and heated temperature groups (r^2 adj.= 0.987 and 0.975 respectively) with no significant differences between groups ($F_{1,197}$ =0.802; P>0.05) (Fig 2.12a).

Muscle fibre recruitment

The relationship between fibre number and total muscle cross-sectional area (TCA) is shown in Fig.2.13 and indicates higher fibre recruitment to reach a given TCA in the ambient group. From first feeding to October 1997, muscle fibre recruitment rate was $294d^{-1}$ and $289d^{-1}$ in the heated and ambient groups respectively. Fibre recruitment increased markedly between October 1997 and May 1998 to 512 d^{-1} in the heated group and $429d^{-1}$ in the ambient group. Overall, between the S1 parr stage and sea water transfer in June 1998, the heated group had experienced a 3-fold increase in fibre number while the ambient group had increased it's fibre number by almost 8 times. (Fig.2.11b Table 2.5). Following seawater transfer in June 1998, fibre number increased rapidly in both groups with a 1.5-fold increase in the heated group and a 2.5 fold increase in the ambient group. White muscle fibre number was significantly higher in the heated groups at all sample points from August 1997 until sea water transfer in June 1998 (Fig.2.11b). Following considerable compensatory

fibre recruitment in the ambient group after sea water transfer, there were no significant differences ($F_{1,8}$=3.64, P>0.05) between fibre number in the heated (321,182±8973) and ambient groups (285,516±10356) (mean±SE, n=10) by November 1998,

	TCA		Fibre number		Fibre diameter	
Date	$F_{1,18}$	P	$F_{1,18}$	P	$F_{1,18}$	P
Hatch	21.18	0.044	31.19	0.031	10.18	0.086
First feed	7.21	0.115	16.55	0.055	8.24	0.102
Aug. 1997	183.05	0.005	439.27	0.002	10.51	0.083
Oct. 1997	18.88	0.049	25.98	0.036	10.27	0.085
Dec. 1997	360.79	0.002	49.72	0.019	5.22	0.149
Feb. 1998	443.16	0.002	388.83	0.002	0.99	0.423
Apr. 1998	796.74	0.001	71.42	0.013	0.02	0.897
May 1998	29.017	0.032	601.83	0.001	2.30	0.268
Nov. 1998	57.29	0.017	3.64	0.196	81.57	0.012

Table 2.4 Results of two-way nested ANOVA for total white muscle cross-sectional area, white fibre number and white fibre diameter in Atlantic salmon fry hatched from eggs incubated at heated temperature and ambient temperature (Significance level P<0.05)

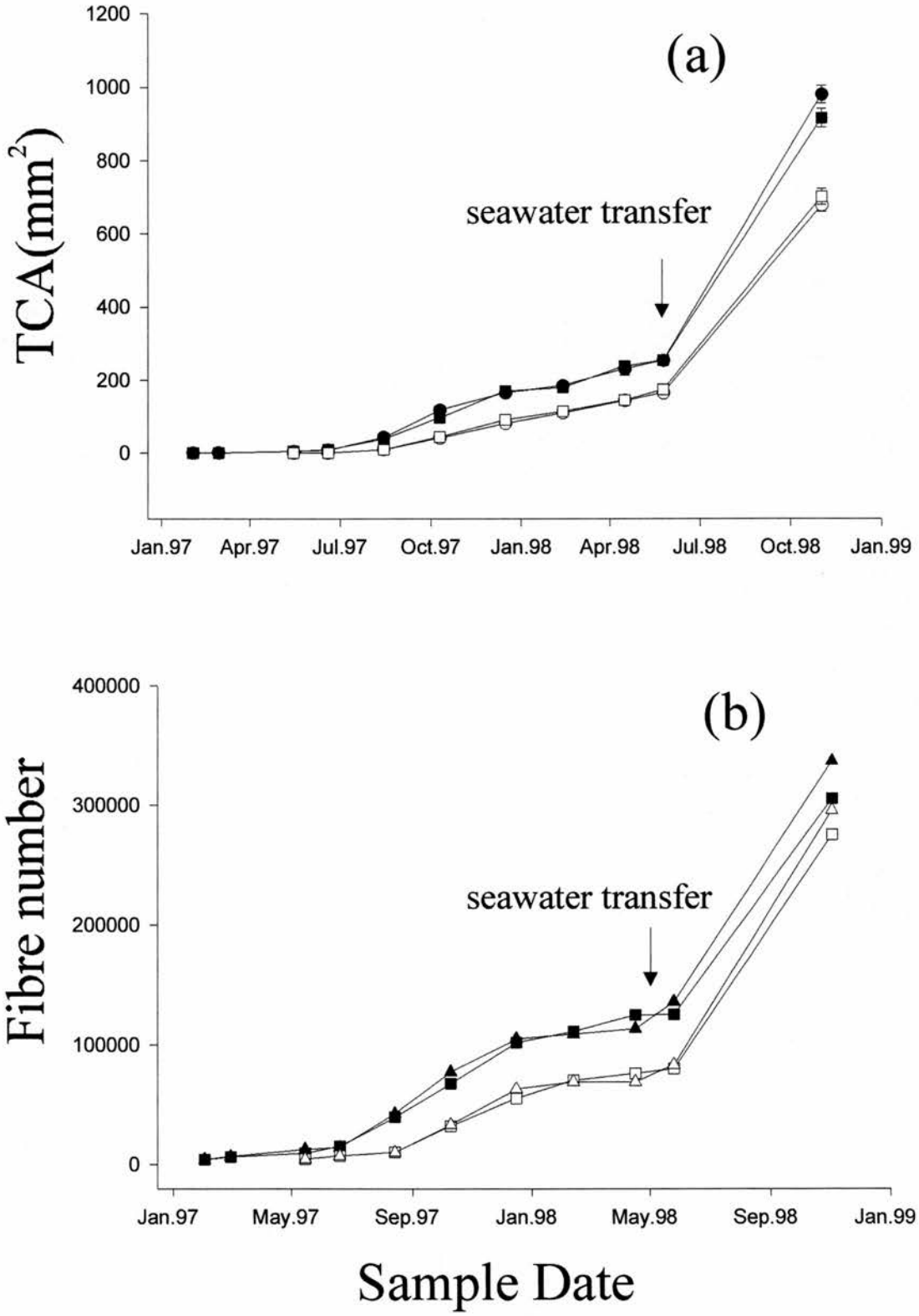


Fig. 2.11 (a) Total cross-sectional area and (b) muscle fibre number in Atlantic salmon incubated at heated (solid symbols) and ambient (open symbols) temperatures.

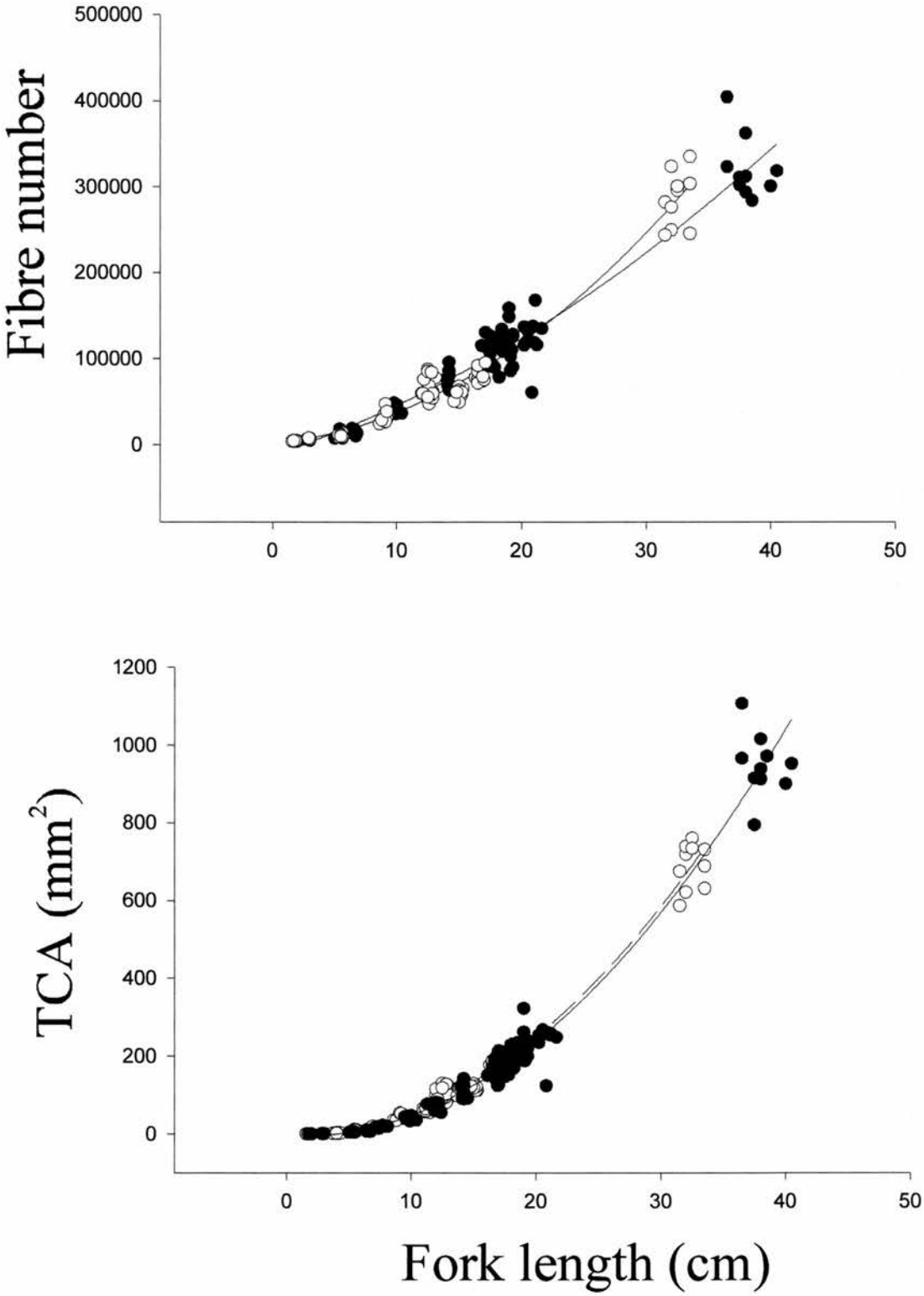


Fig. 2.12 Fork length plotted against (a) muscle fibre number and (b) total cross-sectional area (TCA) in Atlantic salmon incubated in heated (closed symbols) and ambient (open symbols) water temperatures

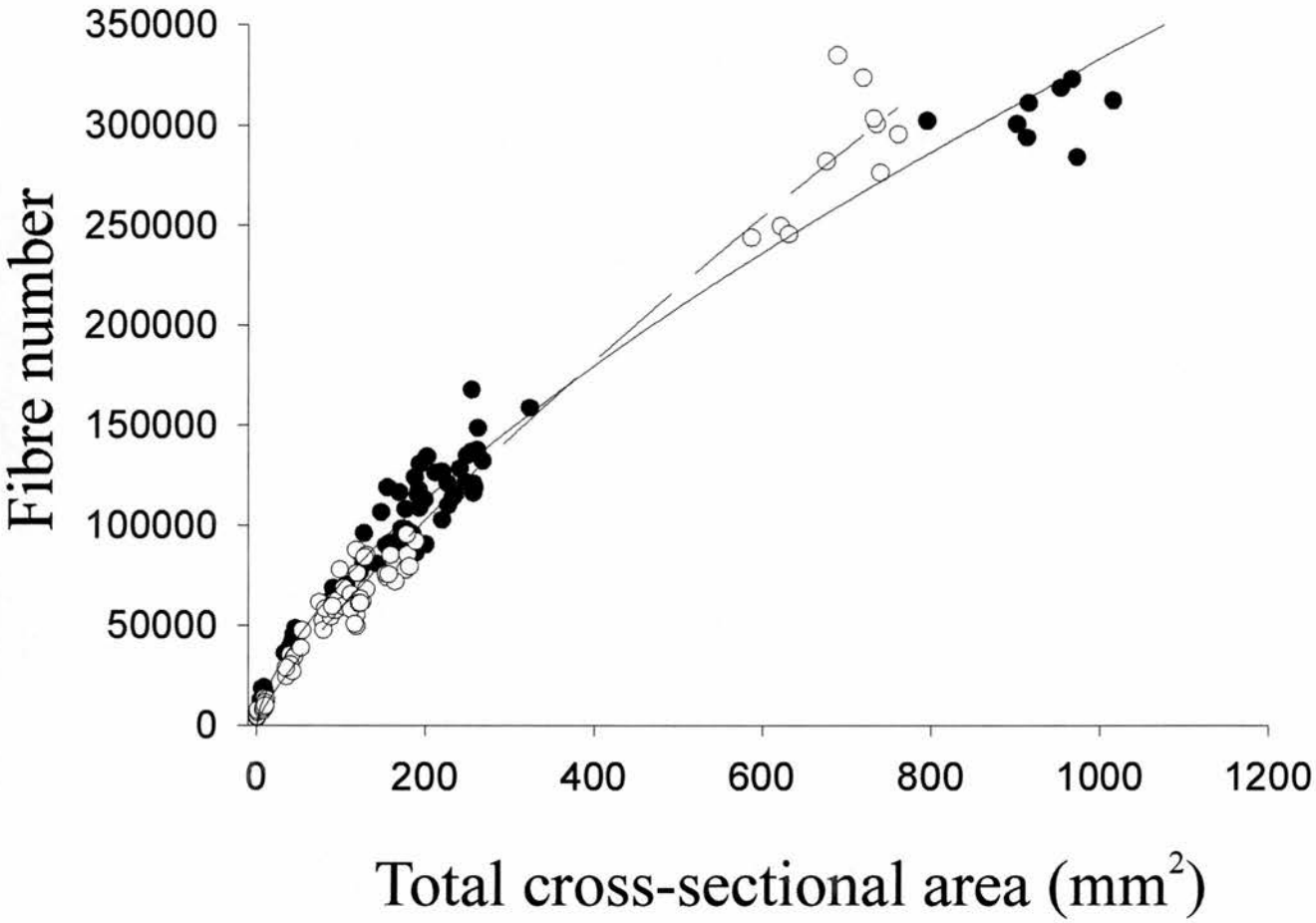


Fig. 2.13 Total cross-sectional area plotted against muscle fibre number in Atlantic salmon incubated in heated (closed symbols) and ambient (open symbols) water temperatures

Stage	Heated incubation		Ambient incubation	
	Replicate 1 [fork length]	Replicate 2	Replicate 1	Replicate 2
S1-Parr	39,538±1655 [9.4 cm]	42,463±1019 [9.7 cm]	10,382±878 [5.46 cm]	10,283±355 [5.59 cm]
1st winter in fresh water	110,753±6019 [17.7 cm]	108,841±6343 [18.0cm]	70,014±3944 [12.7 cm]	68,854±4620 [13.1 cm]
Smolt (prior to transfer)	124,992±2899 [20.1 cm]	135,806±6371 [20.9 cm]	79,738±1708 [16.5 cm]	82,970±3285 [16.9 cm]
6 months following transfer	305,477±13,116 [34.1 cm]	336,888±14,831 [36.0 cm]	275,419±9902 [29.8 cm]	295,614±10,356 [28.8 cm]

Table 2.5 Number of white muscle fibres per myotome at various stages of the life cycle of Atlantic salmon (*Salmo salar* L.) hatched from eggs incubated in heated and ambient temperature water. Values represent mean ±SE (n=5). All measurements were made at the level of the adipose fin. The average fork length for each stage is shown in square brackets. Results of ANOVA given in Table 2.2.

Muscle fibre hypertrophy

Hypertrophic growth was investigated by calculating the smoothed probability density function (pdf) of muscle fibre diameter for each fish using a sample size of 400 fibres (Figs 2.14 –2.16). For further statistical analysis, 10 fish from each treatment group were compared and the mean smoothing parameter calculated (values for the mean smoothing parameters used are given in Table 2.6). The mean probability density function was calculated for fish at each sample date and is shown as the solid line (Figs.2.14 – 2.16). Boot-strap techniques were used to construct a variability band (Figs. 2.17-2.19) (Johnston *et al.*, 1999) in order to provide evidence on the structure of the underlying fibre distributions. At hatch, the peak muscle fibre diameter was between 10 and 12μm in both heated and ambient groups (Figs 2.20a,b). By first feed, fish in the ambient group had a thicker right tail in the fibre distribution and analysis of the 50^{th} and 95^{th} percentiles revealed significantly higher diameters in the ambient group when compared to the heated group (Wilcoxon rank-sum test $P<0.01$) (Table 2.7).

Muscle fibre distributions were similar between groups by August 1997 with peak muscle fibre diameters of 28-30μm (Fig.2.20c). Increased hypertrophy in the right tail of the distribution was apparent in the heated groups by October 1997 (Fig 2.21a) with a significantly higher 50^{th} percentile (Fig 2.24a.) of 39.1μm compared to 36.0μm in the ambient group (Table 2.7). The mean diameter of all percentiles increased steadily from hatch until October 1997 indicating that fibre hypertrophy was the dominant muscle growth process during this period (Figures 2.23-2.24). The values of the 5^{th},10^{th},50^{th} and 95th percentiles of muscle fibre diameter decreased markedly as water temperature fell between November 1997 and May 1998(Figs 2.23 and 2.24). During the winter period (November to April), the 50^{th} percentile fell from

39.09μm to 22.82μm in the heated and from 36.00μm to 21.30μm in the ambient group indicating a substantial shift away from hypertrophy and towards fibre recruitment as the dominant muscle growth process (Fig. 2.24a)

Following seawater transfer in June 1998 and a seasonal rise in water temperature, hypertrophy became the dominant muscle growth process with mean diameters in all percentiles increasing rapidly by November (Figs. 2.23 and 2.24). Using evidence of the probability plots, muscle fibre distributions were unimodal from hatch until the final sample in November 1998 (Figs 2.14 to 2.16). Some evidence did suggest however, pre-smoltification bi-modality appearing in the distribution of fibres in the heated group at 382d post hatch (Fig.2.15f) and in the ambient group at 375d post hatch (May 1998, Fig. 2.16c)

Sample data	Number of fish per group	Smoothing parameter (h)	
		Heated	Ambient
Hatch	10	0.090	0.090
First feed	10	0.137	0.136
August 1997	10	0.081	0.081
October 1997	10	0.100	0.104
December 1997	10	0.106	0.115
February 1998	10	0.113	0.116
April 1998	10	0.108	0.116
May 1998	10	0.121	0.131
November 1998	10	0.114	0.105

Table 2.6 Samples sizes and values of mean smoothing parameter (h) used in the analysis of muscle fibre distributions

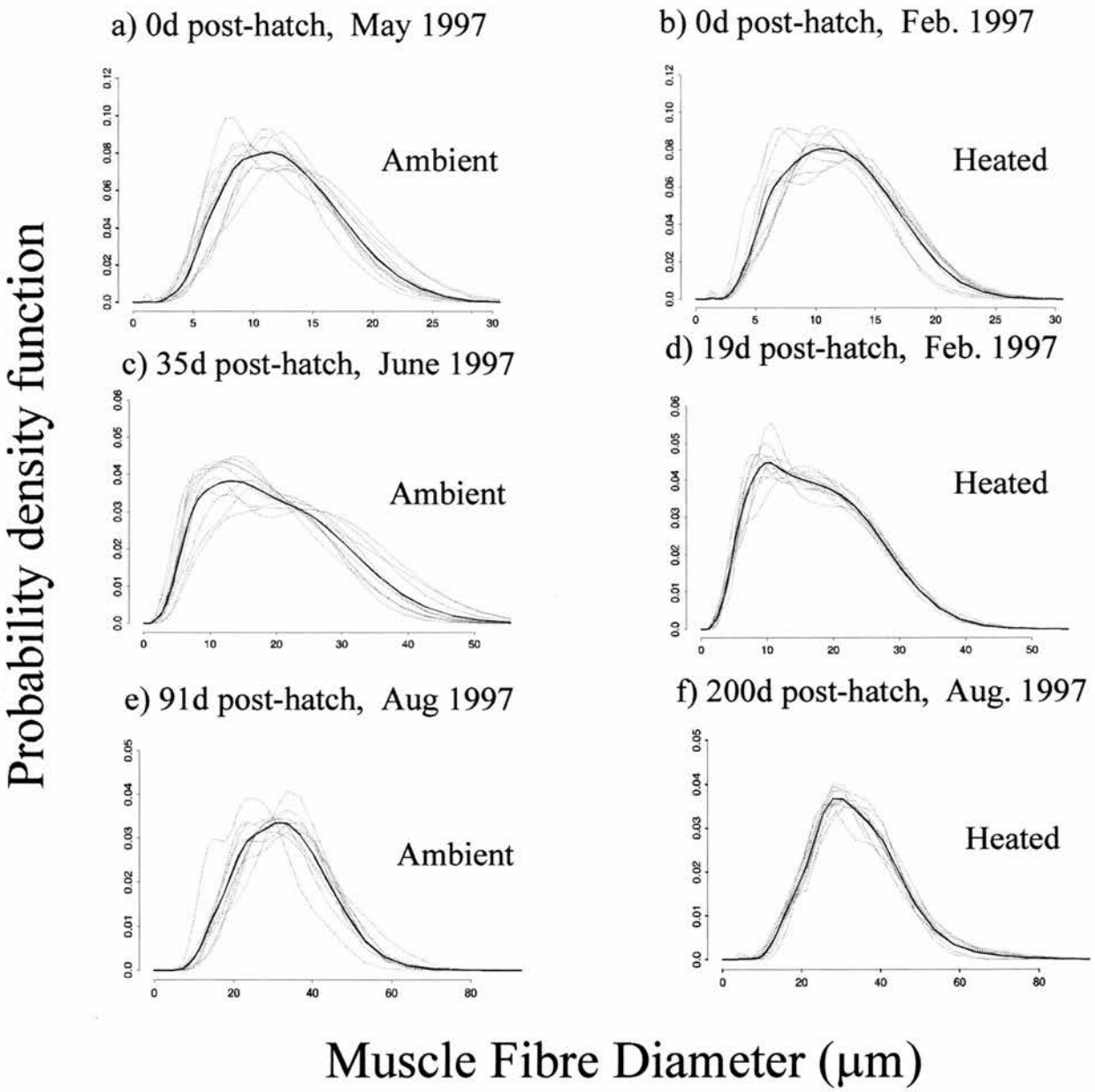


Fig. 2.14 Probability density function of white muscle fibre diameter for Atlantic salmon salmon incubated at heated and ambient water temperatures. The dotted lines show individual fish and the solid line the average probability density function calculated using the mean smoothing parameter (see Table 2.6).

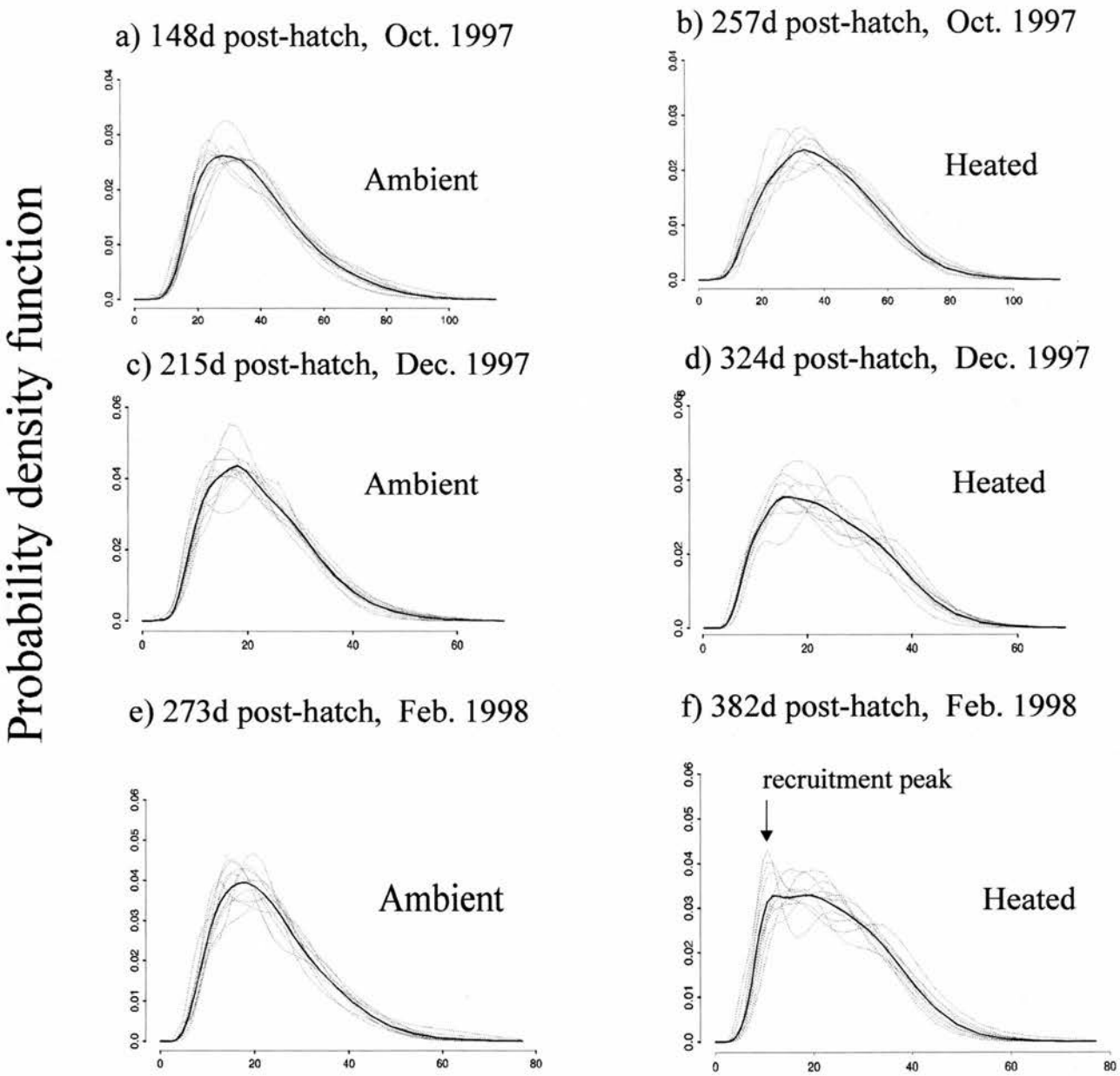


Fig. 2.15 Probability density function of white muscle fibre diameter for Atlantic salmon salmon incubated at heated and ambient water temperatures. The dotted lines show individual fish and the solid line the average probability density function calculated using the mean smoothing parameter (see table 2.6)

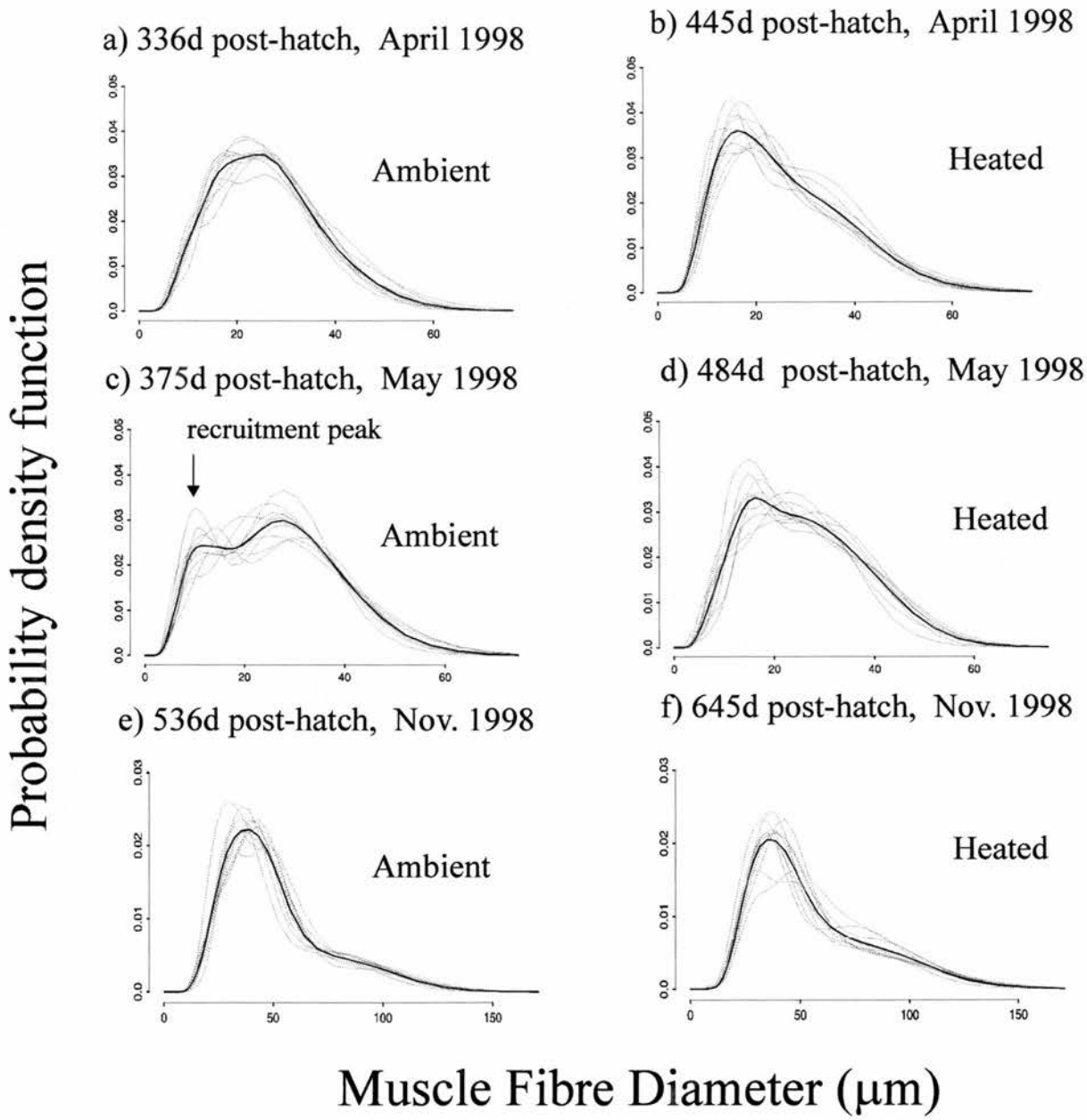


Fig. 2.16 Probability density function of white muscle fibre diameter for Atlantic salmon salmon incubated at heated and ambient water temperatures. The dotted lines show individual fish and the solid line the average probability density function calculated using the mean smoothing parameter (see Table 2.6)

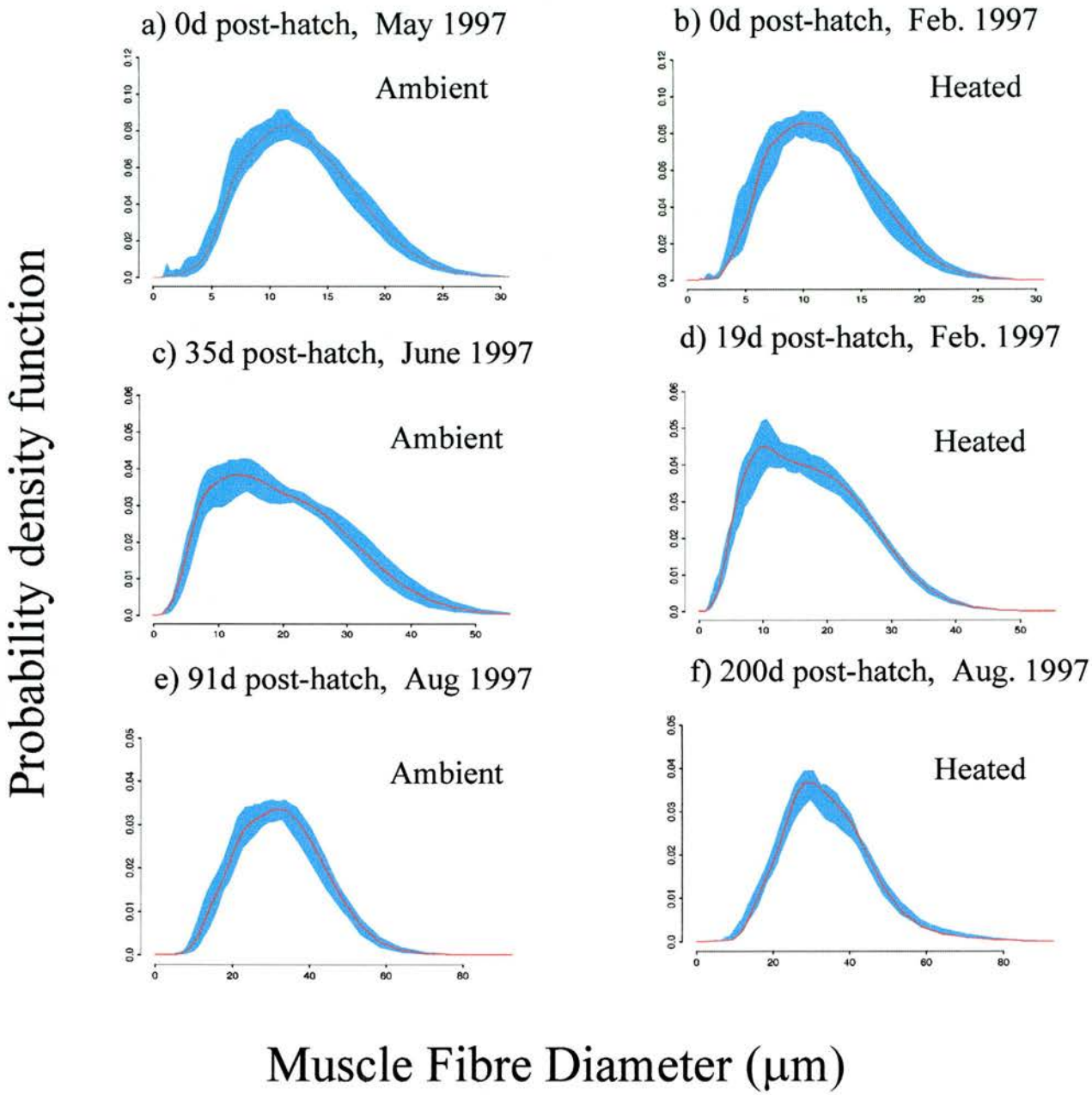


Fig. 2.17 Bootstrap estimates of the variance of probability density function of white muscle fibre diameter for Atlantic salmon incubated at heated and ambient water temperature. The blue shaded area corresponds to the variability band of the 100 boot strap estimates and the red line the mean probability density function.

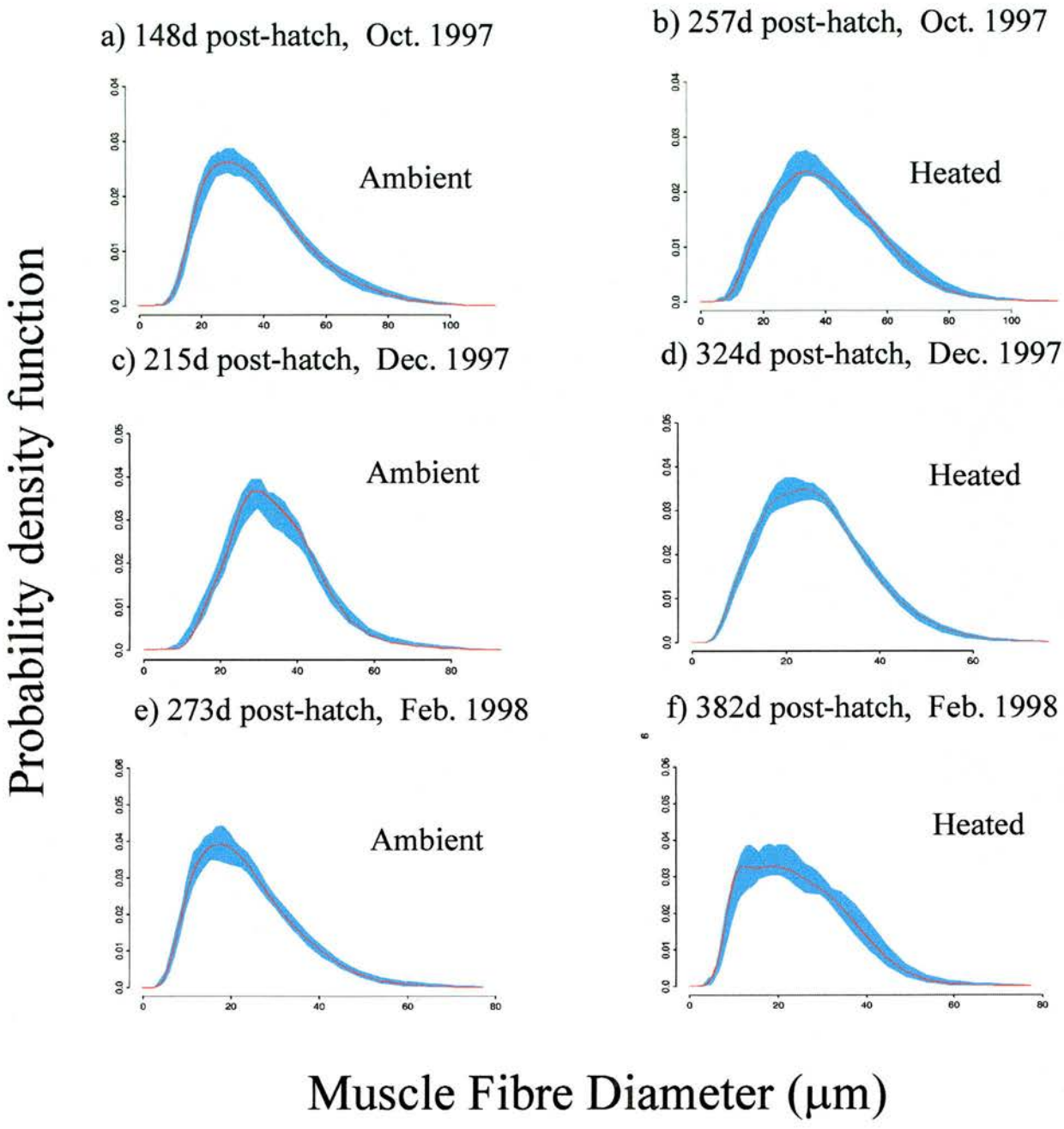


Fig. 2.18 Bootstrap estimates of the variance of probability density function of white muscle fibre diameter for Atlantic salmon incubated at heated and ambient water temperature. The blue shaded area corresponds to the variability band of the 100 boot strap estimates and the red line the mean probability density function.

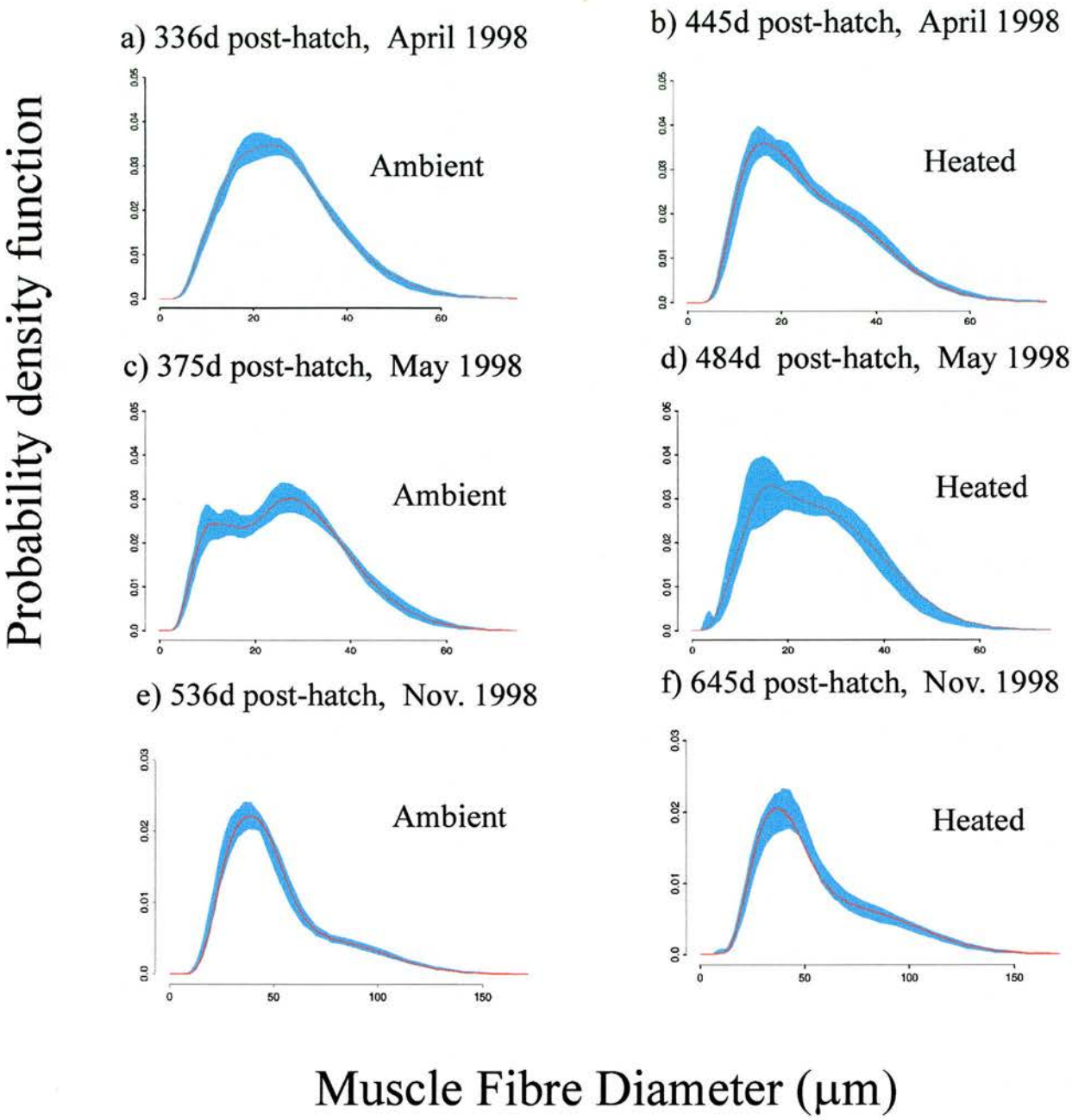


Fig.2.19 Bootstrap estimates of the variance of probability density function of white muscle fibre diameter for Atlantic salmon incubated at heated and ambient water temperature. The blue shaded area corresponds to the variability band of the 100 boot strap estimates and the red line the mean probability density function.

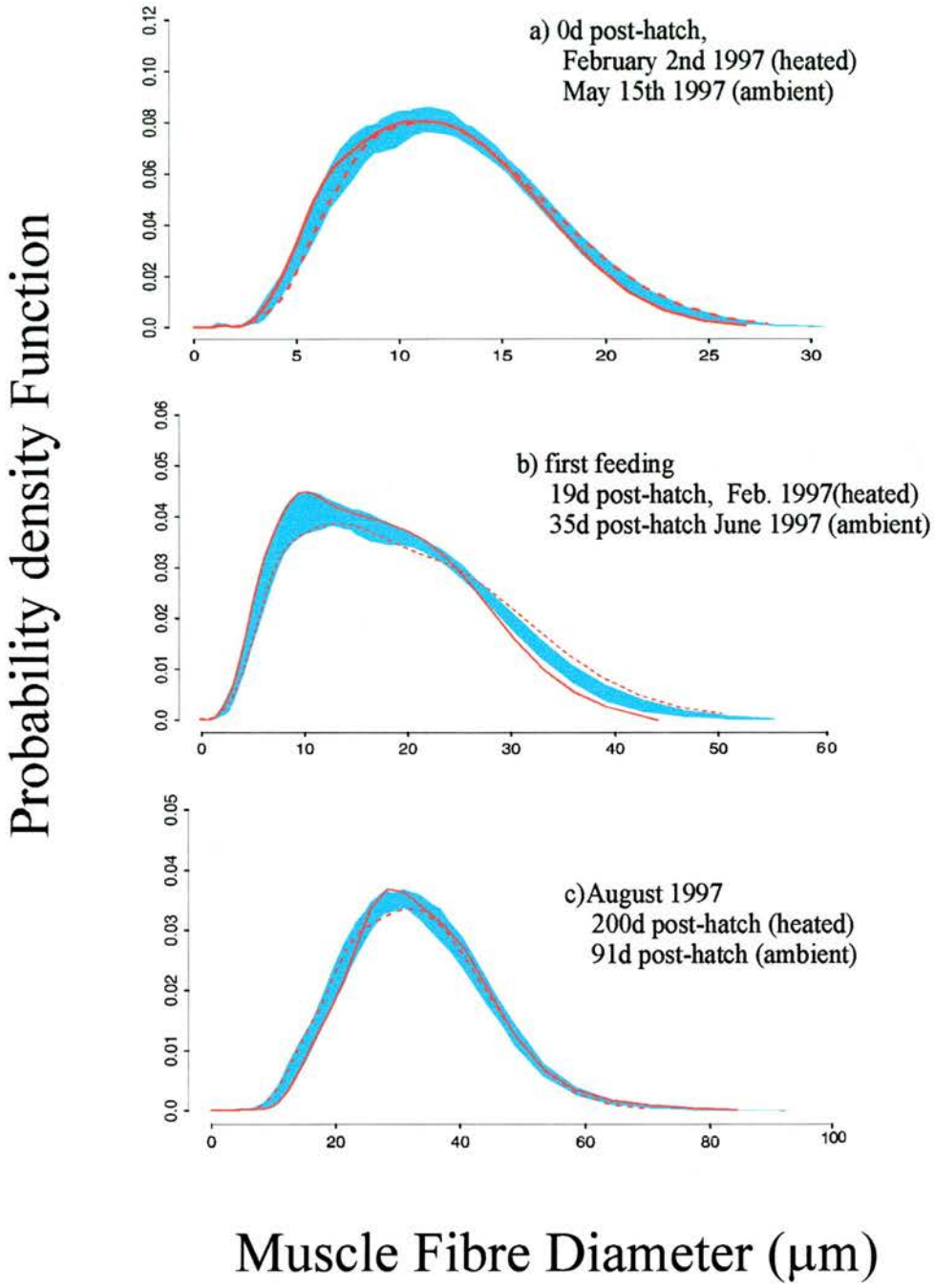


Fig.2.20 Reference band (shaded area) representing the variation band for the combined probability density functions of muscle fibre diameter in Atlantic salmon incubated in heated (solid line) and ambient (dotted line) water temperatures

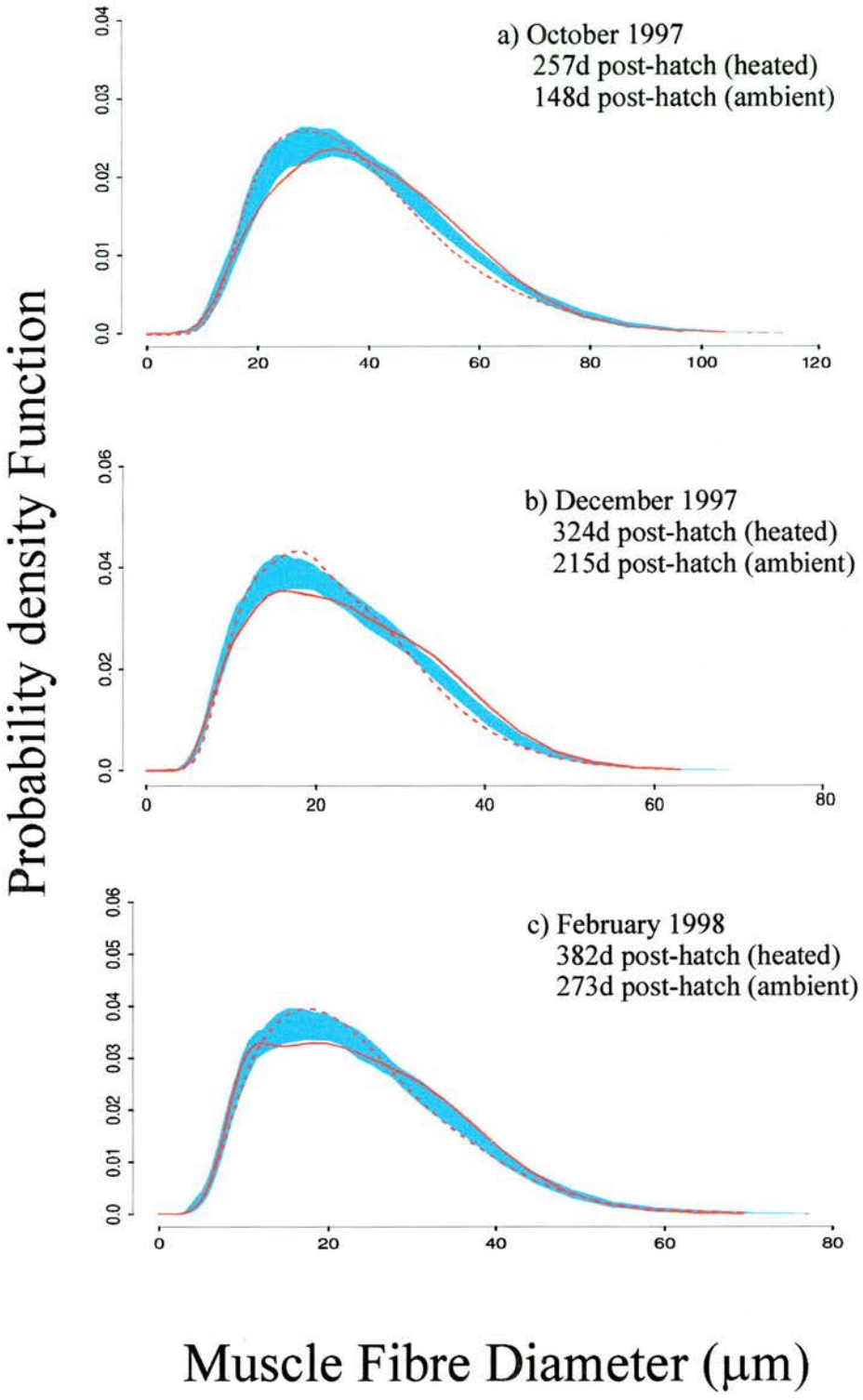


Fig.2.21 Reference band (shaded area) representing the variation band for the combined probability density functions of muscle fibre diameter in Atlantic salmon incubated in heated (solid line) and ambient (dotted line) water temperatures

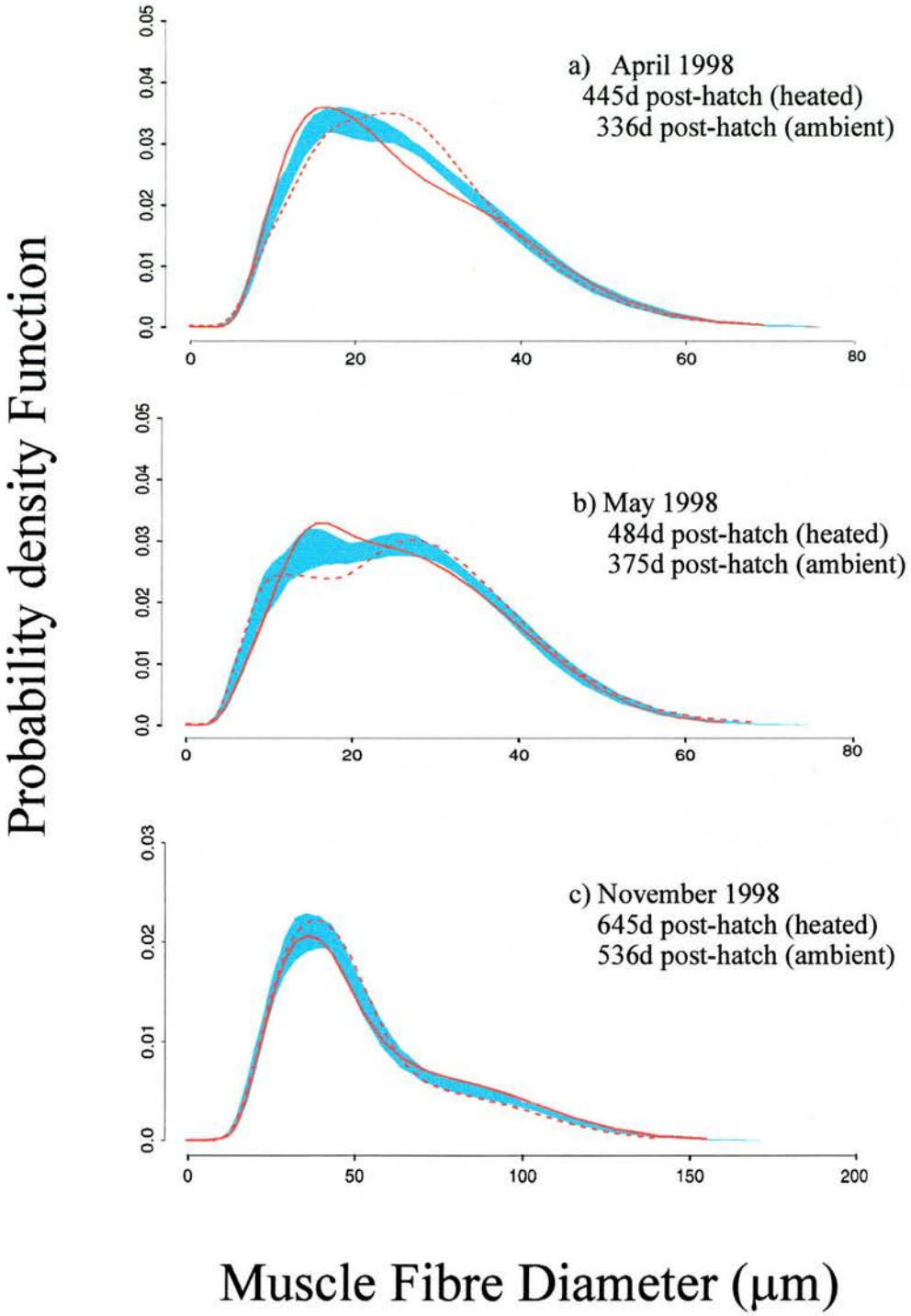


Fig.2.22 Reference band (shaded area) representing the variation band for the combined probability density functions of muscle fibre diameter in Atlantic salmon incubated in heated (solid line) and ambient (dotted line) water temperatures

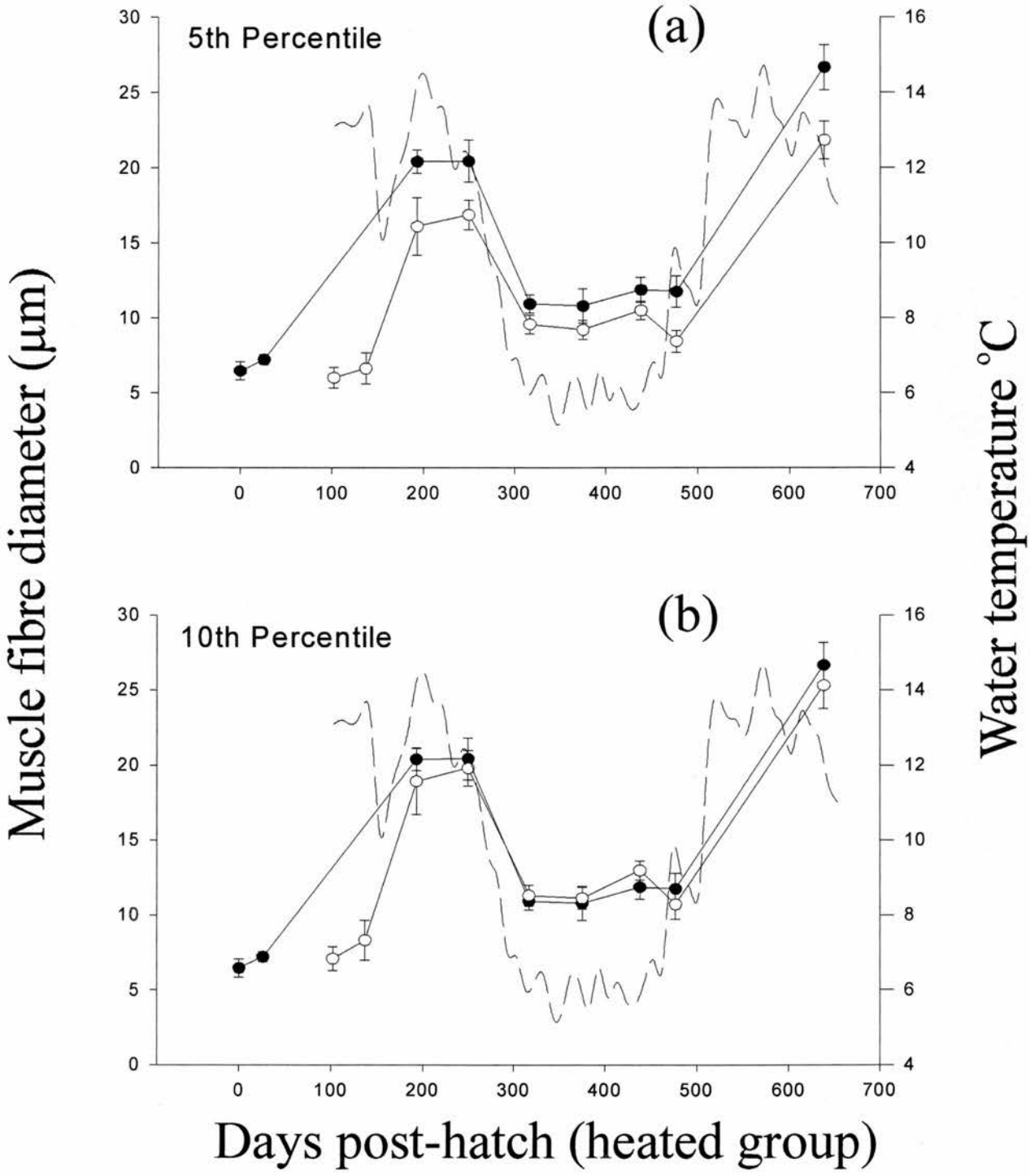


Fig.2.23 The (a) 5th and (b) 10th percentiles of muscle fibre diameter calculated from the probability density functions of individual fish incubated at heated (filled circles) and ambient (open circles) water temperatures. Significant differences ($P<0.05$) are denoted for each sample point in Table 2.7.

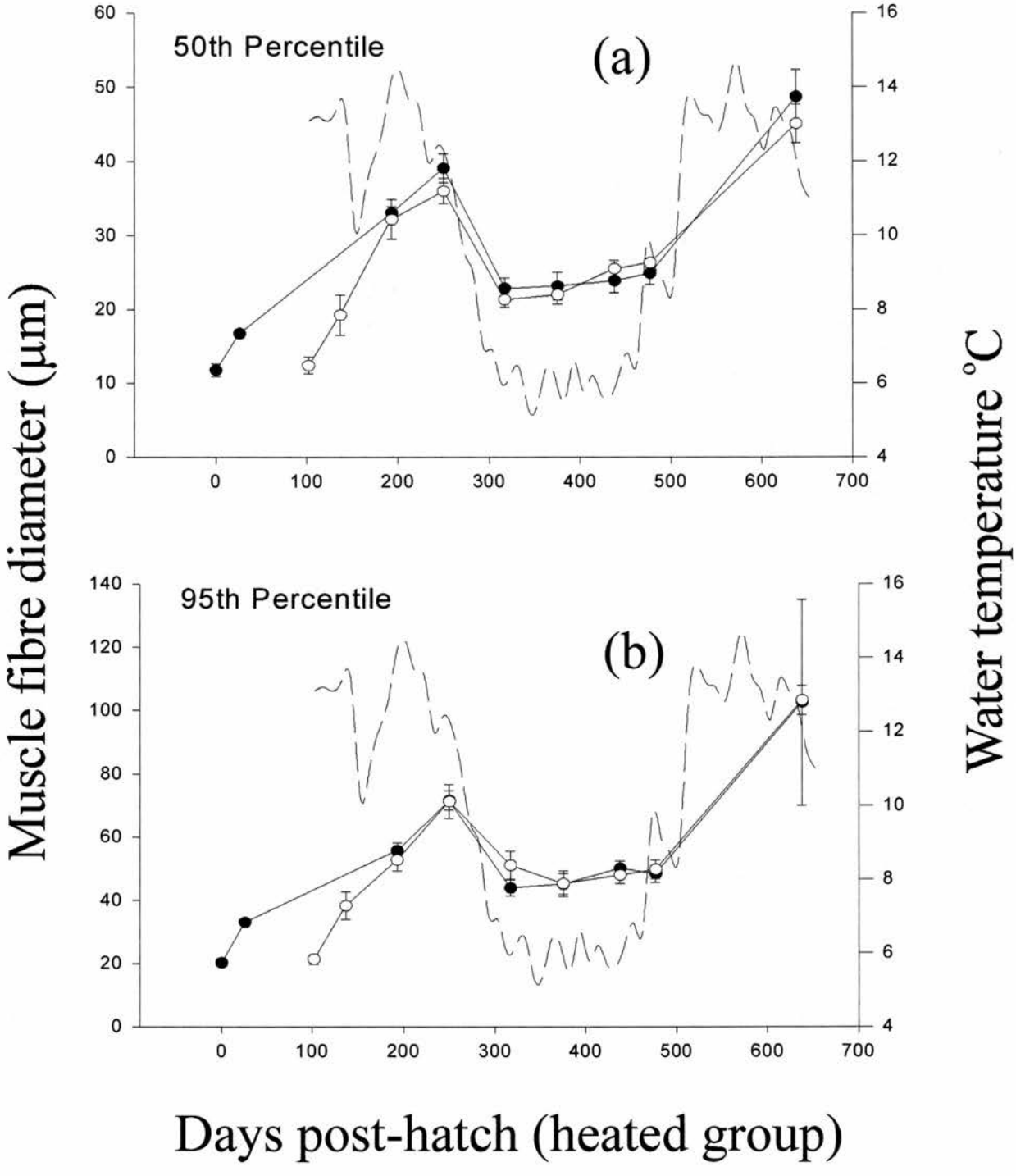


Fig.2.24 The (a) 50th and (b) 95th percentiles of muscle fibre diameter calculated from the probability density functions of individual fish incubated at heated (filled circles) and ambient (open circles) water temperatures. Significant differences ($P<0.05$) are denoted for each sample point in Table 2.7.

Age (d) post-hatch Heated	Ambient	Percentile	Wilcoxon Test P-value	Percentile for f^H	Percentile for f^A
0	0	.05	0.04	5.43	4.32
		.10	0.06	6.46	5.18
		.50	0.19	11.77	10.05
		.95	0.09	20.27	18.03
19	35	.05	0.01	5.68	6.61
		.10	0.02	7.21	8.32
		.50	0.01	16.74	19.25
		.95	0.01	33.08	38.32
200	91	.05	0.07	17.36	16.08
		.10	0.06	20.39	18.92
		.50	0.34	33.05	32.18
		.95	0.06	55.69	52.83
257	148	.05	0.90	16.90	16.85
		.10	0.29	20.42	19.79
		.50	0.001	39.09	36.00
		.95	0.88	71.60	71.29
324	215	.05	0.03	8.93	9.56
		.10	0.20	10.91	11.30
		.50	0.01	22.82	21.30
		.95	0.001	43.89	51.05
382	273	.05	0.57	8.97	9.19
		.10	0.43	10.78	11.13
		.50	0.13	23.13	21.97
		.95	0.92	45.09	45.26
445	336	.05	0.05	9.86	10.48
		.10	0.004	11.86	12.97
		.50	0.02	23.88	25.48
		.95	0.08	50.09	47.99
484	375	.05	0.02	9.52	8.42
		.10	0.03	11.74	10.70
		.50	0.01	24.87	26.30
		.95	0.29	48.40	49.78
645	536	.05	0.11	22.76	21.81
		.10	0.06	26.65	25.30
		.50	0.02	48.69	45.07
		.95	0.01	102.56	103.21

Table 2.7 Comparison of the percentiles for the average probability density functions of muscle fibre diameter in heated incubation (f^H) and ambient incubation (f^A)Atlantic salmon.

2.4 Discussion

Temperature has been shown to exert a dramatic influence on fibre recruitment during hatch (Stickland *et al* 1988, Johnston and McLay 1997) and first feeding Atlantic salmon (Usher *et al* 1994). The main finding of the present study was that the influence of egg incubation temperature extends to the satellite cell phase and may affect muscle fibre growth in seawater up to 2 years following hatch. Fish reared at higher water temperatures hatched earlier and had fewer fibres although with a similar diameter to those reared at ambient temperatures. Similar findings were reported for other populations of the same species by Johnston and McLay (1997) and Stickland *et al.* (1988). Usher *et al.* (1994), referring to a paper by Cheek and Hill (1970), suggested that hyperplasia was favoured at lower temperatures because a lower metabolic rate would allow re-allocation of energy reserves to drive the metabolically expensive process of hyperplasia, requiring both protein synthesis and nuclear divisions. Following exhaustion of the yolk sac and the onset of exogenous feeding in the current experiment, fibre number and diameter in the heated and ambient groups were not significantly different, indicating a "catch up" in muscle fibre recruitment in the ambient group. Johnston and McLay (1997) reported similar findings for Atlantic salmon and also found family variation in response to incubation temperature.

Following first-feeding, marked differences in the patterns of fibre recruitment and hypertrophy were apparent between treatments during fresh water and sea water growth. The ambient group displayed increased fibre recruitment between the S1 parr stage in October 1997 and sea water transfer in June 1998 relative to the heated group, indicating a significant "catch up" in fibre recruitment. However, in absolute terms,

the heated fish had more muscle fibres per myotome at smoltification (Table 2.5). The number of white muscle fibres per myotome had increased more in both groups 6 months following sea water transfer than in the whole of fresh water growth. A similar, rapid increase in muscle fibre number in Atlantic salmon following transfer to sea water was found by Johnston *et al.*, (1999b). Following seawater transfer however, greater fibre recruitment in the ambient group than the heated group resulted in a similar number of muscle fibres per myotome by the end of the experiment. Higher body mass in the heated group may be accounted for by earlier fibre recruitment followed by rapid hypertrophy in seawater. The heated group fish therefore had the same number of fibres per myotome as the ambient group but greater hypertrophy in the heated group following transfer resulted in a higher average fibre diameter contributing towards greater body mass. Weatherley *et al.*, (1988) suggested that nutrient assimilative capacity by fibres is a function of their surface areas. High surface area to volume ratios will enable amino acid assimilation to occur at high rates and small fibres will therefore hypertrophy more rapidly than larger ones and result in higher growth rates. An earlier recruitment of fibres in the heated group would, therefore, provide a large surface area to volume ratio in the myotome, providing the basis for increased assimilative capacity and rapid somatic growth by hypertrophy.

Higgins and Thorpe (1990) found a distinct period of recruitment prior to smoltification in S1 salmon parr. Smoltification is associated with an increase in growth hormone (Hoar, 1988) and Fauconneau *et al.*, (1997), found that growth hormone administration in rainbow trout increased the proportion of small diameter fibres suggesting a possible role in regulating fibre recruitment. In the present study,

ambient group fish displayed a peak of fibres of around 10μm in May 1998 prior to smoltification (Fig.2.15f) while a similar peak was seen three months earlier in the heated group (Fig.2.16c). In larger salmon, a distinct modality can be found in the muscle fibre frequencies as smaller, newer fibres enter the left side of the distribution and larger, older fibres hypertrophy and move to the right (Johnston *et al.*, 2000a,b). However, in the smaller fish studied in the current experiment, a lack of distinct modality in the distributions suggests that once recruited, new fibres are subject to hypertrophy and quickly move towards the right tail of the distribution of diameters (Johnston *et al* 1999b, Weatherley *et al.*, 1988).

In rainbow trout (*Oncorhyncus mykiss*) and bluntnose minnow (*Pimephales notatus Rafinesque*), experimentally induced differences in somatic growth rate are matched by proportional adjustments in recruitment and rates of hypertrophy of the growing muscle (Weatherley and Gill, 1988). It has been hypothesized that the relationship between muscle fibre frequency and body size ensures muscle tissues meet the functional and structural requirements imposed by a particular body size (Gill *et al.*, 1989). For any given fish length, the frequency distribution pattern of fibre diameter classes is conserved (Weatherley *et al.,* 1979, Weatherley and Gill, 1984). However, this is not the case in Atlantic salmon subjected to ploidy manipulation. For any given fork length, triploid Atlantic salmon have fewer but larger muscle fibres than normal diploid fish (Johnston *et al.,* 1999) while ambient group fish in the present study had more muscle fibres per cross-sectional area at the end of the experiment than heated group fish. This suggests that the response of muscle growth to environmental influences may be more plastic than suggested by Weatherley *et al.,* (1988). Variation in key environmental variables such as temperature during specific

ontogenic 'windows' may therefore lead to significant changes in muscle growth pattern during later life stages.

It is known that appetite, feeding and growth all increase with temperature and specifically the number of degree-days post-hatch (Austreng *et al.*, 1987; Holmefjord *et al.,* 1995). Body mass and the relative timing of hypertrophy and hyperplasia in the two groups suggests that both the somatic and muscle growth program were 'advanced' in the heated group. Evidence for seasonal cycles of recruitment and expansion of muscle fibres have been found in Atlantic salmon (Suresh and Sheehan 1998, Johnston *et al.,* 1999a, 1999b) and in fish of the genus Mugilidae (Carpene and Veggetti, 1981). Muscle fibre frequency distributions have been used widely to infer distinct cycles of muscle fibre hypertrophy and hyperplasia in fish (Weatherley *et al.,* 1980; Stickland, 1983; Higgins and Thorpe, 1990; Kiessling *et al.,* 1991). Periods of phased hyperplastic growth have been described in the sea bream (*Sparus aurata*) Rowlerson *et al.,* (1995), rainbow trout (*Oncorhyncus mykiss*) (Weatherley and Rogers, 1979; Stickland, 1983), eel (*Anguilla anguilla*) (Romanello *et al.*, 1987). Percentile plots derived from muscle fibre frequency distributions in the current experiment reveal a distinct seasonality in the muscle growth process. The decrease in the 5^{th} and 10^{th} percentile fibre diameter in both groups during winter and spring, indicate a relative increase in the contribution of new fibre recruitment associated with seasonal lows in water temperature and day-length. The absolute increase in fibre number during this period was, however small and the reduction in percentile values probably reflects a relative increase in fibre recruitment relative to hypertrophy. Higgins and Talbot (1985) found no measurable weight gain during the winter months in upper modal group Atlantic salmon, although an increase in body length was found during winter. Lengthening of the skeletal frame and a continual

'background' recruitment of muscle fibres during this winter period may be seen as a preparatory growth phase, which lays the foundations for future growth. A decreased contribution of fibre hypertrophy and a subsequently reduced growth rate are in agreement with the finding of Kiessling *et al.*, (1991), who concluded in a long term study of muscle growth in the rainbow trout, that hypertrophy was closely linked to periods of rapid growth and higher temperatures.

Kiessling *et al.*, (1991) concluded that the white muscle as well as the epaxial muscle as a whole, grows linearly with the whole fish. The deviations from the linear relationship, which denote a high specific growth rate suggest a more rapid fibre enlargement in fast than in slow growing fish. Valente *et al.*, 1998 however, found a higher concentration of muscle DNA in a fast growing strain of rainbow trout suggesting a smaller cell size and larger number of cells per unit weight of tissue. A faster decrease of DNA concentration, with increasing body weight in a slower growing strain of rainbow trout reflected a lower percentage of hyperplasia and consequently a lower capacity for growth. The results of the current experiment are in agreement with those of Kiessling *et al.*, (1991) and suggest that cross-sectional area growth of muscle in Atlantic salmon is highly seasonal and takes place by phased fibre recruitment followed by rapid hypertrophy. Future studies on fish muscle should take this take this into account since the sampling period could significantly influence results.

The nuclei required for muscle growth arise from the muscle stem cell population (Johnston, 1999). Each muscle stem cell (satellite cell) is thought to undergo an asymmetric division to regenerate the stem cell and produce a myoblast

committed to differentiation after a limited number of further divisions (Quinn *et al.* 1988, Schultz, 1996). The myoblasts are absorbed into existing fibres to maintain the nuclear to cytoplasmic ratio as they hypertrophy (Koumans *et al.*, 1994;Johnston *et al.*, 1998). The regulation of hypertrophic growth is thought to be controlled by processes governing the availability of myonuclei (Johnston, 1999). These include the number of times myoblasts divide prior to terminal differentiation and/or their cell cycle time. *In vitro* studies by Matschak and Stickland (1995) found that increased temperature led to an increase in differentiation rate and growth of isolated myosatellite cells. Raised water temperatures and therefore metabolic rates during late summer would decrease cell cycle times allowing greater proliferation of myoblasts and, therefore, increased hypertrophy similar to that seen in the heated group in sea water.

There is evidence therefore, for an endogenous programme of muscle growth in the Atlantic salmon with distinct periods of recruitment and hypertrophy (Fauconneau *et al.,* 1997, Suresh and Sheehan, 1998; Beattie *et al.,* 2000; Johnston *et al.,* 2000a,b). This program is plastic to changes during specific ontogenic windows such as egg incubation by environmental variables including temperature. Hurling *et al.,* 1996, reported a positive correlation between average muscle fibre diameter and sensoric firmness in several species of fish. Johnston and co-workers found that muscle fibre density in Atlantic salmon accounted for up to 40% of the total variation in visual pigment score (Roche SalmoFan™) and concluded that fibre density was linked with a firmer texture (Johnston *et al.,* 1999a; 1999b). Hurling *et al.*, (1996) reported a positive correlation between average muscle fibre diameter and sensoric firmness in several species of fish. Johnston *et al.*, (2000b) found that muscle fibre

density in Atlantic salmon accounted for up to 40% of the total variation in visual pigment score (Roche SalmoFan™) and that there was a significant positive correlation between fibre density as measured by taste panel and the 'firmness' of the final product.

The manipulation of hatchery temperature may produce long term changes in phenotype which impact on flesh quality, particularly textural characteristics. Furthermore, seasonal variations in flesh pigmentation are known to occur in farmed salmon. The so-called 'spring drop' results in a reduction of pigmentation in salmon following their first winter at sea, leading to downgrading at harvest. The findings of the current experiment suggest that this phenomenon could be a result of increased muscle recruitment during winter and early spring which somehow 'masks' pigment already laid down in existing muscle fibres. The manipulation of muscle hypertrophy through changes in diet, broodstock selection or husbandry may, therefore provide a possible solution to this costly problem.

However, the ecology of the Atlantic salmon is not driven by temperature alone. Temperature is a highly variable and therefore 'noisy' environmental cue and salmon require a more reliable means of synchronising their physiological and behavioural cycles. The additional cue is photoperiod, which may be manipulated in a number of ways to affect the development and therefore production of Atlantic salmon.

Chapter 3

ADVANCED PHOTOPPERIOD TREATMENT AFFECTS SMOLTIFICATION AND MUSCLE GROWTH IN ATLANTIC SALMON (*Salmo salar* L.)

3.1 Introduction

Salmonids, like many other temperate zone animals have a seasonally changing physiology that is manifest in cycles of growth, precisely timed migrations and seasons of reproduction (Hoar, 1965). Atlantic salmon undergo a remarkable metamorphosis, the parr-smolt transformation when fresh water juveniles become prepared for ocean migration and a marine existence (for review see Hoar, 1988; Wedemeyer *et al.*, 1980). The parr-smolt transformation of Atlantic salmon involves fundamental behavioural, morphological, biochemical and physiological changes in addition to a pronounced increase in growth, which transforms the dark bottom dwelling parr to a silvery pelagic smolt prepared for life in the ocean (Dickhoff and Sullivan, 1987; McCormick and Saunders, 1987; McCormick *et al.*, 1987 and Thorpe, 1987).

Smolting is usually completed in spring and is generally the only time of the year that juvenile salmon (parr) can be safely transferred to seawater. The efficiency of Atlantic salmon culture is restricted by the seasonal availability of smolts and for practical reasons, the abbreviation of the parr stage remains a major objective of salmon farmers. To stabilise annual production and reduce hatchery rearing time, photoperiod manipulation is increasingly used to produce under-yearling or (0+) smolt capable of

being transferred to sea water in autumn following hatch (Clarke, 1989; Duston and Saunders, 1990b; Gaignon and Quemener, 1992; Duston and Saunders, 1995).

Photoperiod is the primary entraining factor of the endogenous rhythm controlling growth and smoltification in Atlantic salmon via the 'light-pituitary axis' which initiates endocrinal changes associated with parr-smolt transformation (Komourdjian, 1976; Villareal *et al.,* 1988). Transduction of light signals takes place through peripheral detectors (retina, pineal and mid-brain surface) to the nucleus *lateralis tuberis* of the hypothalamus, from which neurendocrine transmissions via the pituitary induce physiological responses to light change (Lundqvist, 1983). Although growth rates are improved by rearing the fish under artificially extended photoperiods, a decrease in photoperiod is necessary to initiate the smolting process which is completed under increasing daylength (Bjornsson *et al.,* 1989; Berge *et al.,* 1995; Skilbrei *et al.*, 1997).

Weatherley and Gill (1988) concluded that experimentally induced differences in somatic growth rate are closely matched by proportional adjustments in muscle fibre recruitment and rates of hypertrophy of the growing muscle. Johnston *et al.,* (1998) however, found that changes in environmental variables can drastically alter the cellularity of muscle in the Atlantic herring (*Clupea harengus*). Teleost fish display a different pattern of muscle growth, which distinguishes them from that in higher vertebrates (Greer-Walker, 1970). In contrast to mammals, teleost muscle may grow by both increasing the size of existing fibres (hypertrophy) and by the recruitment of new fibres (hyperplasia) (Willemse, 1976; Weatherley and Rogers, 1978; Weatherley *et al.*,

1979; Stickland 1983). New fibres are formed on the surface of larger diameter fibres formed during a previous development stage, giving the muscle a mosaic appearance in transverse section (Weatherley *et al.*, 1979; Carpené and Vegetti, 1981; Stickland 1983; Romanello *et al.*, 1987 ; Talesera and Urfi , 1979; Scaopolo *et al.*, 1988; Vegetti *et al.*, 1993).

Little is known of the changes in muscle growth which result from increased growth rates induced by advanced photoperiod treatment. It is known, however, that muscle fibre cellularity can influence final product texture in Atlantic salmon (Johnston *et al.*, 2000a,b) and a variety of marine species (Hurling *et al.,* 1996). With increasing production of salmon from 0+ smolt stock in the Scottish salmon farming industry, increased growth rates and changes in muscle cellularity related to the use of advanced photoperiods may have a significant impact upon harvest quality. This study sets out to investigate the changes in growth and muscle cellularity which result from the use of advanced photoperiods under semi- commercial farming conditions.

3.2 Materials and Methods

3.2.1 Fish stock and rearing conditions

Atlantic salmon eggs from the hatchery stock at Matre Aquaculture Research Station, Norway (61°N) hatched in January 1997, and fry were reared on continuous light (24L) from first feeding in March until the start of the experiment in July 1997. The experimental tanks were $1m^2$ square covered fibre-glass tanks with a rearing volume of 350L. Water flow was kept at 15L-min $^{-1}$. Light was supplied by two 18W flourescent

daylight tubes installed in the tank cover producing a light intensity of 960 Lux at the base of the tank. All groups were fed commercial dry feed (T. Skretting Ltd., Stavanger, Norway) in excess from automatic feeders according to temperature and fish size (Austreng 1987).

3.2.2 Experimental design

Fish from duplicated experimental tanks were distributed equally between three different photoperiod treatments on 16th July 1997 (mean body mass 18g ± 0.80 SE). One group (CL) remained on constant light throughout the experiment while two further groups, short winter (SW) and long winter (LW) were given a reduced daylength (12h light: 12h dark) for six weeks until 30th August 1997. SW fish were then exposed to a constant light treatment for a further 14 weeks; LW fish remained on short daylength (12h light: 12h dark) until 8th October 1997 and thereafter on constant light, until the termination of the experiment on 4th December 1997, (Fig. 3.1).

At monthly intervals, 100 fish from each treatment were anaesthetised using metomidate hydrochloride (Wildlife Pharmaceuticals Co. USA) and measured for fork length (nearest 0.1cm) and body mass (nearest 0.1g). Fulton's condition factor (K) was calculated according to Busacker *et al.* (1990) while specific growth rate was calculated according to Houde and Scheckter (1981) (see Chapter 2). Length growth (mm/d^{-1}) was calculated using the formula $lg=(l_2-l_1)/(t_2-t_1)$. Individuals with a body mass close to the average for 100 fish weighed, were sampled to investigate muscle growth. A photograph of each fish sampled was taken and later used to assess smolt status through the presence

or absence of parr markings, darkening of fin margins, the development of a marine pelagic smolt shading pattern and lengthening of the caudal peduncle (anal to caudal fin region) (Stefansson, 1990; Winans and Nishioka, 1987). Precocious sexual maturity was assessed in all fish taken for muscle sampling by running a finger along the ventral surface of the fish and checking for the release of milt. All fish sampled came from the upper modal group in each tank and no sign of precocious maturity was detected in any fish sampled for muscle analysis.

3.2.3 Quantification of muscle fibre growth

Fish sampled for muscle analysis were anaesthetised and killed by a sharp blow to the head. Transverse sections of the trunk were taken from a point immediately anterior to the adipose fin and photographed on a background scale to determine total white muscle cross-sectional area. The right side epaxial and hypaxial quadrants were then isolated, covered in Tissue-Tek (Shandon, Pitsburgh, PA 15275, USA) and frozen in 2-Methylbutane (iso-pentane) cooled to it's melting point in liquid nitrogen. Frozen muscle blocks were wrapped in aluminium foil, placed in sealed plastic bags and stored at -80°C until sectioning. Transverse sections were cut at 10-12μm on a Bright Starlet Microtome set to -20°C and mounted on poly-L-lysine coated slides (Sigma, Poole, Dorset UK). Muscle sections were air dried and then stained using Carb-aniline Red (Scarba red) before mounting in glycerol gelatin (Sigma, Poole, Dorset, UK).

The cross-sectional areas of white muscle fibres were measured at four main sites on the myotome (see Chapter 2 Materials and Methods). Measurements of fibre area were

acquired using an Image Pro 3.0 analysis system (Caltech. Co. USA) attached to a light microscope with X10 objective, and approximately 100 fibres per steak area (400 Fibres per fish) were measured. Total white muscle cross sectional area was plotted for each sample date while white muscle fibre number was estimated by dividing total white muscle cross sectional area by mean white muscle fibre cross-sectional area.

3.2.4 Statistics

Data were analysed using Statistica 5.1 software package (StatSoft Inc., Tulsa, USA). Data were checked for normal distribution using normal distribution plots, and for homogeneity in variance by Levenes test (Sokal and Rohlf, 1995). The effects of photoperiod treatment on length, body mass, condition factor and fibre number were tested using a one-way analysis of variance (ANOVA). Where significant differences were detected between treatments groups, a Post-hoc Tukey Kramer multiple comparison test was applied (Heath, 1995).

For details of muscle fibre frequency analysis, see Chapter 2: Materials and Methods

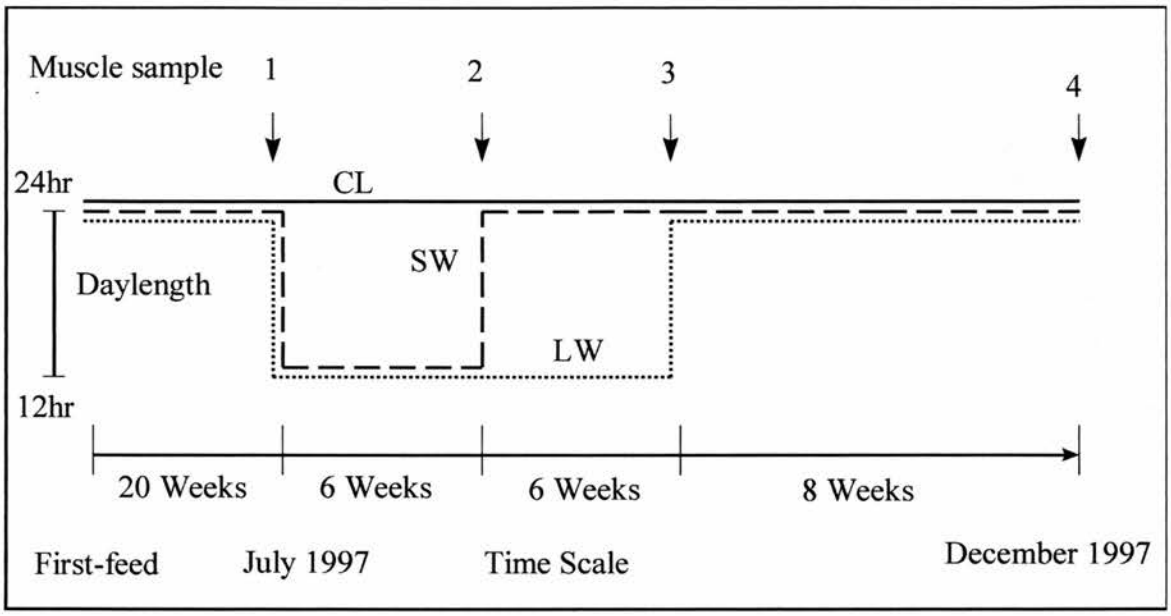


Figure 3.1 Outline of experimental rearing photoperiods, **CL**: 24hr light for 26 weeks (solid line) **SW**: 24hr light for 6 weeks, 12hr light for 6 weeks, 24hr light for 14 weeks (dashed line) and **LW**: 24hr light for 6 weeks, 12hr light for 12 weeks, 24hr light for 8 weeks (dotted line). Muscle sample points are indicated at throughout the rearing period.

3.3 Results

3.3.1 Somatic Growth

Fork length

Fish with a mean fork length (11.48cm ± 0.16) and body mass (18.41cm ± 0.73) (mean ± SE) were distributed evenly between tanks at the start of the experiment. Fork length of fish in all treatment groups then increased in a similar fashion with no significant differences until October (Fig. 3.2a), when the CL and SW group were significantly longer than the LW group ($F_{(2,198)}$=108.23, P<0.001). Following a return to constant light in October, the SW group displayed a marked increase in fork length, which was significantly higher than the other two groups by the end of the experiment ($F_{(2,198)}$ = 47.65, P<0.001). Length growth showed a close relationship with water temperature in fish held under CL with a gradual decrease as water temperatures fell (Fig.3.4a). Length growth in the SW and LW groups was not closely related to temperature however, and increased following a return to constant light in August and October respectively.

Body mass

Body mass increased with age in all groups from 9th July to 2nd December 1997 (Fig. 3.2b). Fish reared under constant light initially had a higher body mass than either of the simulated winter groups. However, a marked increase in body mass took place in SW fish following a return to constant light, surpassing that of the CL and LW groups. This difference in body mass persisted until the end of the experiment when the SW groups were significantly heavier (92.26g ± 2.99) than the CL (76.52g ± 3.62) or LW

(59.61g ± 2.62) groups (mean ± SE, n=100), ($F_{(2,198)}$ =34.25, P<0.001). Specific growth rate was initially higher in the CL group but decreased as water temperature fell towards the end of the experiment (Fig. 3.4b). In contrast, SGR in the SW and LW groups stabilised at points in the light program coincidental with an abrupt increase in daylength in late August and early October respectively.

Condition Factor

Condition factor showed an initial increase in all groups until September 1997 when condition factor fell markedly from around 1.3 to 1.1 in the SW group after a return to constant light following the simulated winter period (Fig. 3.3). Following a return to constant light in the LW group in October, condition factor decreased dramatically and showed a similar pattern of development to that seen several weeks earlier in the SW group. Condition factor in the constant light group remained relatively stable and decreased only slightly towards the end of the experiment.

3.3.2 Indicators of Smoltification

Table 3.1 shows indicators of smoltification in each group following 18 weeks of light treatment. Fish exposed to a short winter period displayed a significantly lower condition factor and higher mean anal to caudal fin length than fish from the two other treatment groups. Short winter fish also showed well developed silvering and a total absence of freshwater parr markings in October while the CL and LW groups maintained a 'brownish green' freshwater appearance. (Figs 3.5–3.7). At the final sample in December SW and LW fish showed a well-developed marine shading pattern with

substantial silvering due to the deposition of guanine and hypoxanthine crystals. The CL group, although larger in terms of length and mass, retained juvenile parr markings (Fig.3.5)

Treatment	CF	X ACFL	PPM	MPS	Silvering
Cl	1.29b	25a	+	-	1
SW	1,16c	34b	-	+	3
LW	1.32a	25a	+	-	2

Table 3.1 Indicators of smoltification after 18 weeks on light programme (CL,SW and LW). (CF= condition factor, X ACFL = mean anal to caudal fin length, PPM=presence of parr markings, MPS= marine pelagic shading, Silvering. = silvering of scales. Significant differences (ANOVA, $P<0.05$) between groups are indicated by different letters a,c and c.

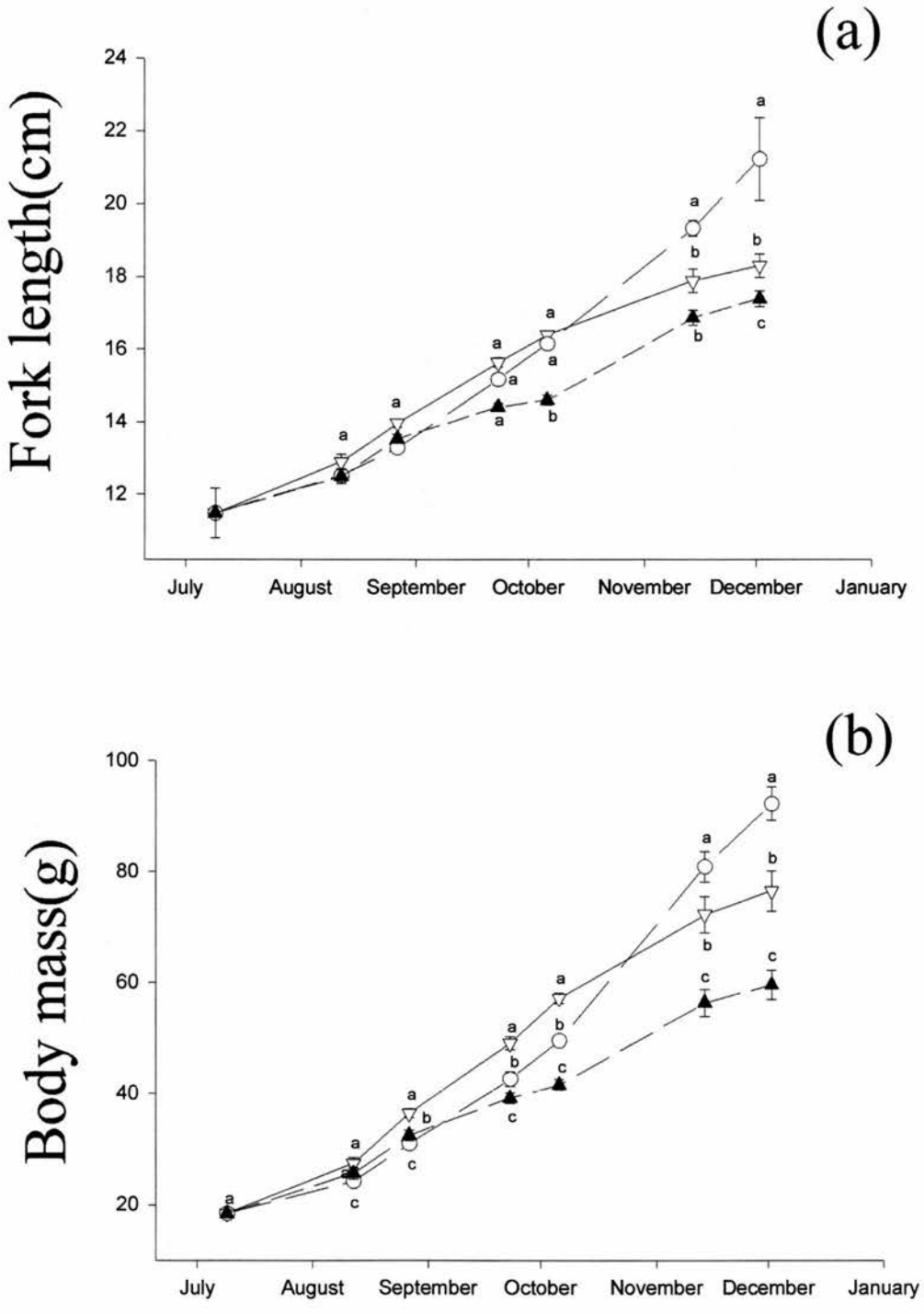


Fig 3.2 (a) Fork length and (b) body mass in juvenile Atlantic salmon reared under constant light treatment (CL) (open triangles, solid line) , SW (open circles, long dashed line) and LW (solid triangles, short dashed line) from July to December 1997.Significant differences (ANOVA, $P<0.05$) between groups at each sample point are indicated by different letters (a,b and c)

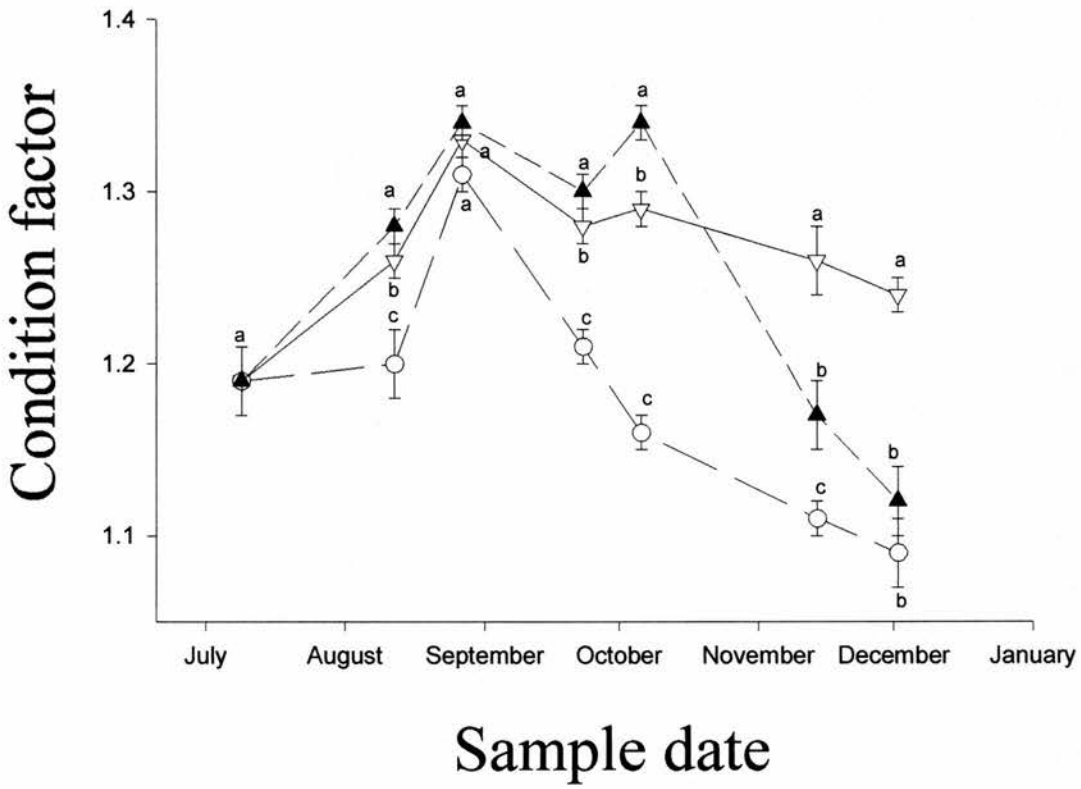


Fig.3.3 Condition factor in juvenile Atlantic salmon reared under light treatment CL (open triangle, solid line) , SW (open circle, long dashed line) and LW (solid triangle, short dashed line) from July to December 1997.Significant differences (ANOVA, $P<0.05$) between groups at each sample point are indicated by different letters (a,b and c)

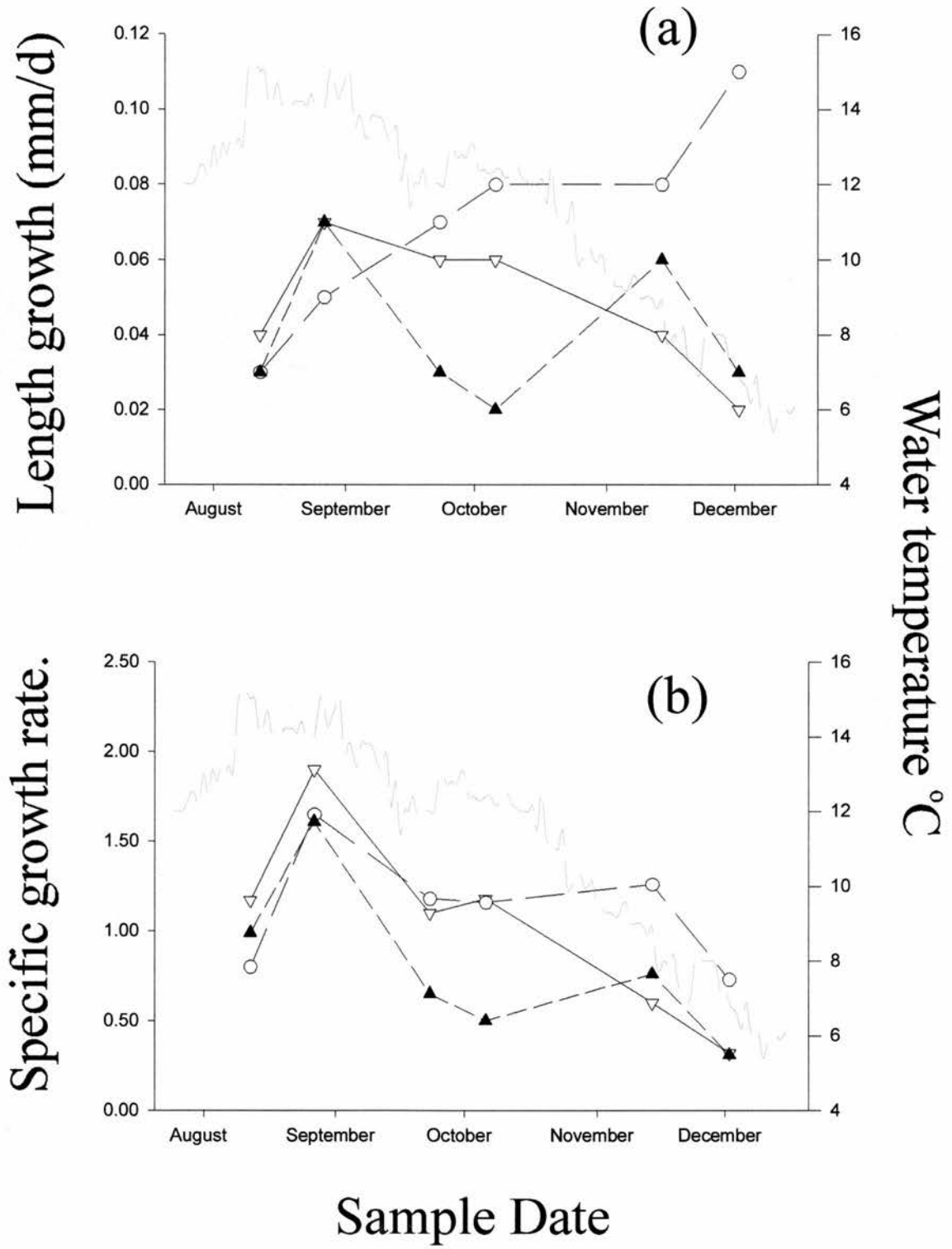
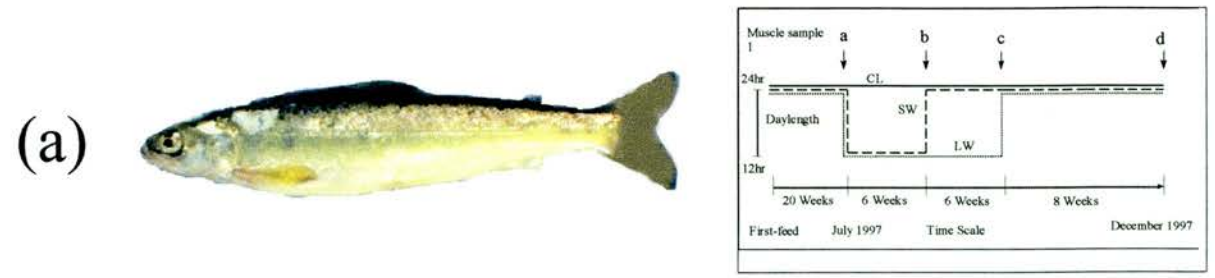
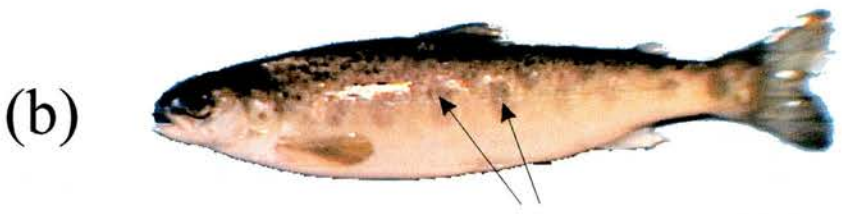
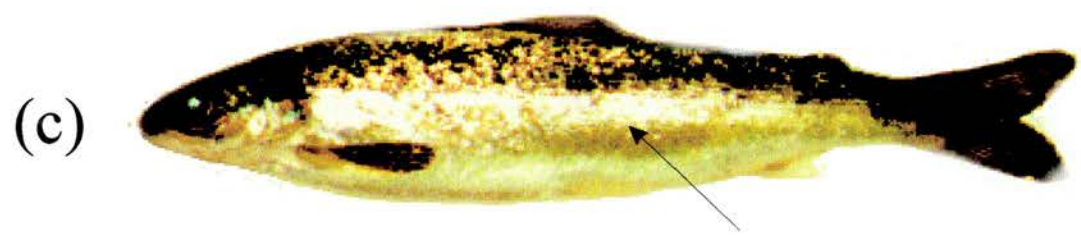


Fig.3.4 (a) Length growth and (b) specific growth rate in juvenile Atlantic salmon reared under light treatment CL (open triangles, solid line) , SW (open circles, long dashed line) and LW (solid triangles, short dashed line) from July to December 1997. Mean water temperature is shown as a dashed grey line.

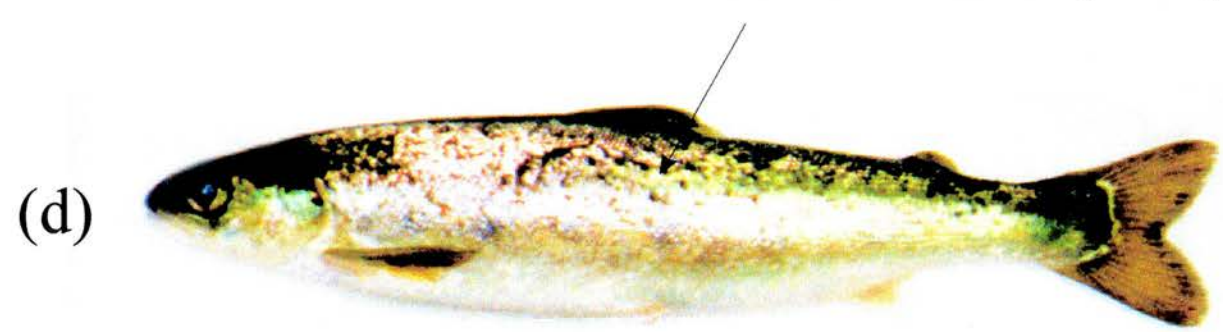




Freshwater parr markings

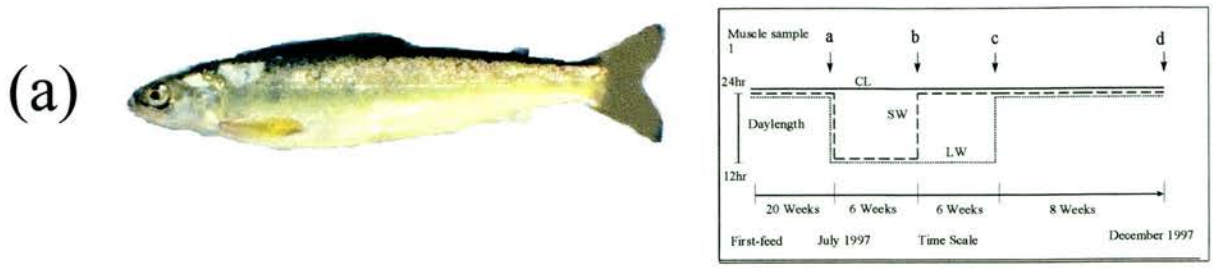


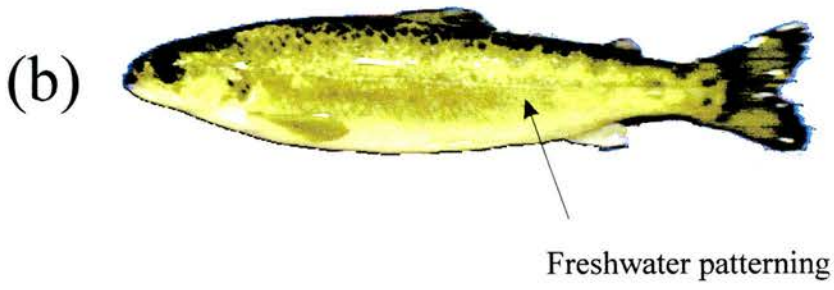
Maintenance of freshwater patterning

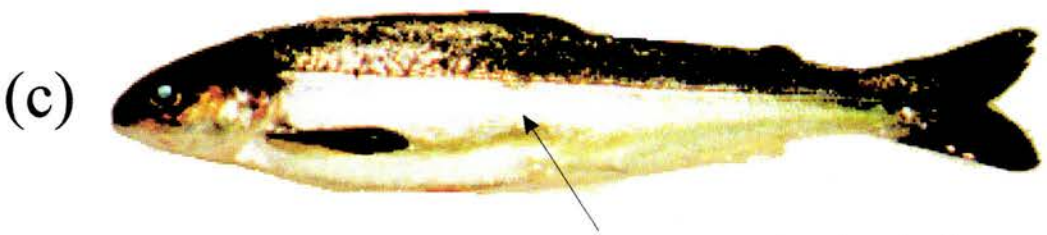


25cm

Fig 3.5 Juvenile Atlantic salmon reared under light program CL in (a) July 1997 (11.48cm ± 0.16), (b) August (1997) (13.96cm ± 0.67), (c) October 1997 (16.40cm ± 0.97) and (d) December 1997 (18.40cm ± 1.19) (mean fork length ± SE, n>100). Details of light program inset.







25cm

Fig. 3.6 Juvenile Atlantic salmon reared under light program SW in (a) July 1997 (11.48cm ± 0.16), (b) August (1997) (13.28cm ± 0.14), (c) October 1997 (16.15cm ± 0.14) and (d) December 1997 (21.23cm ± 1.14) (mean fork length ± SE, n>100). Details of light program inset

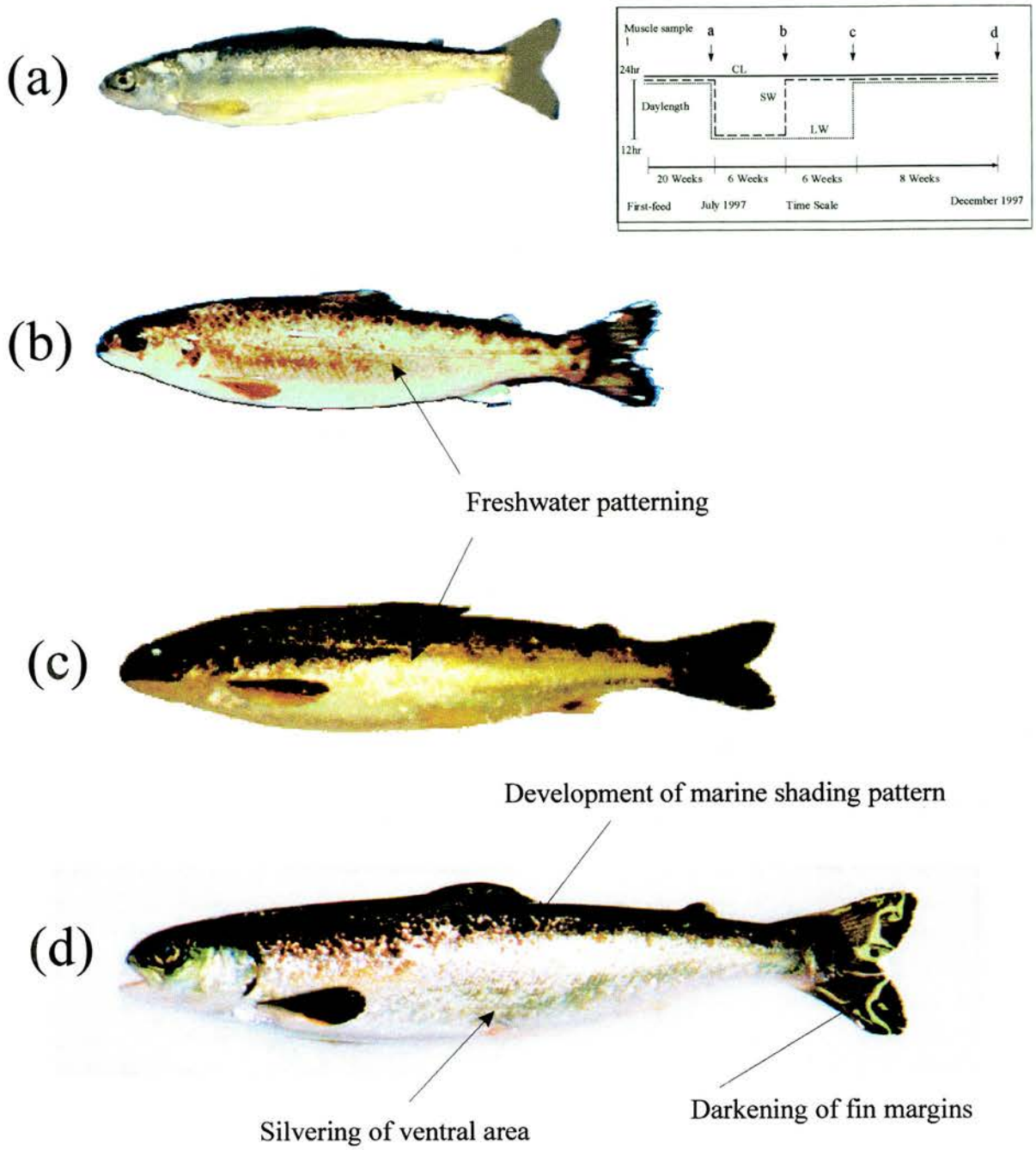


Fig. 3.7 Juvenile Atlantic salmon reared under light program LW in (a) July 1997 (11.48cm ± 0.16), (b) August (1997) (13.53cm ± 0.68), (c) October 1997 (14.60cm ± 0.14) and (d) December 1997 (17.39 ± 0.22) (mean fork length±SE, n>100). Details of light program inset.

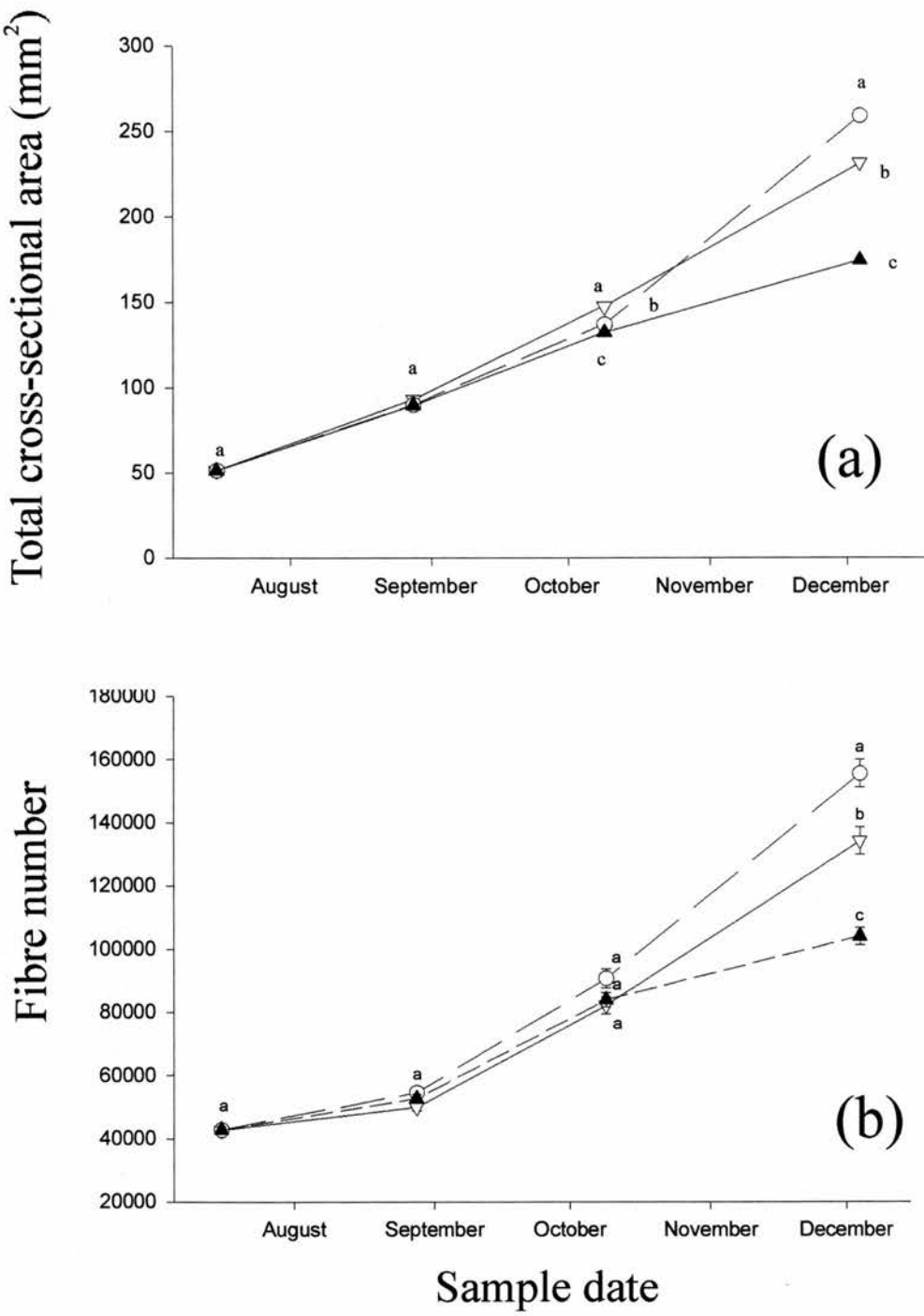


Fig. 3.8(a) Mean total cross-sectional area and (b) number of white muscle fibres per myotome in Atlantic salmon reared under light treatment CL (open triangles, solid line), short winter (open circles, long dashed line) or long winter LW (closed triangles, short dashed line) from July to December 1997. Significant differences (ANOVA, $P<0.05$) at each sample point are indicated by different letters (a,b and c).

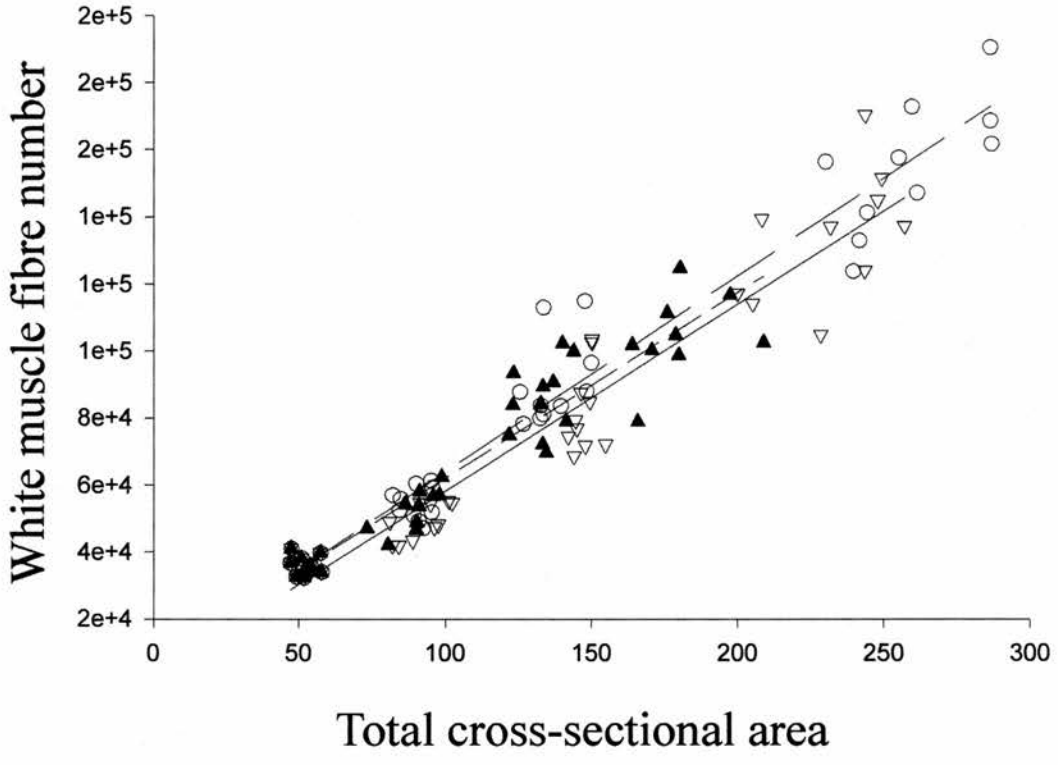


Fig. 3.9 Linear regression plot of total cross-sectional area (mm^2) vs fibre number per myotome in Atlantic salmon reared under light treatment CL (open triangle, solid line, r^2=0.927), SW (open circles, long dashed line, r^2=0.956) and LW (solid triangles, short dashed line r^2=0.903) from July to December 1997.

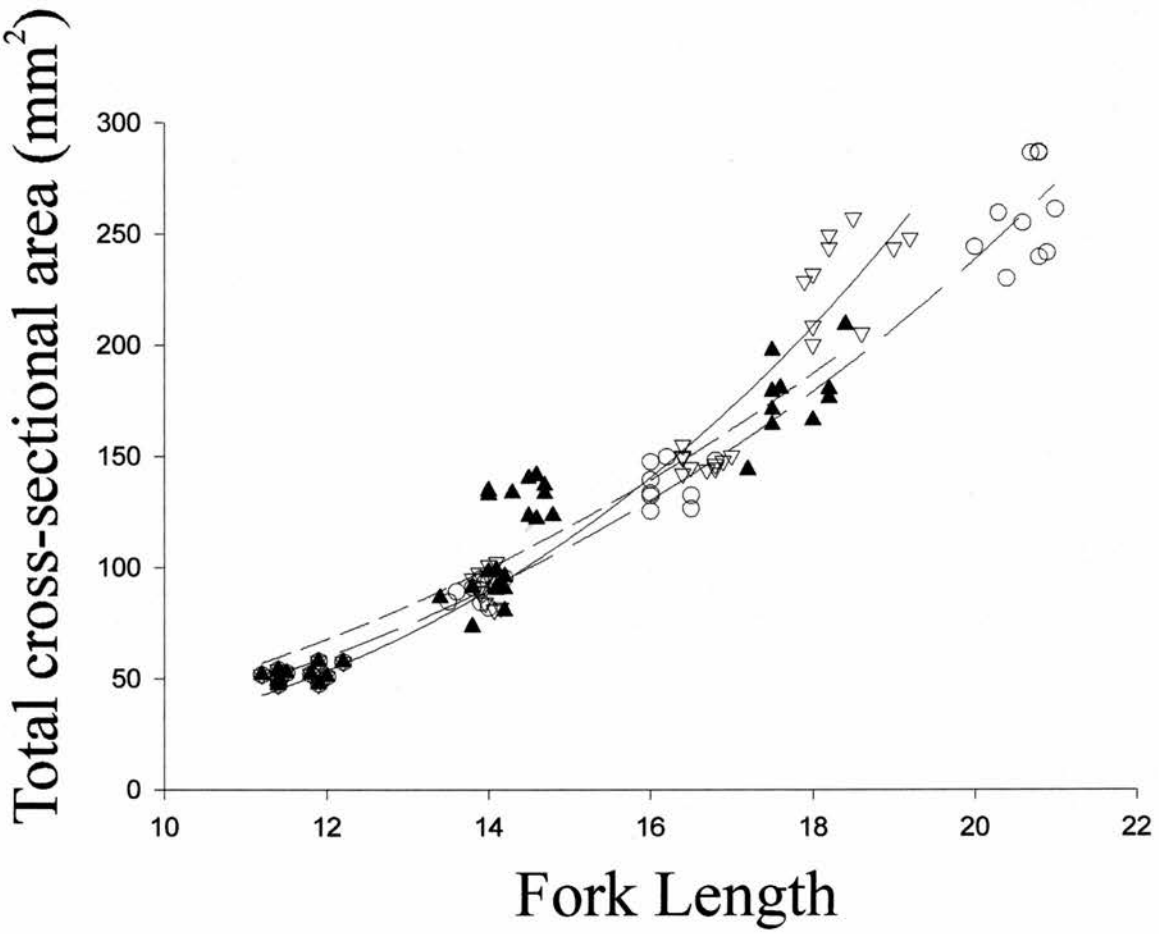


Fig. 3.10 Regression plots of total white muscle cross-sectional area (mm^2) against fork length in Atlantic salmon reared under light treatment CL (open triangles, solid line, r^2=0.872) SW (open circles, long dashed line, r^2=0.979) and LW (solid triangles, short dashed line, r^2 =0.956)

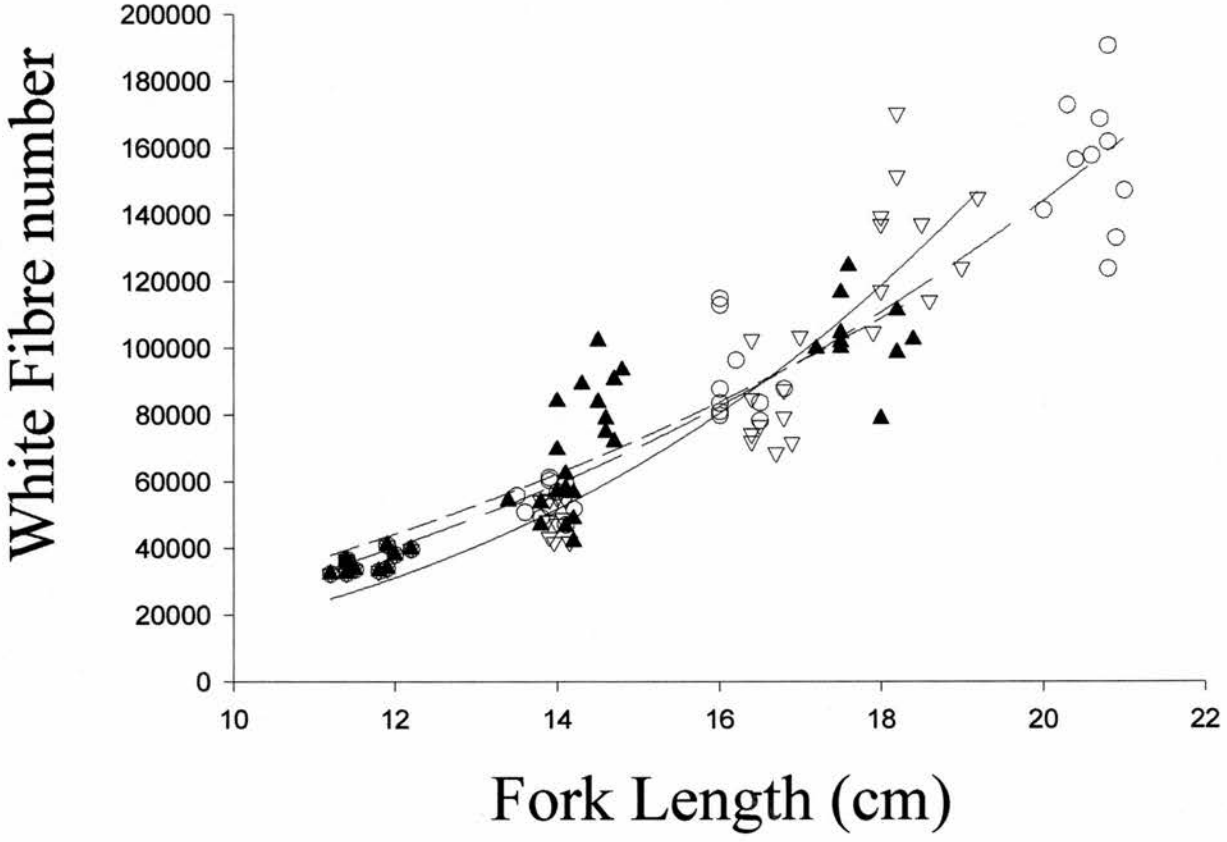


Fig 3.11 Regression plots of white muscle fibre number per myotome against fork length in Atlantic salmon reared under light treatment CL (open triangles, solid line, r^2=0.874) SW (open circles, long dashed line, r^2=0.923) and LW (solid triangles, short dashed line, r^2=0.761)

3.3.3 Muscle growth

Total cross-sectional area (TCA)

TCA was linearly related to muscle fibre number in all groups (Fig. 3.9a) with no significant differences between treatments ($F_{2,27}$=0.782; P>0.05). TCA was significantly higher ($F_{(2,27)}$ =12.95 P<0.001) in the CL group following 18 weeks of light treatment after an initial high growth rate. At the end of the experiment in December 1997 (Fig. 3.8a), however the SW group displayed the highest TCA followed by that of the constant light (CL) group and then the long winter group (LW) ($F_{(2,27)}$= 44.33 P<0.001). Plots of TCA and fibre number against fork length revealed significant differences between treatment groups (Fig. 3.10 and 3.11) with the constant light group having higher TCA and fibre numbers than the SW and LW groups for any given fork length ($F_{2,27}$ =8.56, P<0.01; $F_{2,27}$ = 11.32 , P<0.05)

Muscle fibre hypertrophy

Hypertrophic growth was investigated by calculating the smoothed probability density function (pdf) of muscle fibre diameter for each fish using a sample size of 400 fibres (Figs 3.12 to 3.20; smoothing parameters are given in Table 3.2) and the values of the 5th, 10th, 50th and 95th percentiles calculated (Fig. 3.19 and 3.20; see Chapter 2: Temperature effects). Values of the 50th percentile were significantly higher ($F_{2,27}$=28.28, P<0.01; $F_{2,27}$=7.42, P<0.01) in fish reared under the CL light program in August and October, indicating a greater contribution of hypertrophy to muscle growth in this group. The value of the 95th percentile was significantly higher ($F_{2,27}$=3.84, P<0.05) in the CL group after 18 weeks of continuous light in October (Fig. 3.20), indicating a greater

hypertrophy of the largest fibres in the distribution. The 50th and 95th percentile values then decreased in the CL and LW group as water temperature fell towards winter. The value of the 95th percentile in the SW group however, increased over the same period, suggesting a relative rise in the contribution of hypertrophy to growth in this group over the last two months of the study concomitant with a return to constant light conditions.

Muscle Fibre recruitment

Fibre number increased in all groups throughout the experiment (Fig.3.8b) with a four-fold increase in the SW group. Fibre number was significantly higher ($F_{(2,27)}$=21.78, P<0.001) in the SW group (155,381 ±140) at the termination of the experiment compared to the CL (134,143 ±139) and LW (103,927 ±110) groups (mean±SE, n=10). Recruitment of new fibres increased from July to October in all groups (Table 3.3) with the greatest recruitment (1154 fibres per day) in the short winter group taking place between October and December. This finding is mirrored in a decrease in the values of the 5th,10th percentiles of muscle fibre diameter in fish reared under light program SW which suggest a switch in muscle fibre recruitment (Fig. 3.19). CL fish also recruited new fibres during this period although at a much lower rate (928 fibres per day), while in the LW group, the 5th and 10th percentiles remain relatively constant with no apparent shift to greater fibre recruitment.

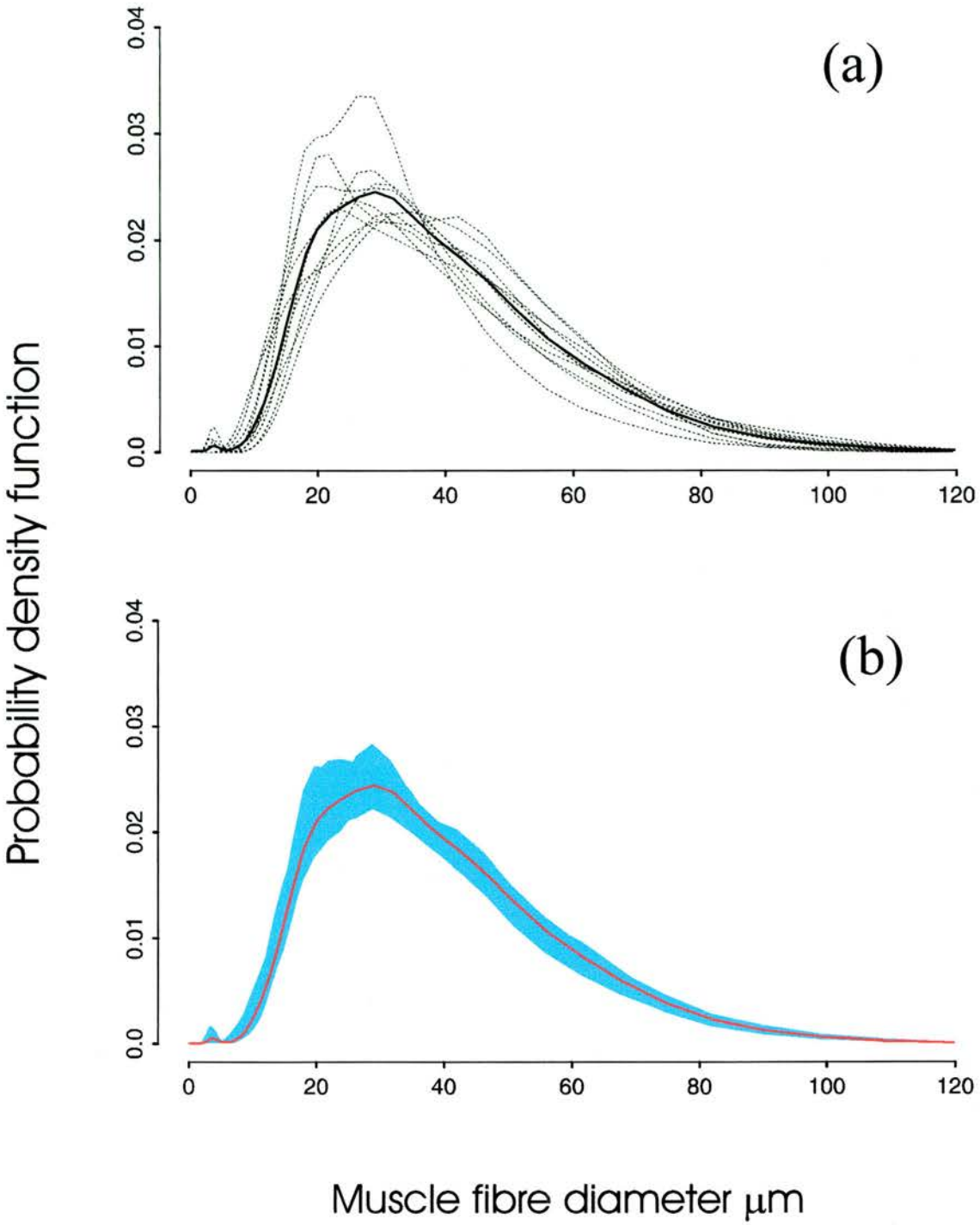


Fig. 3.12 (a) Probability density function of white muscle fibre diameter for all groups at the beginning of the experiment (July 1997). The dotted lines show individual fish and the solid line the average probability density function calculated using the mean smoothing parameter (see Table 3.2) and (b) bootstrap estimates of the variance of probability density function of white muscle fibre diameter for salmon at the same sample point. The blue shaded area corresponds to the variability band of the 100 boot strap estimates and the red line the mean probability density function.

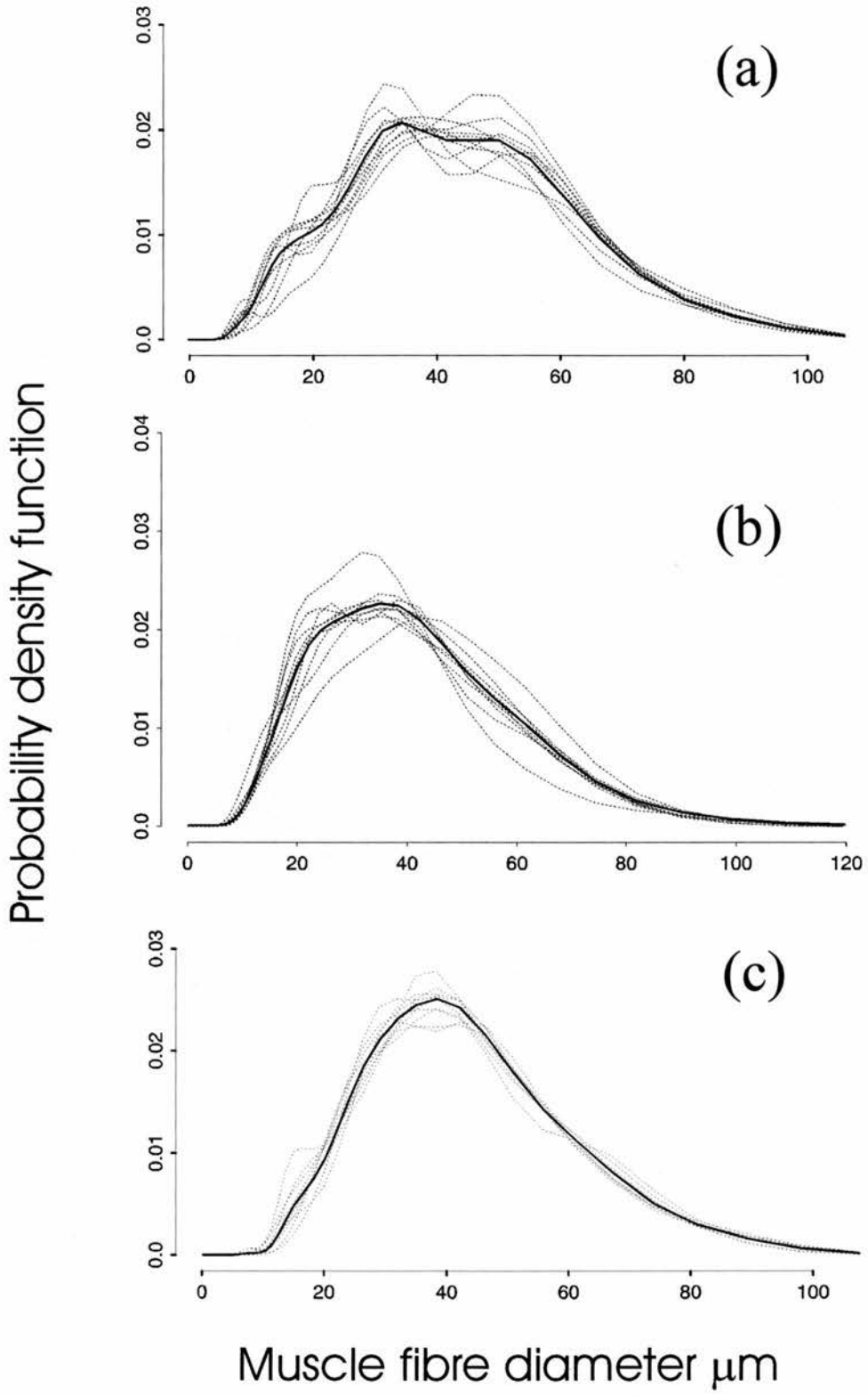


Fig. 3.13 Probability density function of white muscle fibre diameter for salmon reared under light program (a) CL, (b) SW (b) and (c) LW in September 1997. The dotted lines show individual fish and the solid line the average probability density function calculated using the mean smoothing parameter (see Table 3.2)

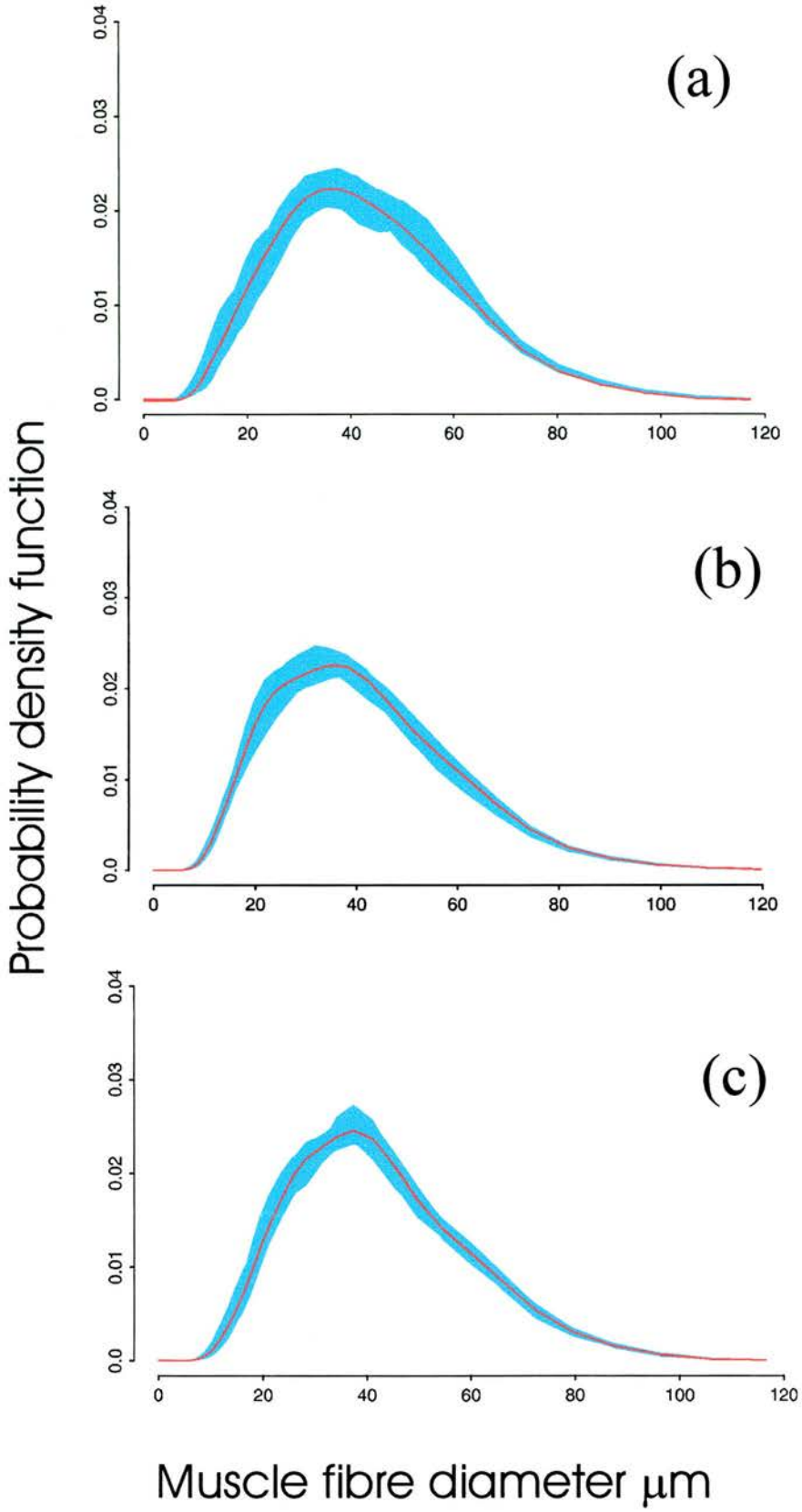


Fig. 3.14 Bootstrap estimates of the variance of probability density function of white muscle fibre diameter for salmon reared under light program (a) CL, (b) SW and (c) LW sampled in September 1997. The blue shaded area corresponds to the variability band of the 100 boot strap estimates and the red line the mean probability density function.

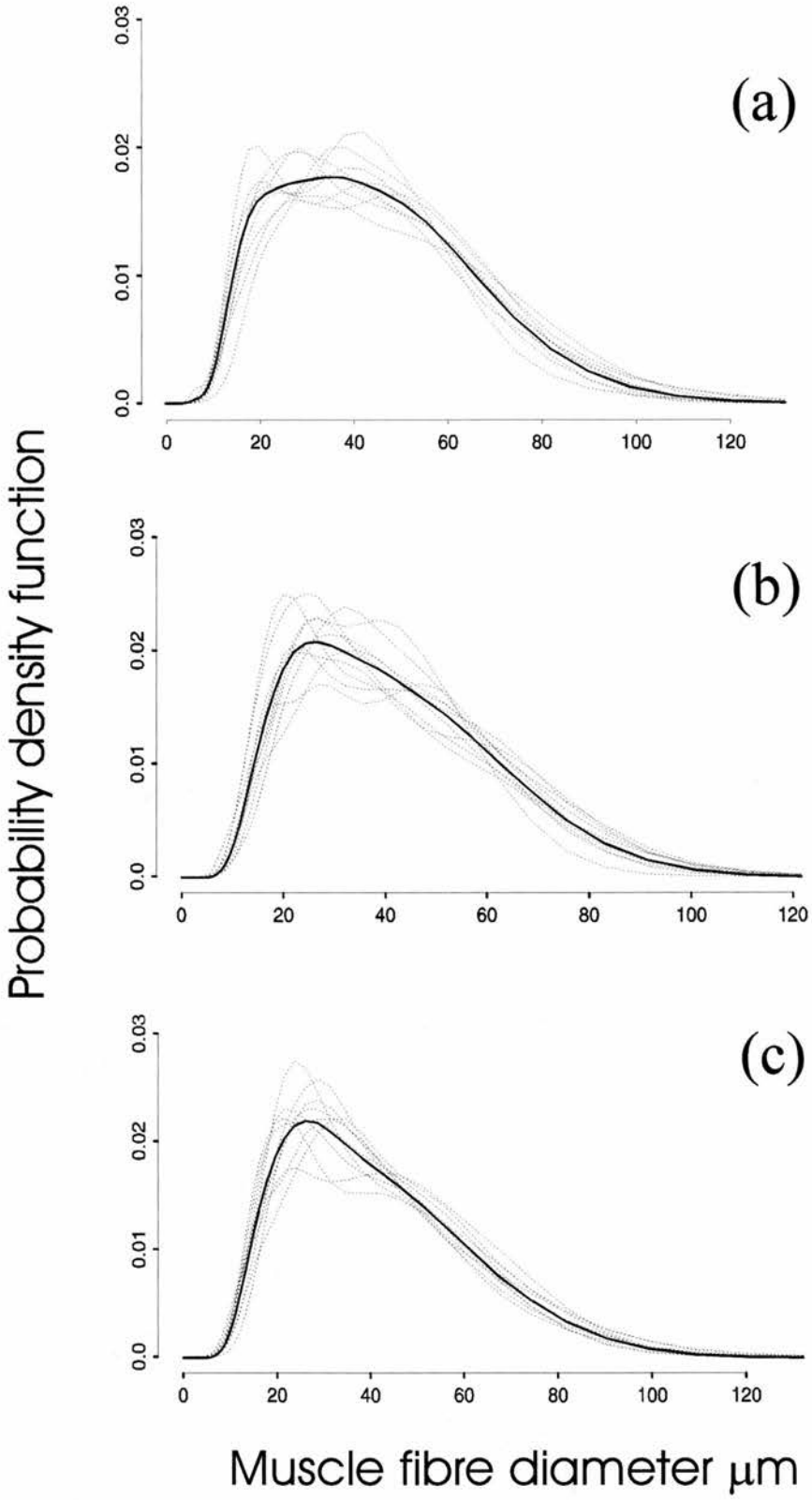


Fig. 3.15 Probability density function of white muscle fibre diameter for salmon reared under light program (a) CL, (b) SW and (c) LW in October 1997. The dotted lines show individual fish and the solid line the average probability density function calculated using the mean smoothing parameter (see Table 3.2)

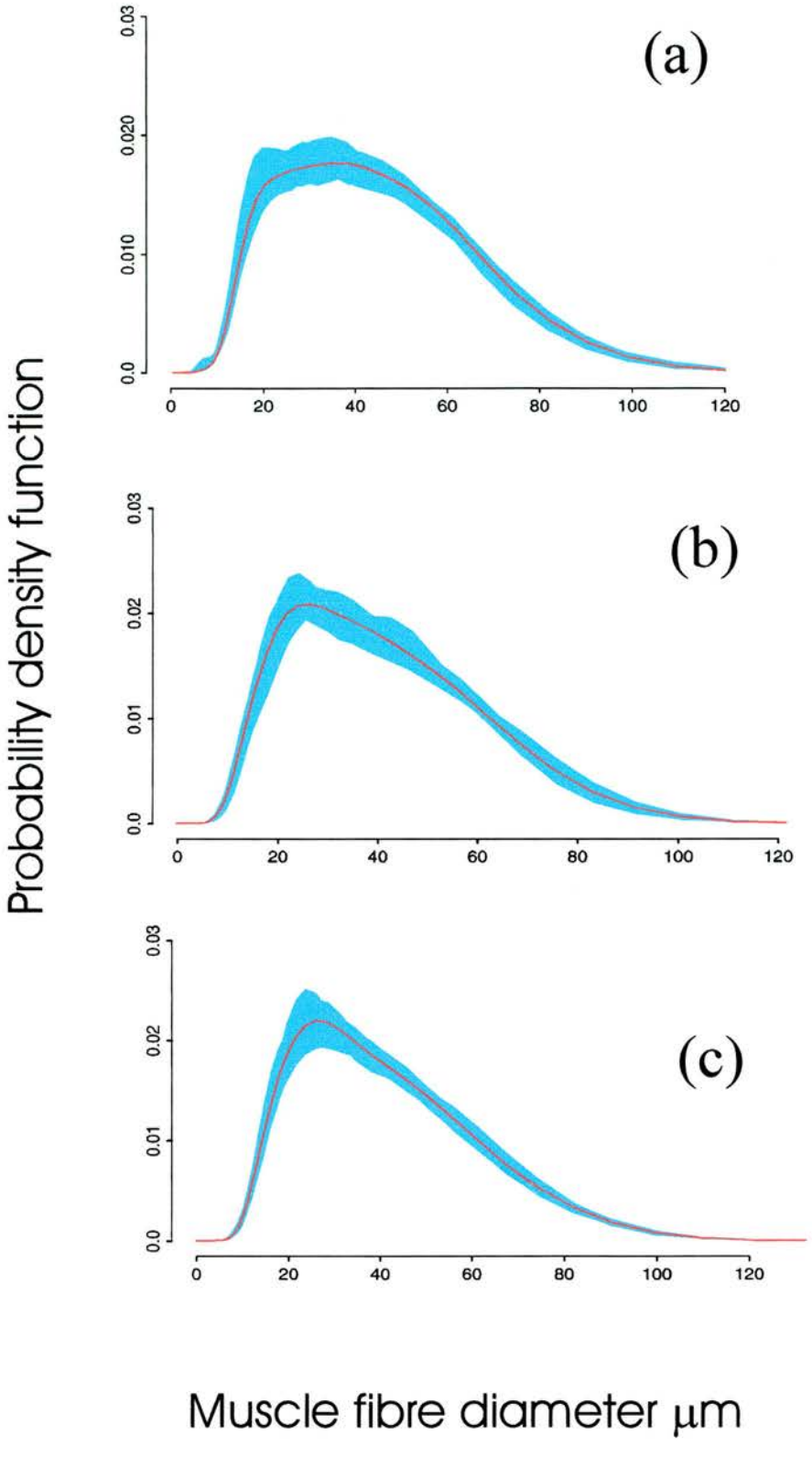


Fig. 3.16 Bootstrap estimates of the variance of probability density function of white muscle fibre diameter for salmon reared under light program (a) CL, (b) SW and (c) LW sampled in October 1997. The blue shaded area corresponds to the variability band of the 100 boot strap estimates and the red line the mean probability density function.

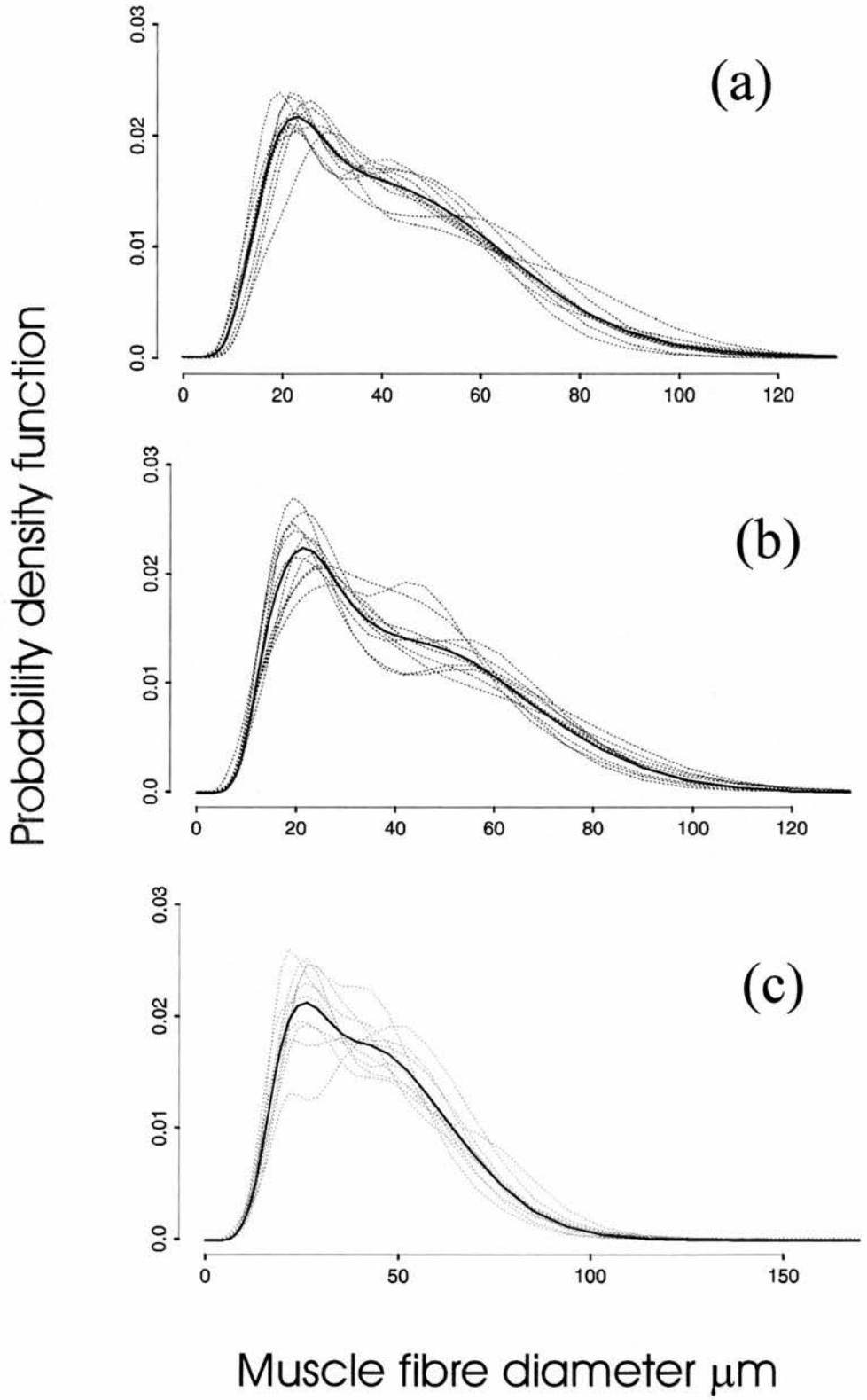


Fig. 3.17 Probability density function of white muscle fibre diameter for salmon reared under light program (a) CL, (b) SW and (c) LW in December 1997. The dotted lines show individual fish and the solid line the average probability density function calculated using the mean smoothing parameter (see Table 3.2)

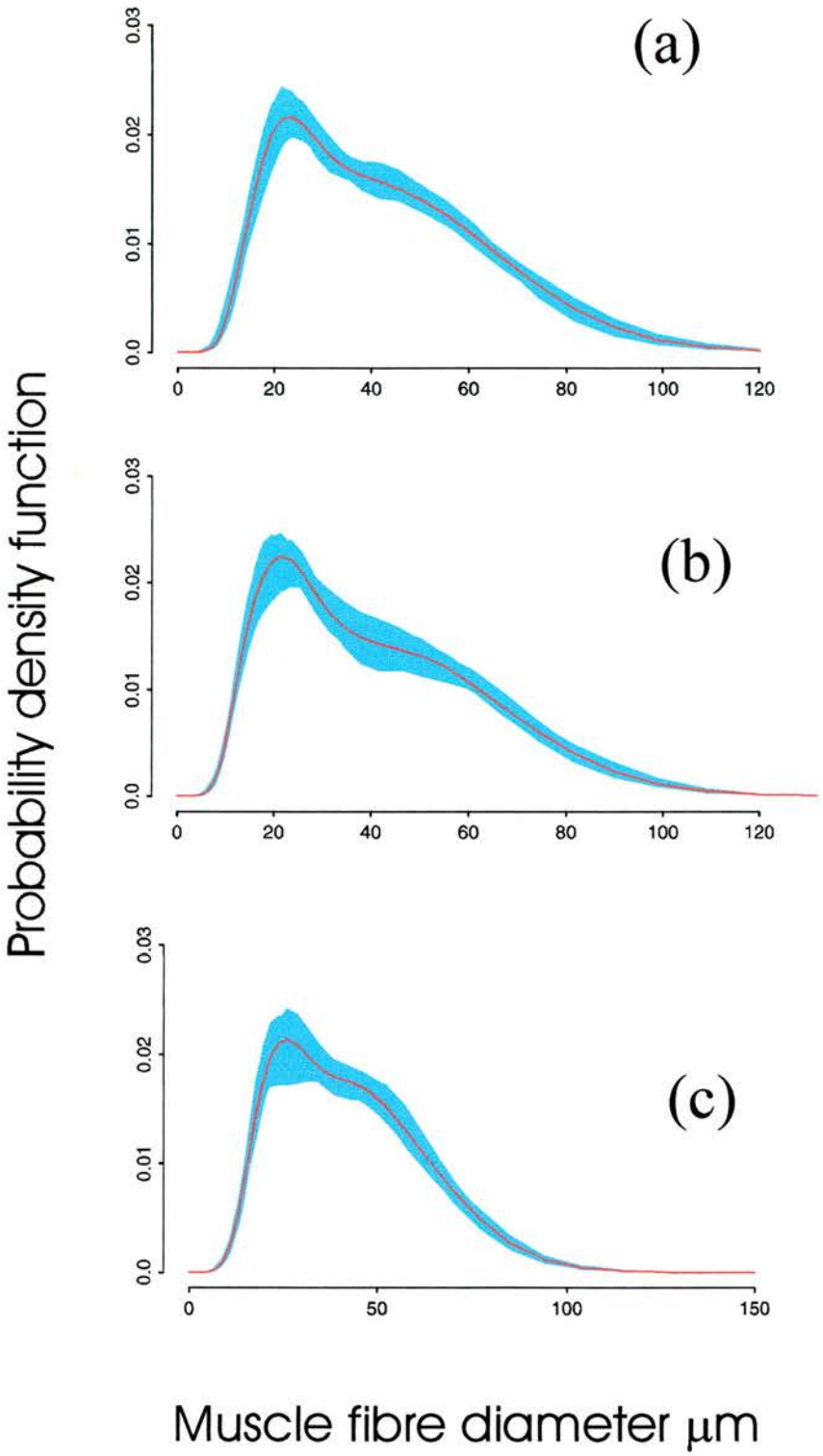


Fig. 3.18 Bootstrap estimates of the variance of probability density function of white muscle fibre diameter for salmon reared under light program (a) CL, (b) SW and (c) LW sampled in December 1997. The blue shaded area corresponds to the variability band of the 100 boot strap estimates and the red line the mean probability density function.

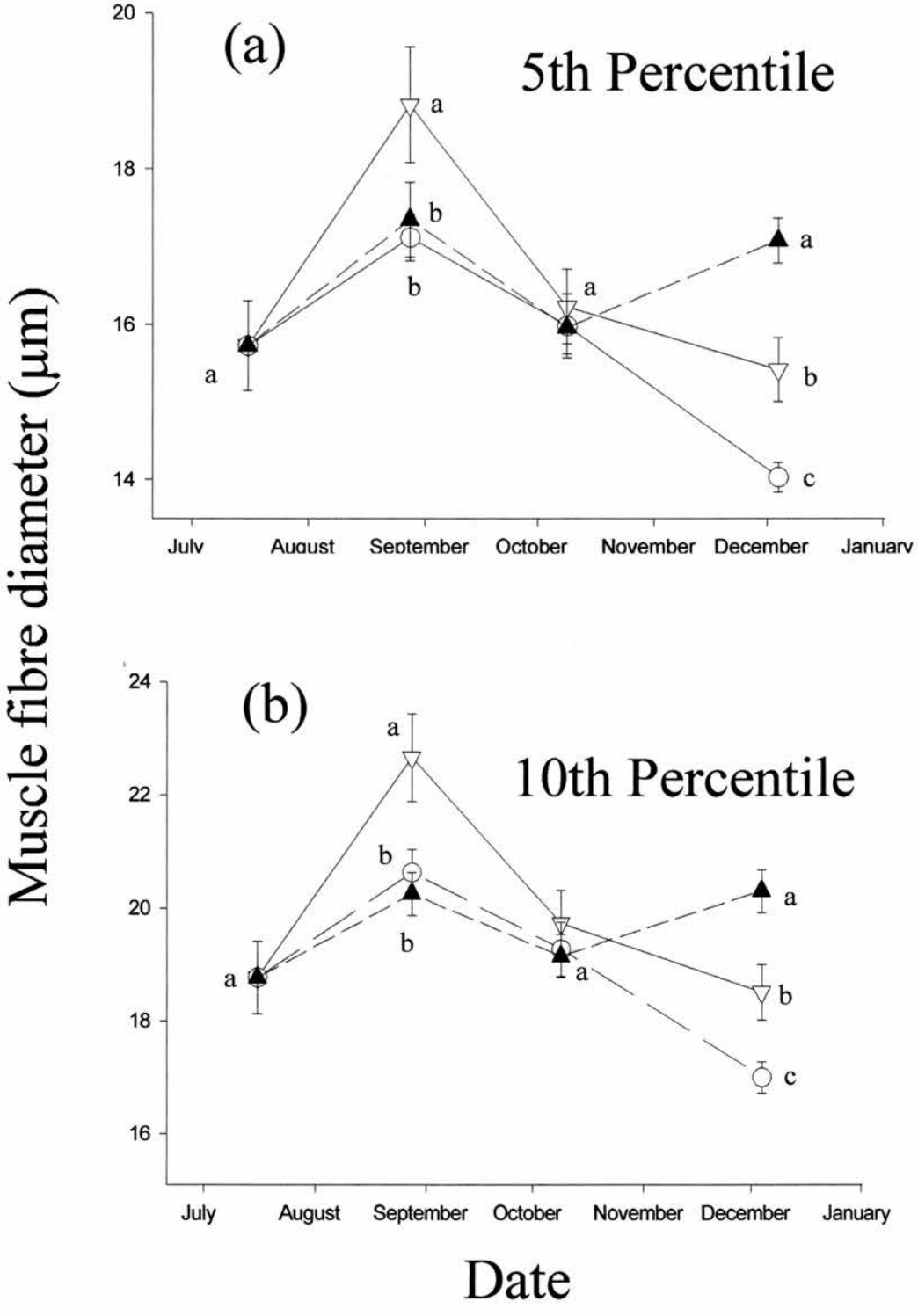


Fig. 3.19 The (a) 5th and (b) 10th percentiles of muscle fibre diameter calculated from the probability density functions of individual fish reared under constant light CL (open triangles, solid line) short winter SW (open circles,long dashed line) or long winter (LW). (solid triangles, short dashed line). Significant differences (Wilcoxon two-sample non-parametric test, $P<0.05$) between groups at each sample point are denoted by different letters a,b and c (see Table 3.4.)

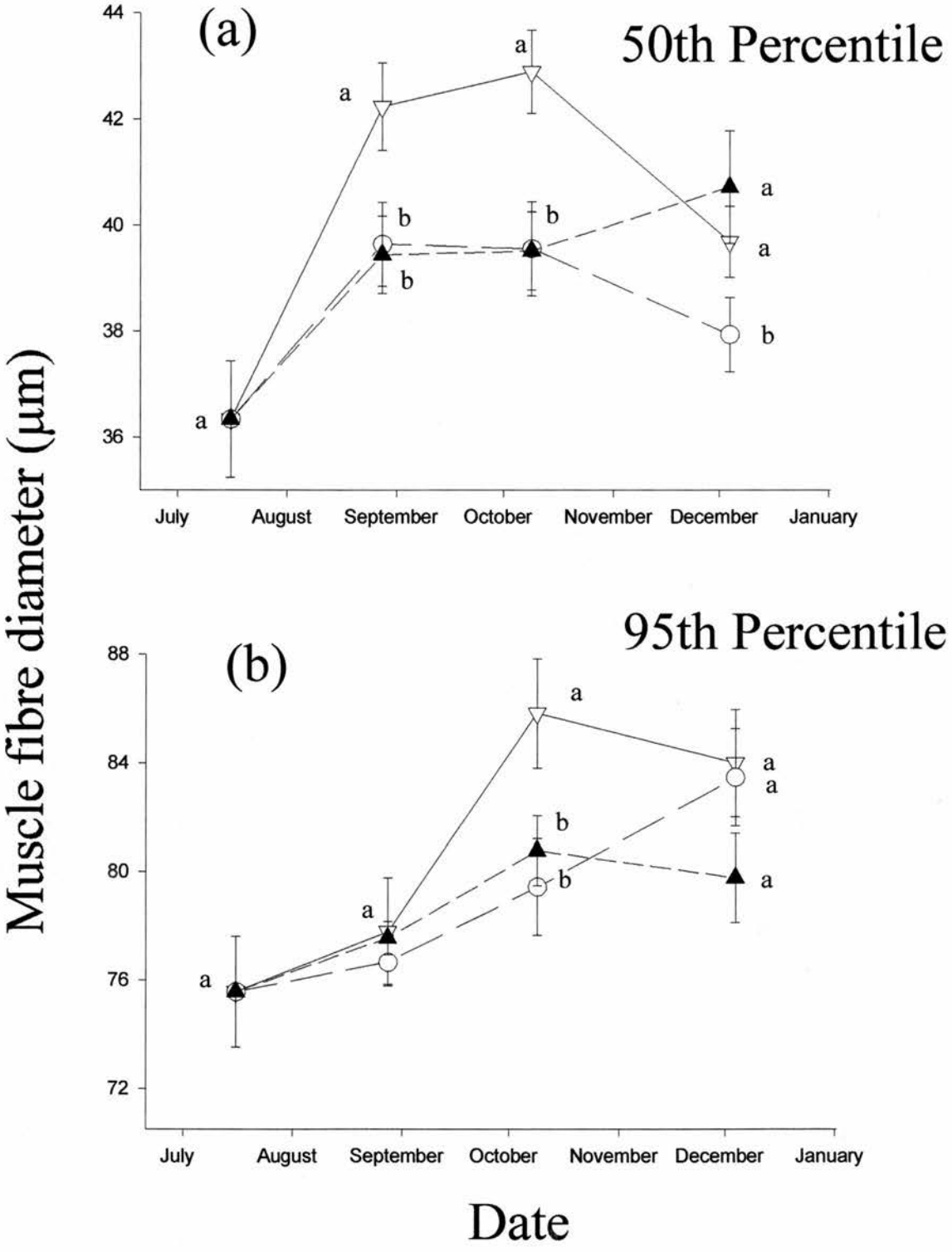


Fig. 3.20 The (a) 50th and (b) 95th percentiles of muscle fibre diameter calculated from the probability density functions of individual fish reared under constant light CL (open triangles, solid line) short winter SW (open circles,long dashed line) or long winter (LW). (solid triangles, short dashed line). Significant differences (Wicoxon's two-sample non-parametric test, $P<0.05$) between groups at each sample point are denoted by different letters a,b and c (see Table 3.4).

Date	Light treatment		
	CL	SW	LW
July	0.117	0.117	0.117
August	0.097	0.104	0.090
October	0.120	0.114	0.114
December	0.123	0.130	0.111

Table 3.2 Smoothing parameter (h mean) for muscle fibre distributions of Atlantic salmon reared under light constant light (CL), short winter (SW) and long winter (LW).

Growth Period	Fibre recruited /day^{-1}		
	CL	SW	LW
July to August	326	459	412
August to October	806	881	761
October to December	928	1154	357

Table 3.3 Muscle fibre recruitment in juvenile Atlantic salmon reared under light program CL, SW and LW from July to August 1997

Percentile	*F*-Ratio	df	*P*
August			
5th	3.58	2,27	0.042
10th	5.48	2,27	0.010
50th	28.28	2,27	0.001
95th	6.34	2,27	0.006
October			
5th	0.13	2,27	0.876
10th	0.38	2,27	0.387
50th	7.42	2,27	0.003
95th	3.84	2,27	0.034
December			
5th	24.13	2,27	0.001
10th	17.58	2,27	0.001
50th	2.85	2,27	0.075
95th	1.62	2,27	0.215

Table 3.4 Analysis of variance table for 5th, 10th, 50th and 95th percentile estimates calculated from samples taken in August, October and December 1997.

3.4 Discussion

It has been generally accepted that photoperiod plays an important role in the seasonal adjustment of physiological and behavioural patterns in the life of anadromous salmonids (Hoar, 1976 and Wedemeyer *et al.*, 1980) but the direct effect of photoperiod manipulation on muscle growth has not been demonstrated. The results of the current experiment indicate that light treatment has a significant effect on smoltification and muscle growth rate in Juvenile Atlantic salmon. Muscle growth in fish occurs by both hypertrophy of existing fibres and the recruitment of new fibres (Greer-Walker, 1971; Johnston, 1982). Weatherley and Gill (1988) surmised that changes in somatic growth rate were closely matched by proportional increases in muscle growth. Results from the current experiment suggest that although increased somatic growth is reflected by changes in muscle development, a great deal of plasticity exists within this muscle response.

Fish reared under constant light initially had the highest growth rate, driven mainly by hypertrophy of muscle, evidenced by increasing fibre diameters in the frequency distribution with no significant increase in fibre number. Berge *et al.* (1995) and Gaignon and Quemener (1992) found that extended daylength had a growth promoting effect on juvenile Atlantic salmon while Komourdjian (1989) found that extended daylength temporarily stimulated the pituitary axis and subsequently growth. However, the direct effect of constant illumination on growth in salmon is still relatively unclear. Higgins and Talbot (1985) and Villareal *et al.* (1988) suggested that growth rate

in salmonids is dictated largely by food intake and concluded that salmon feed when they can see food. Feed intake and growth over a given interval are therefore influenced by the total hours of daylight during that time. Fish reared under constant light did achieve an initially higher body mass, mainly through hypertrophy of muscle fibres, but this was not sustained throughout the course of the study. Kiessling *et al.* (1991) found that rainbow trout fed a 200% ration grew faster, mainly by hypertrophy, than fish fed a normal 100% ration. Together with results of the current study, this suggests that hypertrophy can be utilised as a short term response to increased food intake in salmonids, but fibre recruitment is required to sustain high growth rates over long periods. Saunders *et al.* (1985) reported that juvenile Atlantic salmon reared under continuous light grew faster than salmon grown under simulated natural photoperiod, but failed to smolt and did not grow like normal smolts when transferred to sea cages. Higgins and Thorpe (1990) found increased fibre recruitment in upper modal group fish undergoing parr-smolt transformation. This suggests a preparatory phase of hyperplasia, which prepares the parr for a rapid increase in muscle mass prior to seaward migration. Fish reared under constant light did not fully experience this transformation, which could explain the final lower body mass in this group. Although growth rates are improved by rearing salmon under constant light, a decrease in photoperiod is necessary to initiate the smolting process, which is then completed under increasing day length (Bjornsson *et al.*, 1989).

Fish reared with a short winter period showed indications of full smoltification with a significant decrease in condition factor, silvering of the skin and a pronounced lengthening of the skeleton (Stefansson *et al.*, 1991). Condition factor is considered a

reliable parameter indicating smoltification in Atlantic salmon in nature (Hoar, 1976) and in most cases a reduction is seen in cultured salmon undergoing the parr-smolt transformation (Farmer *et al.,* 1978; Saunders *et al.,* 1985; Stefansson *et al.,* 1991). This transformation was associated with a significant increase in not only fibre recruitment, but fibre hypertrophy. Proliferative activity and increased hypertrophy has been found in different body tissues taken from juvenile salmon undergoing parr-smolt transformation (Hoar, 1988). Study of pituitary glands from animals reared under advanced photoperiod regimes has revealed hyperplasia and hypertrophy of somatotrops while chloride cell proliferation in the operculum is apparent in smolting parr (Langdon and Thorpe, 1984).

Bjornsson *et al.* (1995) concluded that photoperiod is a major 'zeitgeber' for increased growth hormone levels found in salmon undergoing the parr-smolt transformation. Growth hormone is also involved in stimulating rapid skeletal growth in smolts (Bjornsson *et al.*, 1989) while growth hormone treatment is known to increase fibre recruitment in rainbow trout (Fauconneau *et al.*, 1997). The lengthening of the skeletal frame provides support for future muscle growth by fibre recruitment, which follows seawater transfer. Bjornsson *et al.,* (1989) also found a small increase in growth hormone levels in salmon reared under constant light, this may account for initially higher levels of growth in the constant light group. The significantly higher TCA and fibre number for any given fork length in the constant light group is most probably linked to a feed intake-related hypertrophy of fibres coupled with the absence of a smoltification associated increase in fork length.

Several authors have linked high growth rates to muscle fibre recruitment (Weatherley *et al.,* 1988; Willemse *et al.,* 1976) while Kiessling *et al.* (1991) related fast growth in rainbow trout (*Oncorhyncus mykiss*) to increased hypertrophy. The results of the current study suggest that recruitment is a pre-requisite for sustained growth, which then progresses by rapid hypertrophy. Small muscle fibres have a higher nutritive assimilative capacity due to their higher surface area to volume ratio (Weatherley *et al.*, 1988). Recruitment of new fibres, therefore, increases surface area of muscle relative to the volume, facilitating the rapid assimilation of nutrients. New fibres are therefore recruited into the myotome and hypertrophy quickly, contributing towards a high growth rate similar to that seen in the SW group.

Light manipulation is increasingly used as a tool to promote growth and reduce the length of the production cycle in aquaculture. Some concern has been voiced in the industry over the effect of light manipulation techniques on smoltification and future growth of salmon smolts. The current study suggests that light manipulation simply advances the program of seasonal muscle development in the salmon.

Chapter 4

CAGE SYSTEMS AND LIGHT MANIPULATION INFLUENCE MUSCLE FIBRE CELLULARITY AND HARVEST QUALITY OF UNDERYEARLING (0+) ATLANTIC SALMON (*Salmo salar* L.) IN SEAWATER

4.1 Introduction

Salmonid fish are inherently active swimmers but when held in captivity in traditional rearing units, are often subjected to poor water current conditions and crowding stress. This may result in an increased susceptibility to diseases and infestation of sea lice (Pickering 1989, Juell 1995). Sea lice treatments result in lost growth, skin damage and involve lengthy withdrawal periods prior to harvest (for review see Costello 1993). Increasing consumer concern over pesticide residues in food and a reluctance on behalf of major supermarkets to accept salmon which have been treated with pesticides and antibiotics has led to the development of rearing systems, which reduce the need for therapeutic treatments. The closed cage system (Fig. 4.1) is a semi-enclosed rearing unit which pumps water from 30m depth, avoiding infestations of sea lice and algals blooms. The pumping of large volumes of water into the system generates a steady current, exercising the fish. A great deal of work has been carried out on exercise in fish (for reviews see Hoar and Randall 1978 and Davison 1997). It is generally agreed that at favourable swimming speeds, exercise training in salmonids leads to reduced aggression, increased growth (Sølsnes *et al.*, 1991, Clarke 1998, Adams *et al.,* 1995, Davison 1989) and has a marked positive effect on feed conversion efficiency (Christiansen *et al.,* 1989, Greer-Walker and Emerson 1978, Leon 1986).

Continuous light or extended photoperiods have been used extensively in salmon farming to enhance growth of smolts following transfer to sea water (Saunders and Harmon, 1988; Endal *et al.*, 1991, Kråkenes *et al.*, 1991; Hansen *et al.*, 1992; Taranger *et al.*, 1995; Oppedal *et al.*, 1997). Additional continuous light has also been used to maximise growth and reduce maturation in autumn transferred 0+ smolt in sea water tanks (Duncan *et al.*, 1999) and in sea cages (Oppedal *et al.*, 1999). Continuous light induces behavioural changes in Atlantic salmon, manifest in increased schooling behaviour, effectively producing an exercise effect (Oppedal Pers. Comm). Beattie *et al.* (2000b) found that light manipulation in 0+ smolts advanced the normal muscle program by up to 6 months.

Several authors have found changes in the number and diameter of muscle fibres in a variety of salmonids subjected to exercise training including Arctic charr (*Salvelinus alpinus*) (Christiansen *et al.*, 1989), brook trout (*Salvelinus fontinalis*) (Johnston and Moon 1980a) and Atlantic salmon (Jørgensen and Jobling 1993). Recent work by Hurling *et al.* (1996) linked muscle fibre cross-sectional area to firmness as assessed by sensory taste panel in a variety of marine species. Johnston *et al.* (2000b) showed a link between muscle fibre density and sensory qualities of smoked salmon as assessed by trained sensory taste panel, while Beattie *et al.* (2000c) found a significant correlation between muscle fibre density and firmness as measured by instrumental analysis. Totland *et al.* (1987) found that Atlantic salmon exercised in a raceway had increased hypertrophy of white muscle cells than non-trained fish and resulted in an increase of 9.2% in industry specified 'superior-quality fish'.

This study represents a first attempt at investigating the influence of light manipulation and cage rearing on muscle growth and flesh quality traits in Atlantic salmon. The information gained was used to improve the design and procedures in subsequent experiments.

4.2 Materials and Methods

4.2.1 Fish husbandry

Approximately 70.000 under yearling Atlantic salmon smolts of Sævareid origin (mean weight 90g) were transferred to sea water in October 1994 at a commercial sea farm (Giga salmon farm Solheim, Western Norway 61°N). Two groups were reared in conventional salmon farming cages (12m x12m x 8m) with tidal through flow of water under natural (OCNL) and additional continuous light (OCACL). The remaining two groups were reared in a circular enclosed pen (11.5m diameter, 14m deep, Gigamerden, Norway) under natural (CPNL) and additional continuous light (CPACL). A constant water flow of 30-40 cm sec^{-1} was created in the closed cages by pumping sea water from 30m depth (see Fig 4.1) while open cages experienced variable current flow between 0-8cm sec^{-1}. All groups were fed to satiation (100% ration) with commercial dry pellets (Skretting Royal, 3-12 mm; 30%oil, 40% protein, 75ppm astaxanthin, T Skretting AS, Norway) using automatic feeders. Water temperature was measured in the inlet of the closed cage and at 0.1 and 7m depth in the sea cages. During the course of the experiment, water temperature fluctuated between 7.5 and 12.5°C in the open sea cages and between 3 to 15°C in the closed cages. Oxygen concentration was measured in both cage types and fluctuated from 6.5 to 10mg ml^{-1} in the open cages and from 7 to 13 mg ml^{-1} in the open cages. Stocking density in all cages increased from 3kg m^{-3} to approximately 30 kg m^{-3} during the course of the experiment. Due to the large-scale commercial nature of the study, tanks were not replicated.

4.2.2 Sampling

One hundred fish from each group were anaesthetised with metomidate hydrochloride (Wild-life Pharmaceuticals Co. U.S.A.) on 15th November 1995 and measured for fork length to the nearest 0.5cm, and body weight to the nearest 10g. Condition factor (CF) was calculated as: $CF = BW \times L^{-3} \times 100$, where BW is body weight (g) and L is fork length (cm). 15 fish from each group were sacrificed and transported to Matre Aquaculture Research Station for estimation of fillet colour, lipid and texture analysis. A sub-sample of 6 fish from each cage, of approximately equal length were taken for measurement of muscle characteristics

4.2.3 Histology

Whole steak sections were taken from adult fish (2.8-3.4 kg) anterior to the dorsal fin and photographed on a background scale to determine total white muscle cross-sectional area. A clear acetate sheet with a reference grid (divisions of $1cm^2$) was placed over the steak at the point of excision and co-ordinates noted so the origin of the muscle block could related back to it's position within the whole steak. Sections from the dorsal and lateral epaxial quadrants were measured to assess fibre homogeneity across the whole cutlet section. Muscle fibre density was calculated for each sample area by calculating the number of fibres of average diameter that would fit into an area of 1 mm^2. Twenty individual muscle blocks were taken from each fish sampled. Muscle blocks approximately $1cm^2$ were excised from the steak and immersed in cryo-protectant (Tissue-Tek, Shandon U.S.A.) and frozen in 2-Methylbutane (iso-pentane) cooled to it's melting point in liquid nitrogen (-196°C). Frozen muscle blocks were wrapped in

aluminium foil, sealed in plastic bags and transported to the Gatty Marine Laboratory, St. Andrews, Scotland, for sectioning. The muscle blocks were sectioned at 10-12μm in a Leica cryostat set to -20°C and stained with carbalanine red (scarbra red) on Poly-L-Lysine coated slides (Sigma).

Muscle cellularity was determined using the Video-Plan Image Analysis System (Kontron Electronics, Basle, Switzerland) connected to a microscope at x40 objective. Approximately 250 individual inner muscle fibres per area from each block were measured randomly. Total white fibre number was calculated by dividing the total white muscle area by mean cross-sectional fibre area.

4.2.4 Flesh Quality Analysis

Colour measurements were taken on the epaxial muscle anterior to the dorsal fin using a Roche Colour card for salmonids (Hoffman La-Roche, Basle, Switzerland), scale 11-18 in a standard illumination box. One whole fillet from each fish was then homgenised with 1ml antioxidant (acetone containing 100ppm of 2,[6]-di-tert-butyl-p-cresol, BHT) and placed in a plastic tub prior to freezing for later analysis of total lipid and dry matter. For texture analysis, whole fillet sides were removed from the refrigerator and allowed to reach a temperature between 8-10°C before texture analysis was carried out. A texture profile-analysis compression test using a ball-probe was carried out (Veland *et al.,* 1997; Mittal *et al.*, 1992) and fillet 'hardness' used to correlate with muscle fibre density.

4.2.5 Total lipid and dry matter analysis

Triplicates of 3g (± 0.0001g) from homogenised fillet sections were added to 20ml pre-weighed glass tubes, dried at 105°C overnight, stored in an exicator at room temperature and weighed again for determination of dry matter. Subsequently, 1.5ml of ethylacetate was added to the samples, shaken for 1 hour, and stored overnight at 4°C. An aliquot of 10ml was collected from each sample and transferred to new preweighed glass tubes, evaporated in a water bath (90°C) for 5 hours, dried at 105°C for 20 minutes, cooled to room temperature in an exicator and weighed (± 0.0001g) for determination of total lipid content.

4.2.6 Statistics.

Two-way analysis of variance was used to assess muscle fibre and harvest quality differences between cage type and light treatment with body mass as a covariate. Least-squares linear regression was fitted to muscle fibre density and harvest quality data. For details of muscle fibre frequency analysis, see chapter 2. Differences in muscle fibre densities within the same steak section were analysed by Paired t-test with a significance level of $p<0.05$.

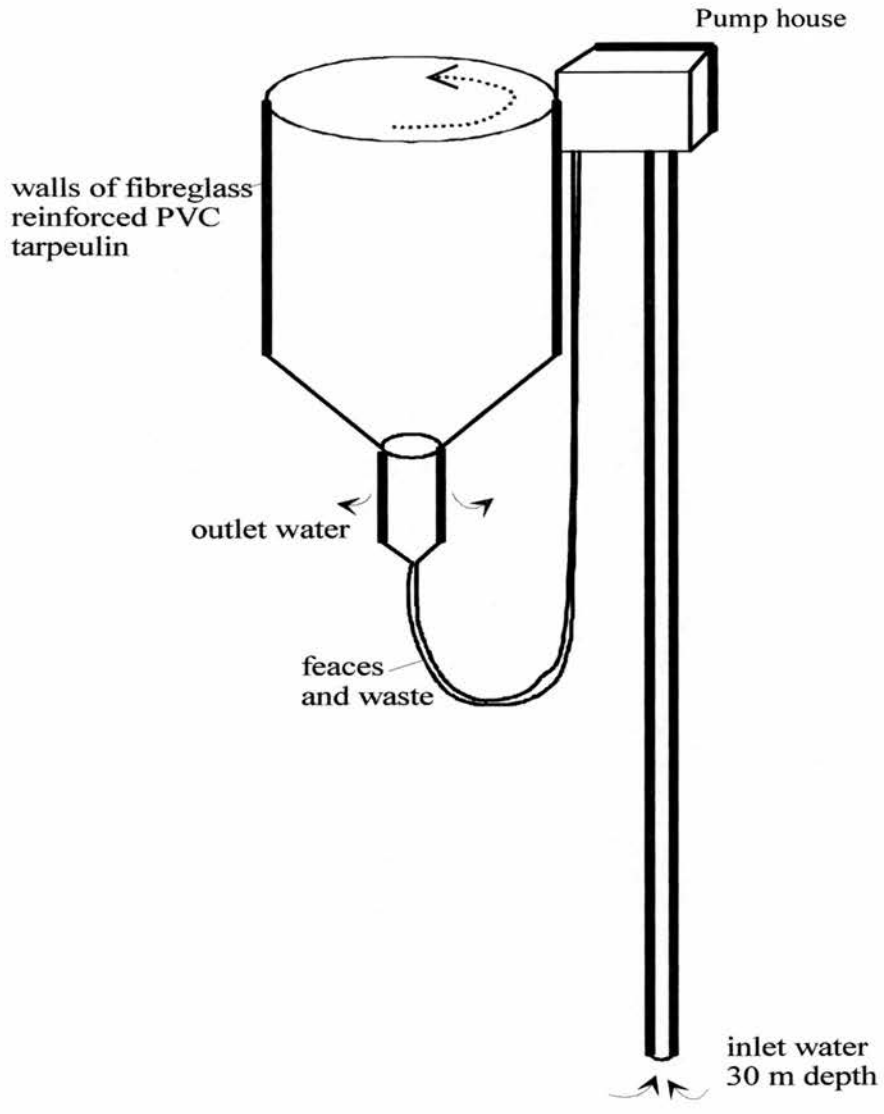


Figure 4.1 Closed pen construction illustrating deep inlet and water circulation (GIGA AS, Storheia 23, 5046 Rådal, After Bjørnevik *et al.*, 2000a).

4.3 Results

4.3.1 Effect of cage and light on muscle fibre frequency distribution

Muscle fibre frequency distributions showed wide variation between treatments with the greatest variation occurring in groups exposed to additional light treatment (Fig.4.2b and fig. 4.3b). There is strong evidence of bi-modality in the distribution of fibre diameters in a number of fish indicating cycles in the intensity of fibre recruitment. Analysis of the 5^{th} and 10^{th} percentiles values of the muscle fibre diameter distributions, however, show no significant differences between light manipulated and non-light manipulated fish (Table 4.1). This effect may have been obscured by the large variation in frequencies seen in the light groups and evidenced by the wide variability bands formed around the bootstrap estimate (Fig. 4.4b and 4.5b). Significant differences were found, however, in the 95^{th} percentile, with light manipulated fish having significantly larger diameter fibres (see Table 3.1) In contrast, fish reared in both open and closed cages under natural light had less variable frequency distribution (Fig. 4.2a and 4.3a) with smaller variability bands around the bootstrap estimate (Figs. 4.4a and 4.5a).

Fish reared in open cages had significantly higher total white muscle cross-sectional areas ($851mm^{-1} \pm 20$ and $768mm^{-1} \pm 19$) compared to fish held in closed pens ($619mm^{-1} \pm 13$ and $692mm^{-1} \pm 27$) (mean ± SE, n =6) ($F_{1,19} = 25.10$ $P<0.001$). Muscle fibre number was significantly correlated with body mass (Fig.4.6) (r^2 adj. = 0.234, $F_{1,23} = 2.47$ $P<0.0001$) and two-way analysis of variance with body mass as a co-variate, revealed no significant differences in fibre number between treatments. Vertical differences in muscle fibre density were found to vary within the cutlet section with a

higher density in the dorsal epaxial region than the lateral epaxial region (Fig. 4.7). However, no significant differences in muscle fibre density were found between treatment groups ($F_{1,19}$ = 1.24 P>0.05) (Table 4.2).

4.3.2 Harvest Quality

No significant differences in texture (hardness) were found between cages or light treatments ($F_{1,19}$ = 1.33, P>0.05) (Table 4.2). Regression analysis using data combined from all treatments revealed no significant relationship between hardness and muscle fibre density (r^2 adj. = 0.06, F1,23 =2.47) (Fig. 4.8). Roche colour card score was not significant between treatment groups (Table. 4.2) and showed no relationship with muscle fibre density (Fig. 4.9). Percentage lipid was significantly higher in the open cage compared to the closed pen groups ((Table 4.2)($F_{1,19}$ = 7.21, P<0.05) although this was related to the higher body mass in this group (Fig. 4.9). No correlation was found between % lipid and hardness of individual fillets (r^2 adj. =0.05 $F_{1,19}$ =0.95 P>0.05) (Fig. 4.10).

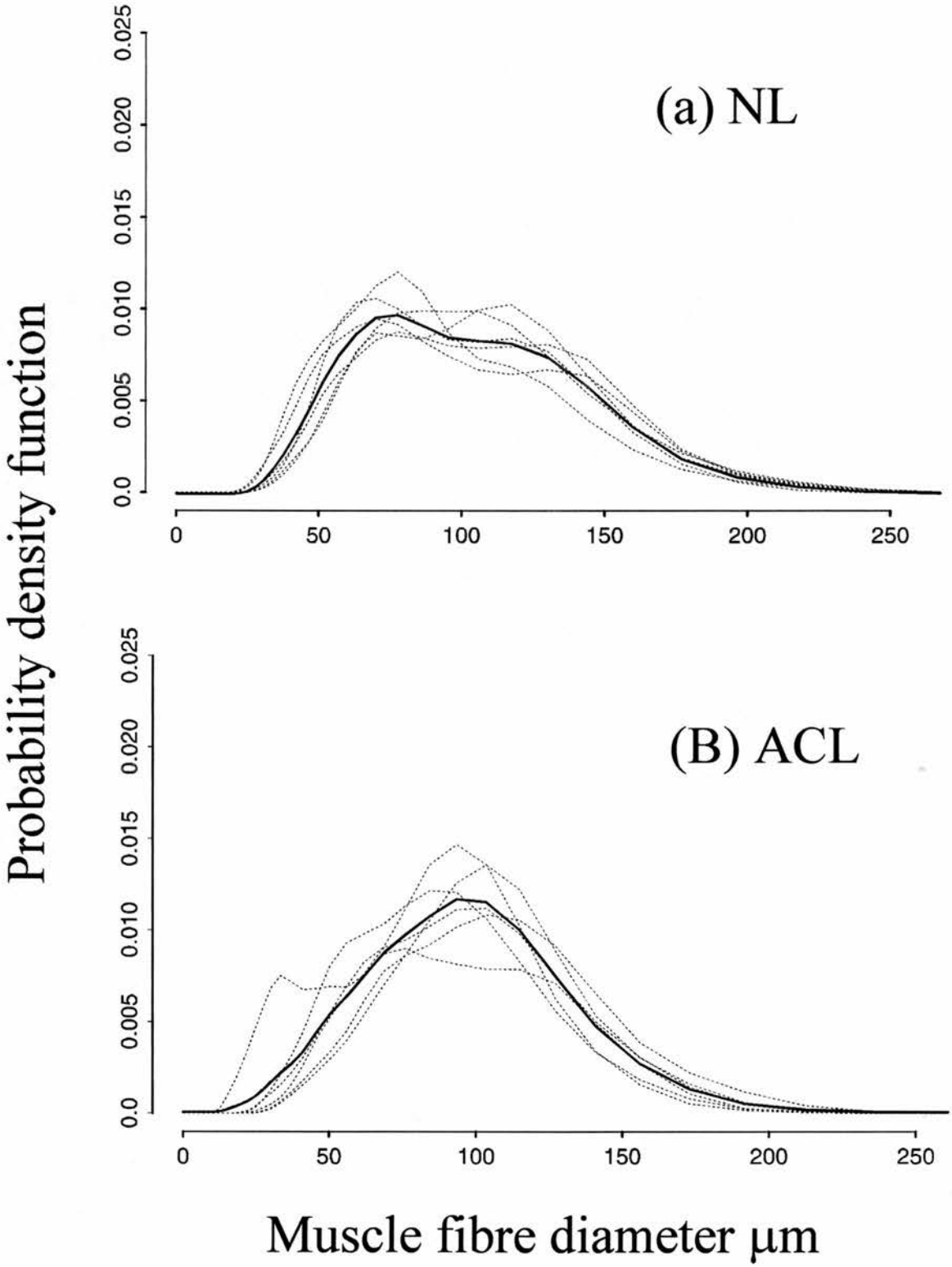


Fig. 4.2 Probability density function of white muscle fibre diameter for Atlantic salmon reared in open sea cages under (a) natural (NL) and (b) additional continuous light (ACL) conditions. The dotted lines show individual fish and the solid line the average probability density fucntion calculated using the mean smoothing parameter.

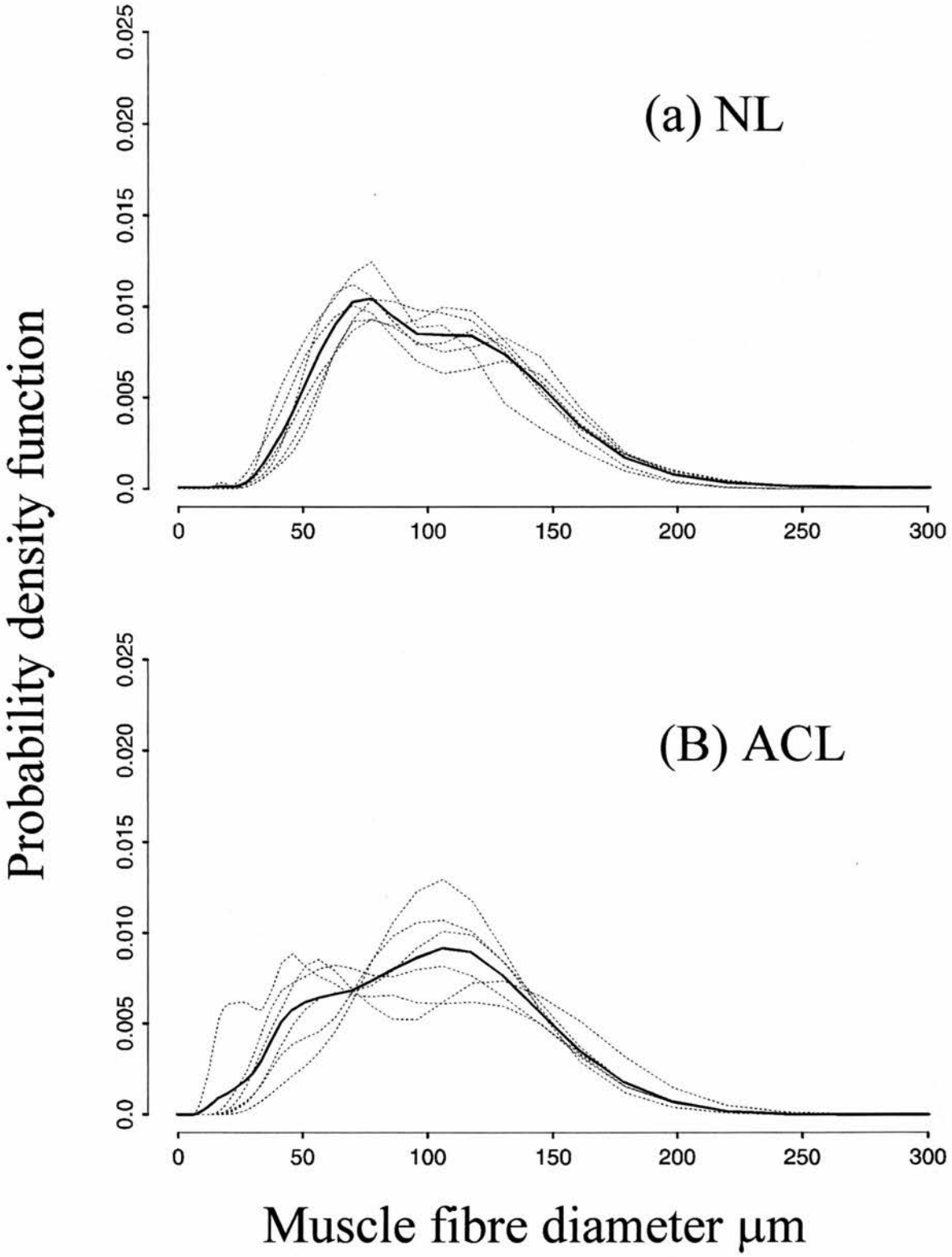


Fig.4.3 Probability density function of white muscle fibre diameter for Atlantic salmon reared in closed pens under (a) natural (NL) and (b) additional continuous light (ACL) conditions. The dotted lines show individual fish and the solid line the average probability density fucntion calculated using the mean smoothing parameter

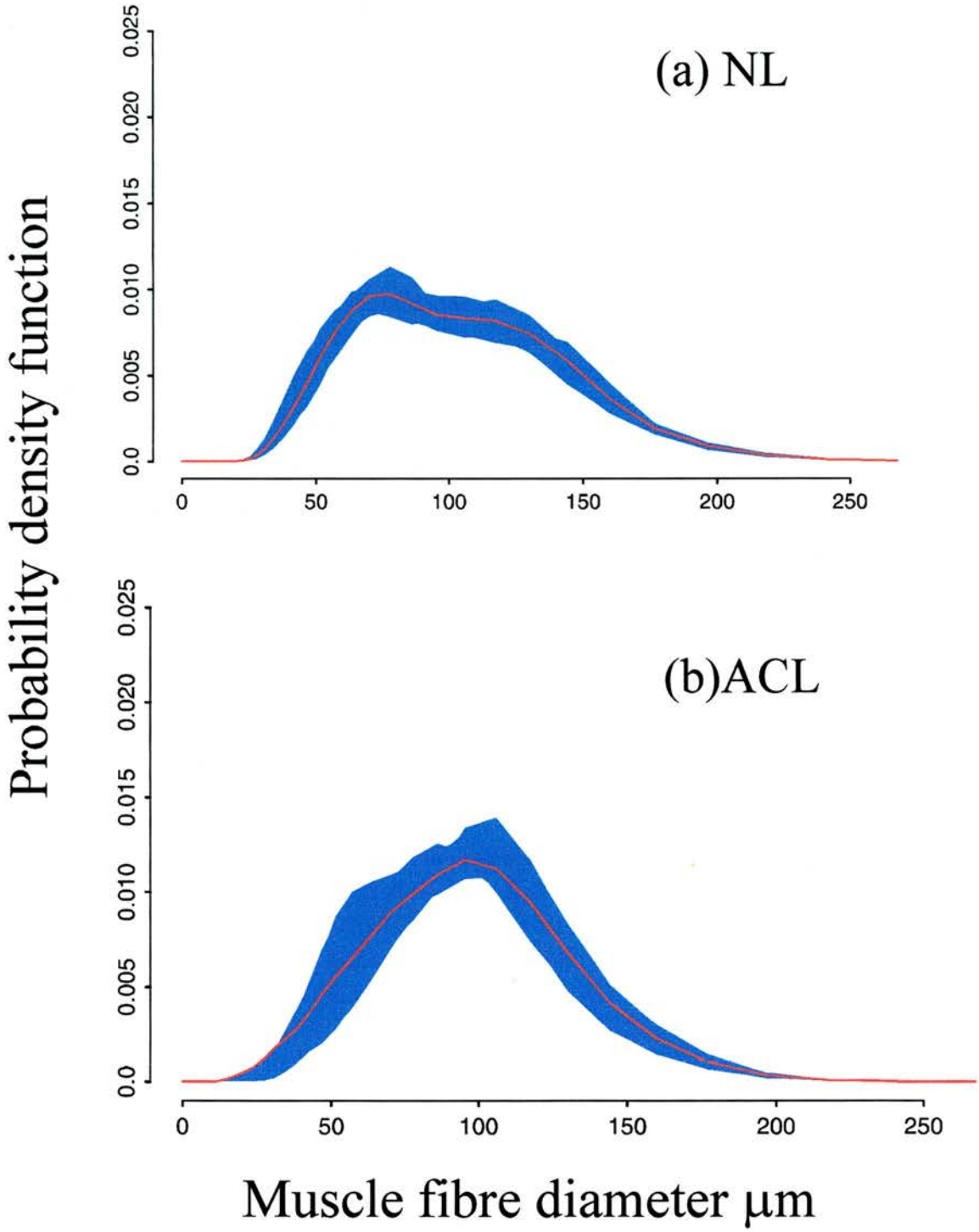


Fig. 4.4 Bootstrap estimates of the variance of probability density function of white muscle fibre diameter for Atlantic salmon reared in open sea cages under (a) natural (NL) and (b) additional continuous light (ACL) conditions. The blue shaded area corresponds to the variability band of the 100 bootstrap estimates and the red line the mean probability density function.

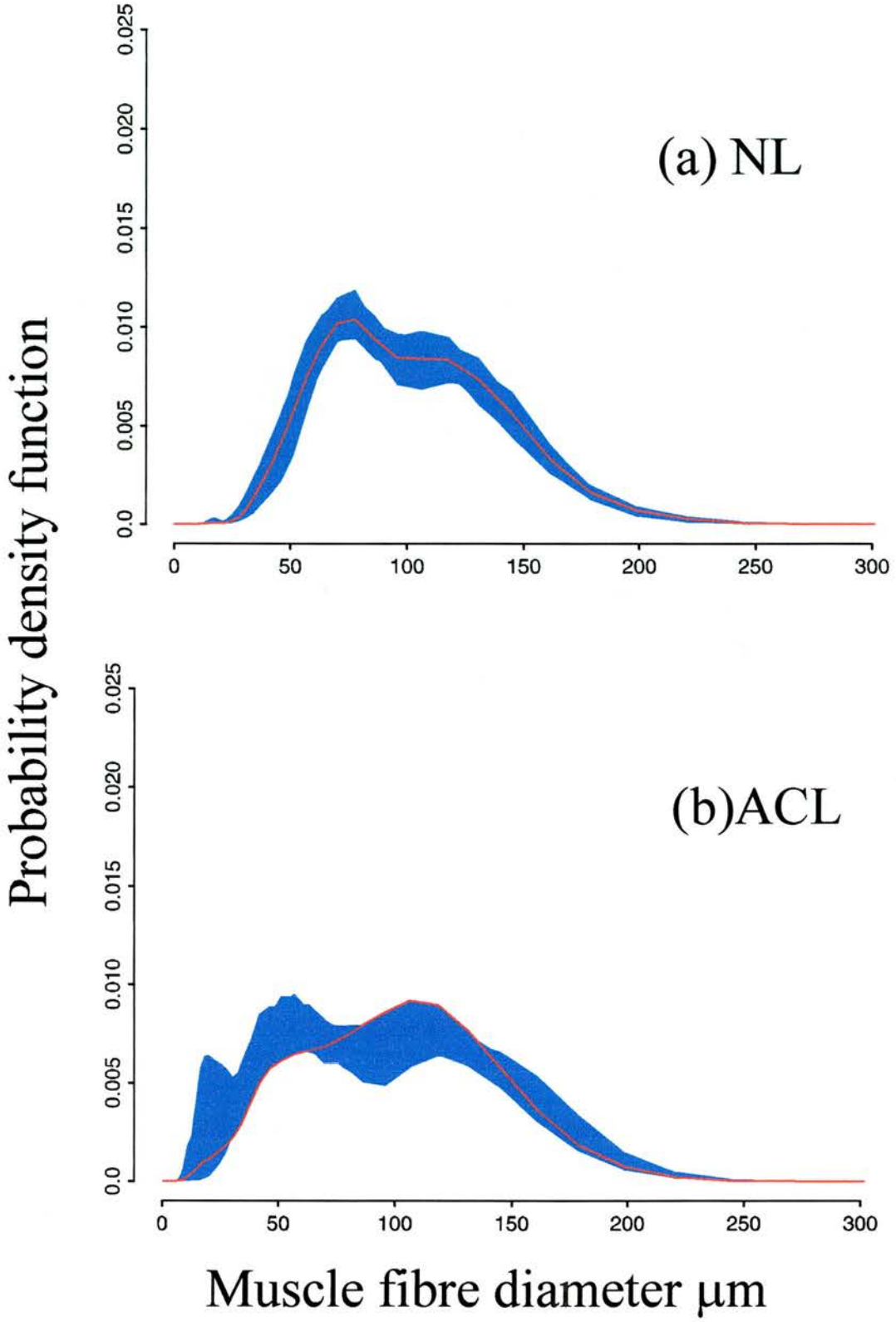


Fig.4.5 Bootstrap estimates of the variance of probability density function of white muscle fibre diameter for Atlantic salmon reared in closed pens under (a) natural (NL) and (b) additional continuous light (ACL) conditions. The blue shaded area corresponds to the variability band of the 100 bootstrap estimates and the red line the mean probability density function.

	Open Cage		Closed Pen	
Percentile	NL (mean±SE,n=6)	ACL (mean±SE,n=6)	NL (mean±SE,n=6)	ACL (mean±SE,n=6)
5th	48.69 ± 1.94	46.58 ± 4.54	49.18 ± 1.21	40.92 ± 2.80
10th	56.66 ± 1.19	55.01 ± 4.92	56.95 ± 1.35	50.27 ± 3.40
50th	101.00 ± 1.92	96.64 ± 3.36	99.96 ± 2.01	101.28 ± 0.59
95th	159.68 ± 3.47	182.18 ± 4.48	162.39 ± 3.18	186.80 ± 4.10

Table.4.1 Percentile values for the average probability density functions of muscle fibre diameter (μm) in Atlantic salmon reared in open cages under natural light (NL) and additional continuous light (ACL), and in closed pens under natural light (NL) and additional continuous light (ACL).

	Open Cage		Closed Pen	
Parameter	NL (mean±SE,n=6)	ACL (mean±SE,n=6)	NL (mean±SE,n=6)	ACL (mean±SE,n=6)
Fibre Density (fibres/mm^2)	105.23 ± 6.06	106.73 ± 6.37	119.45 ± 6.11	117.28 ± 2.54
XS Area (mm^2)	851 ± 20.44	768 ± 19.52	619 ± 13.79	692 ± 27.33
Fibre Number	890774 ± 45640	809559 ± 42033	739661 ± 43325	726907 ± 45270
Lipid %	17.45 ± 0.72	14.92 ± 0.60	11.97 ± 0.70	14.08 ± 0.50
Hardness (g)	1070 ± 211	838 ± 123	1013 ± 99	952 ± 76
Roche Score	15.58±0.08	15.25±0.11	15.50±0.08	15.42±0.08

Table.4.2 Muscle and flesh quality parameters in Atlantic salmon reared in open cages under natural light (NL) and additional continuous light (ACL) and in closed pens under natural (NL) and additional continuous light (ACL).

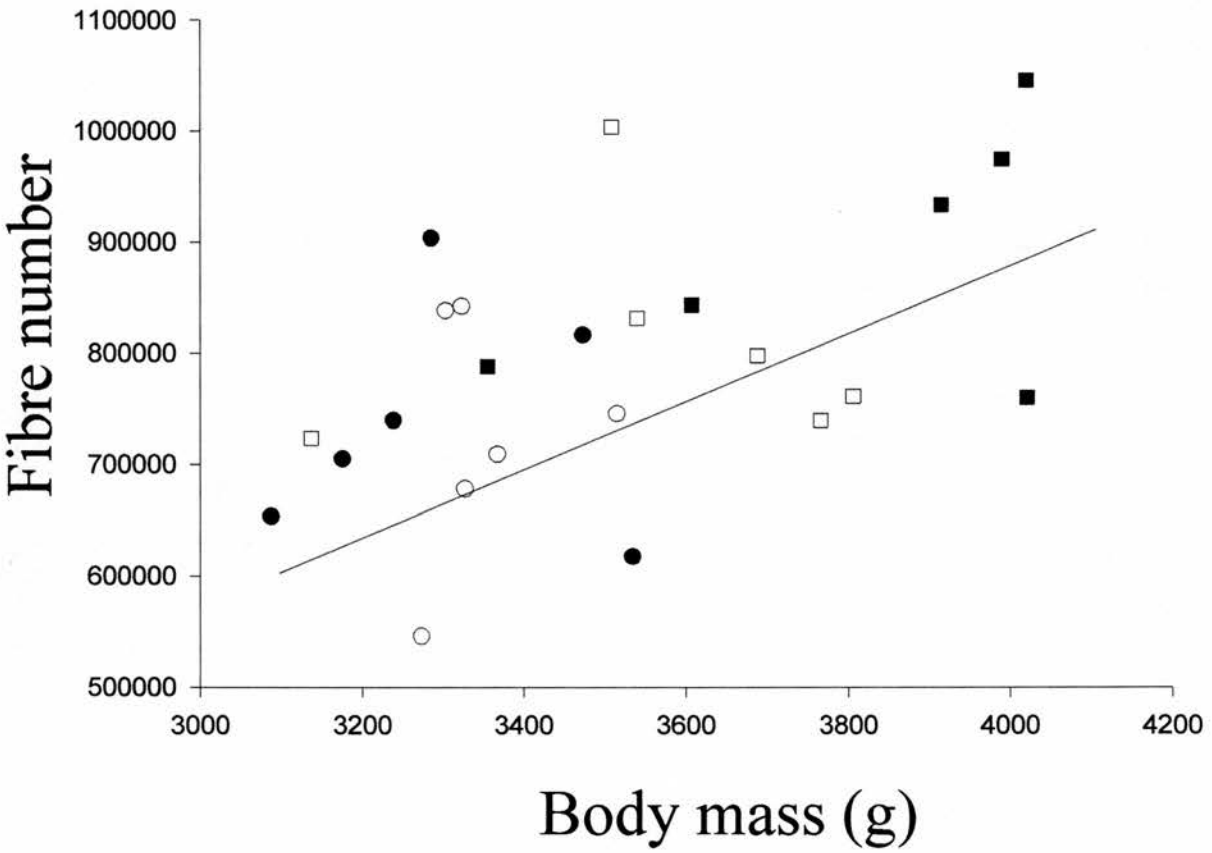


Fig. 4.6 Relationship between body mass and fibre number in Atlantic salmon reared in open cages under natural light (OCNL, filled squares) additional continuous light (OCACL, open squares) and in closed pens under natural (CPNL, filled circles) and additional continuous light (CPACL,open circles) (r^2 adj. = 0.234, P<0.0001)

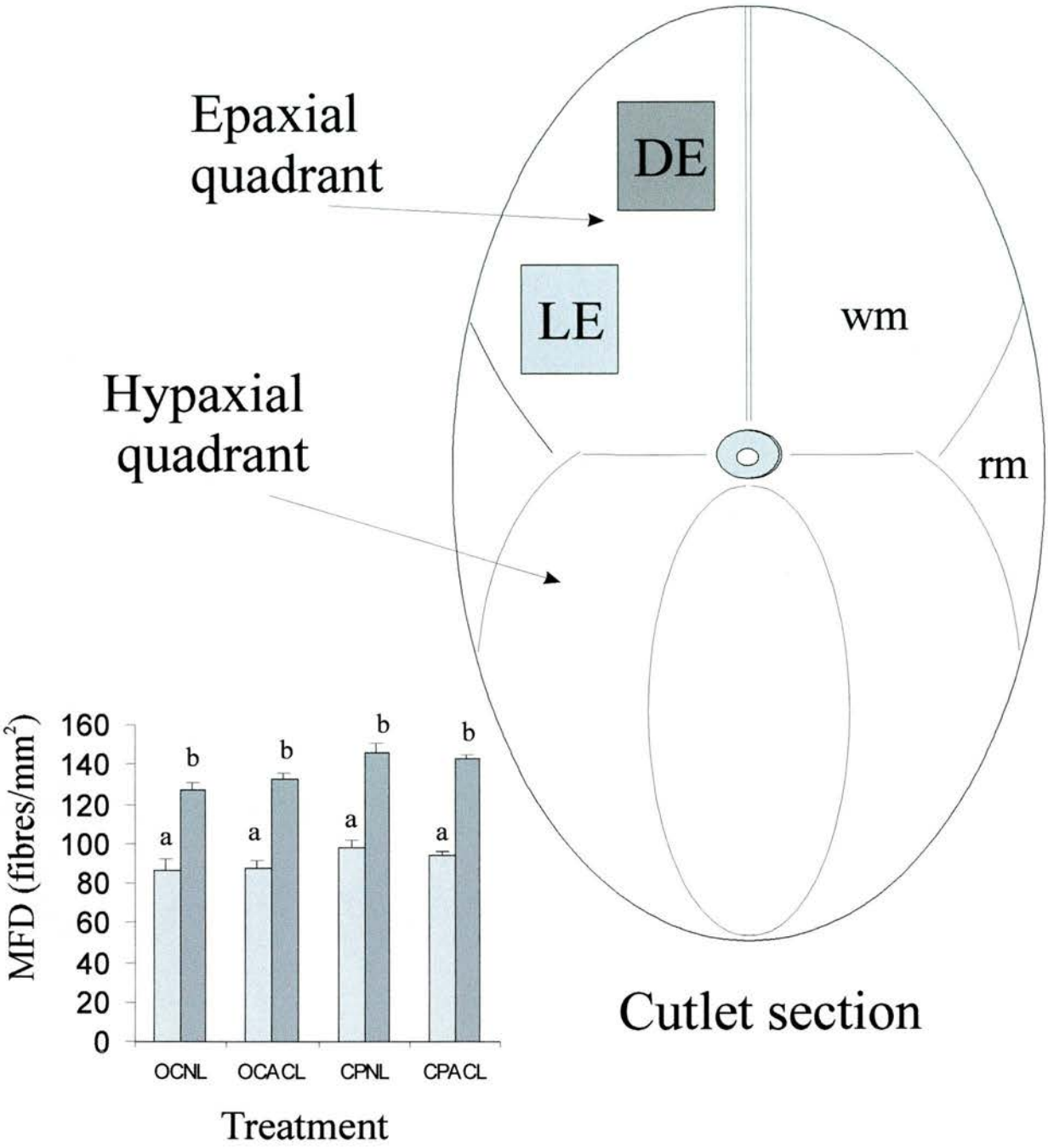


Fig. 4.7 Diagram showing differences in mean muscle fibre density (MFD) between the dorsal region of the epaxial quadrant (DE) and the lateral region (LE) in Atlantic salmon reared in open cages under natural (OCNL) additional continuous light (OCACL) and in closed pens under natural (CPNL) and additional contnuous light (CPACL). Significant differences (t-test, $P<0.0001$) between sample sites are indicated by different letters (a,b) wm =white muscle, rm = red muscle.

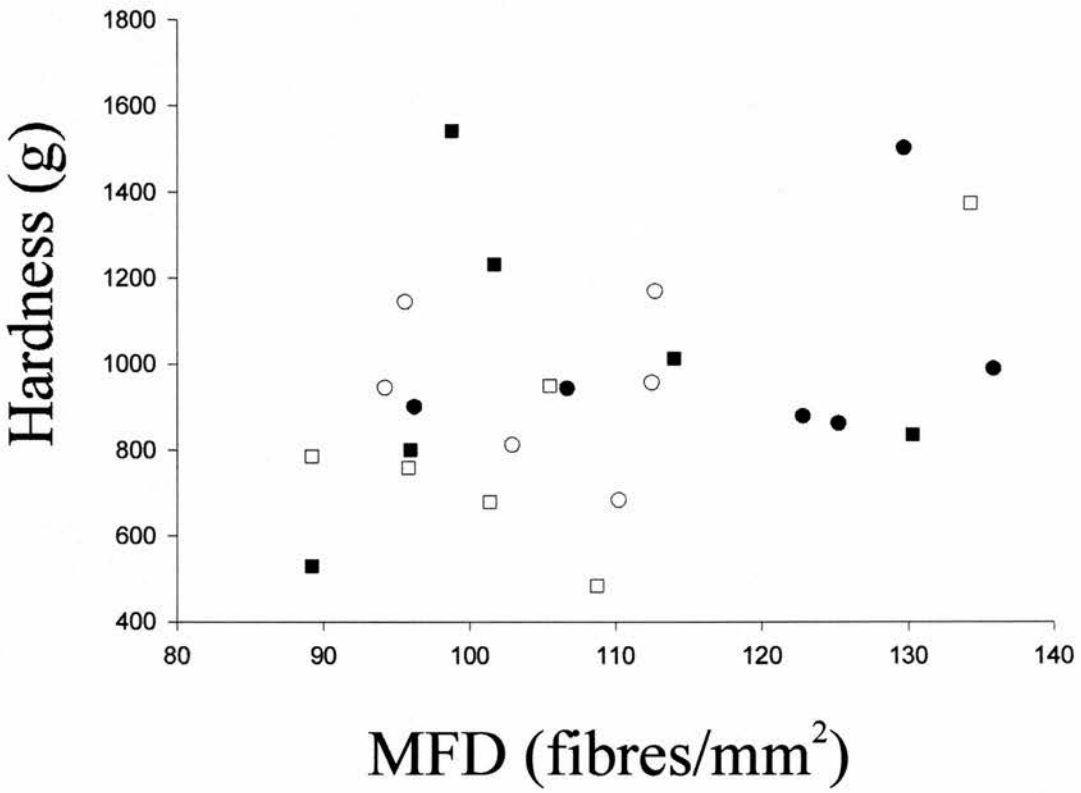


Fig.4.8 Relationship between muscle fibre density (MFD) and hardness in Atlantic salmon reared in open cages under natural light (OCNL, filled squares) additional continuous light (OCACL, open squares) and in closed pens under natural (CPNL, filled circles) and additional continuous light (CPACL,open circles).

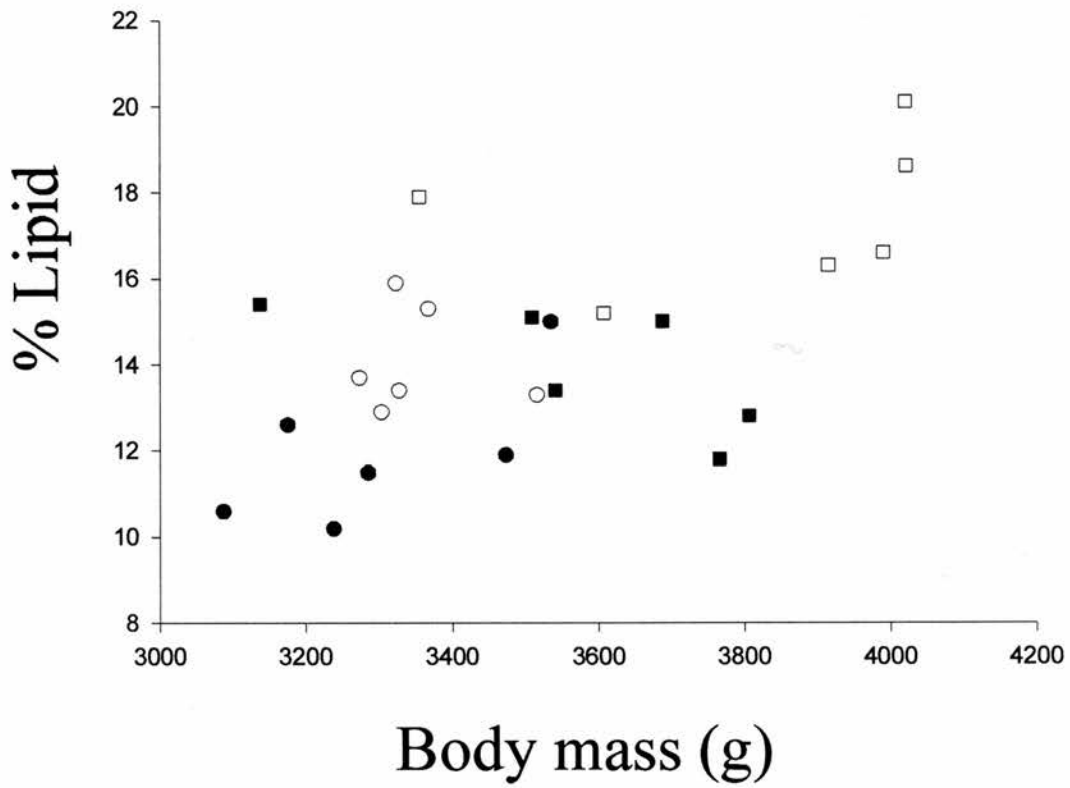


Fig. 4.9 Relationship between body mass and % lipid in Atlantic salmon reared in open cages under natural light (OCNL, filled squares) additional continuous light (OCACL, open squares) and in closed pens under natural (CPNL, filled circles) and additional continuous light (CPACL,open circles).

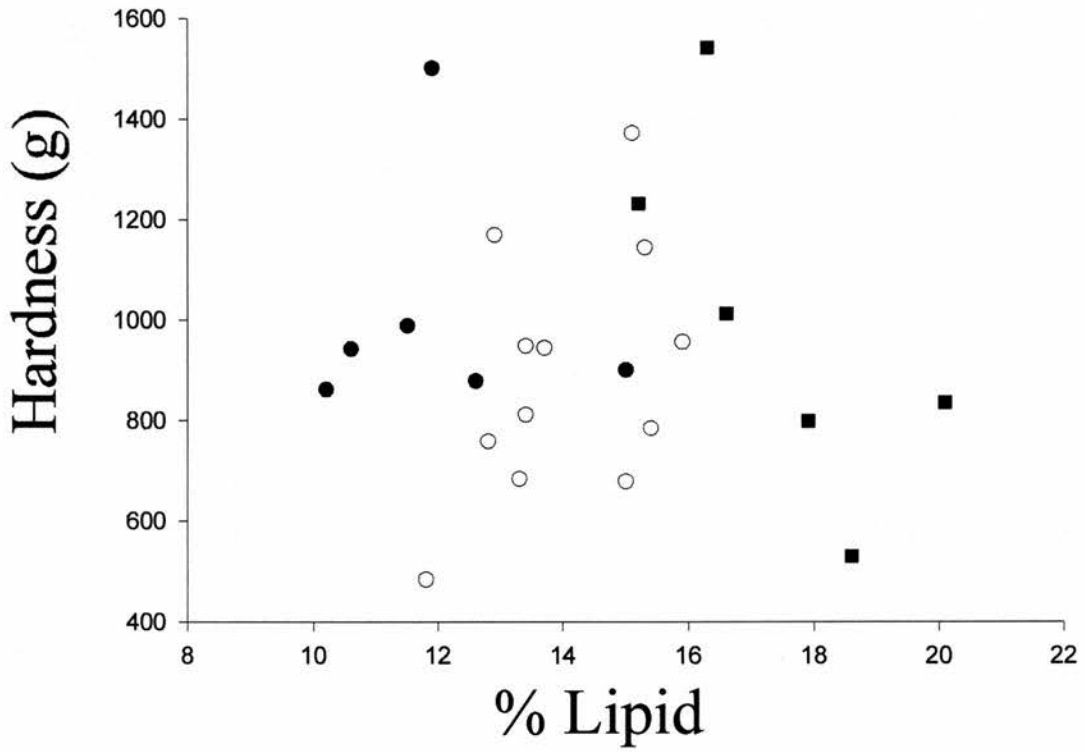


Fig. 4.10 Relationship between % lipid and 'hardness' of filets taken from Atlantic salmon reared in open cages under natural light (OCNL, filled squares) additional continuous light (OCACL, open squares) and in closed pens under natural (CPNL, filled circles) and additional continuous light (CPACL,open circles).

4.4 Discussion

Previous histological studies have shown that when fish are compelled to swim at a certain speed for long periods of time, the muscle fibres used for propulsion become hypertrophied (Greer-Walker, 1971; Greer-Walker and Pull, 1973; Greer-Walker and Emerson, 1978; Johnston and Moon, 1980). The results of the current study found no influence of cage type on muscle cellularity. Values of the 5th and 10th percentiles showed no consistent differences between cage treatments while the significant differences in the 95th percentile values were linked to light treatment. In salmonids, it has been found that swimming speeds of 1.0 to 1.5BL.s-1 (body length per second) results in the highest growth rates (Davison and Goldspink 1977, Jørgensen and Jobling 1993). Totland *et al.* (1987) found that current speeds of 28±11cm/s resulted in hypertrophy of the white muscle in Atlantic salmon. The current velocity in the closed cages varied between 30 and 40cm/s (an equivalent of 1.5BL.s^{-1}) although no effect on hypertrophy of white muscle was apparent. Kiessling *et al.* (1994) found no effect of experimentally induced swimming speeds of 0.3-1.8BL.s^{-1}on hypertrophy of white muscle in all-female Chinook salmon (*Oncorhyncus tshawytscha*) while similar results have been found in rainbow trout (*Oncorhyncus mykiss*) by Gamperl and Stevens (1991). The studies of Johnston and Moon (1980) and Greer-Walker and Pull (1973) were carried out at relatively high swimming speeds and yet only produced modest changes in muscle cellularity. Increased growth rates in exercising fish are dependent on feeding to satiation (Davison and Goldspink 1977, Davie *et al.* 1986). A sub-optimal feeding regime in the closed cages may have restricted growth rates and could, therefore, explain the lack of hypertrophy in the exercised fish.

Total white muscle cross-sectional area was greater in the open cage group compared to the closed pen group, irrespective of body mass. Open cage fish had a significantly higher fat content than closed pen fish although this was related to a higher body mass. Studies of lipid variation in exercised fish have yielded variable results with lipid levels increasing with increased exercise (East *et al.,* 1987), remaining constant (Jørgensen and Jobling 1993, Kiessling *et al.,* 1994) or decreasing (Christiansen *et al.,* 1989). Higher fat content, together with a greater number of muscle fibres linked to body mass (Shearer 1994) may, therefore, explain the higher total cross-sectional area in the open cage group.

Perhaps more interesting, is the variability seen in muscle fibre frequency in fish reared under additional continuous light irrespective of cage type, with evidence of distinct bi-modality and phased recruitment. Johnston *et al.* (1999,2000a) showed distinct seasonal phases of recruitment leading to bi-modal frequency distributions in triploid and diploid salmon. Photoperiod is known to synchronise the endogenous cycle of growth in Atlantic salmon via its effect on the light-pituitary axis (Komourdjian *et al.*, 1986). Beattie *et al.* (2000) showed that light manipulation in juvenile Atlantic salmon advanced smoltification and associated muscle recruitment by six months, leading to an increase in the number of new, small muscle fibres. Continuous light in sea cages has been shown to influence growth rates in seawater stage Atlantic salmon (Taranger *et al.,* 1995; Oppedal *et al.,* 1997) with light manipulated fish out of 'phase' compared to their non-lit counterparts. It possible that the use of continuous light in the open and closed cages 'advanced' the normal muscle growth program of the fish, stimulating a seasonal muscle

fibre recruitment. The value of the 95th percentile was significantly higher in the light manipulated fish indicating increased hypertrophy. The 5th and 10th percentiles were not, however, significantly different between light treatments. Small muscle fibres have a high nutritive assimilative capacity due to their higher surface area:volume ratio and new fibres are, therefore, recruited into the myotome and hypertrophy quickly, making detection of recruitment phases difficult. Periods of intense recruitment provide an opportunity for subsequent rapid growth as the newly formed fibres increase in diameter (Johnston *et al.*, 1999). The fish in each light treatment group were significantly heavier than their non–lit counterparts but all open cage fish were heavier than closed pen fish (Bjørnevik *et al.*, 1999). This suggests the influence of other factors on the growth process in the closed pen fish including water temperature, stocking density and current velocity. The results do indicate, however, that the cellularity but not the overall size of the muscle was affected by light treatment.

Harvest Quality

Hurling *et al.* (1996), Johnston *et al.* (2000b) and Beattie *et al.* (2000c) have all linked muscle fibre density to quality traits in fish as measured by sensoric and instrumental analysis. No relationship was found between muscle fibre density and 'hardness' of the fillet as measured by a compression test in the current experiment. Harvest quality in Atlantic salmon can be significantly affected by pre and post-slaughter treatment (Andersen *et al.*, 1997; Veland, 1997; Wathne, 1995). All fish tested for texture in the current study had been subject to variable treatment prior to texture analysis with some fish 'broken' out of rigor, which could explain the present results. Compression

tests are also subject to variation due to fillet thickness (Veland, 1997), therefore differences in fillet height between treatments would have led to variable results. No relationship was found between muscle fibre density and colour, mainly due to the insensitivity of the method (Roche colour card) which returns non-continuous interval scale results which are open to subjective interpretation by the assessor. Open cage fish did, however, have significantly higher lipid than closed pen fish and this may influence textural properties (Beattie *et al.*, 2000c) by producing a softer flesh. Conversely, exercised fish muscle may contain higher levels of collagen than non-exercised fish (Sato *et al.*, 1986), increasing the 'toughness' of the flesh. Texture analysis in the current study was, however not sensitive enough to detect any differences in texture between treatments.

Significant differences were found between dorsal and lateral epaxial sample sites in the muscle with the dorsal region having a significantly higher muscle fibre density than the lateral region. This is in agreement with the findings of Kiessling *et al.*, (1991) who found a similar pattern of fibre density in the rainbow trout. Beattie *et al.*, (2000c) found longitudinal variation in white muscle fibre density in Atlantic salmon fillets with higher fibre densities in the caudal compared to the rostral region. Future studies should take into account this horizontal and vertical difference when assessing the influence of muscle fibre density and harvest quality parameters.

This first study formed a preliminary investigation into methods for assessing flesh quality and muscle fibre cellularity in Atlantic salmon. Extensive modifications

were made in sample protocols in subsequent studies, particularly in the area of texture analysis (Chapter 5) while the differences in muscle fibre diameter in the dorsal and lateral areas helped to establish the protocol for muscle sampling in Chapters 3 and 4.

Chapter 5

MEASUREMENTS OF FLESH QUALITY AND ITS RELATION TO MUSCLE FIBRE DENSITY IN ATLANTIC SALMON

(*Salmo salar* L.)

5.1 Introduction

The texture of meat is of utmost importance to consumer acceptance and therefore much research effort has been put into this issue in order to be able to understand and control it (Tornberg, 1996). The texture of meat can be defined, according to Szczesniak (1963), as the composite of the structural elements of meat and the manner in which it registers with the physiological senses. The composition of fish meat depends on factors related to fish species, size, maturity, sex and activity of the specimen, as well as environmental conditions encountered by the living organism (Dunajski 1979, Shearer 1994, Aksnes *et al.*, 1986). The fish farmer has some control over physiological factors such as biological age and growth rate and environmental factors such as water temperature, pressure, flow and chemistry and dietary factors such as feeding cycle, starvation, and overfeeding.

Texture of fish meat is influenced by several factors. Among these are the rate and extent of post-mortem muscle shortening (rigor mortis), the rate and extent of post-mortem pH decline and the rate of proteolysis causing myofibril breakdown (Hutllin, 1985; Haard 1990, 1992b; Ando *et al.*, 1991). In species like salmonids, the amount, fatty acid profile and distribution of muscle fat may have an influence on meat texture (Mohr, 1986; Bell *et al.*, 1998 and Waagbø *et al.*, 1993).

In Norway, the production of farmed Atlantic salmon (*Salmo salar* L.) and Rainbow trout (*Oncorhyncus mykiss*) has increased dramatically in the last 5 years from 64,064 tonnes in 1986 to over 380,000 tonnes in 1998 (Data source: Kontali analysis). As overall production rises and prices fall, there is an increasing focus on the quality of salmon. Important attributes of food quality are safety, nutrition, texture, flavour, colour appearance and the suitability of the product for processing and preservation (Haard, 1992). However, flesh quality is a highly subjective and broad term encompassing numerous physico-chemical properties (Love, 1980).

It has been suggested that variations in the quality of the fillet along the length of the body are largely due to variations in the size distributions of muscle fibres (Dunajski, 1979) and are also correlated with the amount of collagen present (Montero and Borderias, 1990). Recent work on flesh quality in fish has related muscle cellularity to different flesh quality traits (Johnston *et al.,* 2000b). Hurling *et al.* (1996) showed that muscle fibre cross sectional area was related to sensory firmness in a number of different species, fish with smaller fibres having a greater sensory firmness. Hatae (1986) proposed a 'lattice' theory of sensory firmness in which he related the textural properties of the fish to the basic structural element of the muscle fibre. Smaller, more closely packed muscle fibres provide a denser, firmer structure than larger, more loosely packed fibres which give a softer texture. Recently, a positive correlation was found in Atlantic salmon between muscle fibre density and the 'firmness' and 'chewiness' of the smoked product as assessed by a trained taste panel (Johnston *et al.,* 1999b). Mean cross sectional area of muscle fibres increase with body length (Weatherley and Gill, 1987 and Kiessling *et al.*,

1991) so it would be expected that sensory firmness would decrease as fish grow and, therefore, the average fibre diameter increases.

Additionally, mean muscle cell diameter decreases rostro-caudally and therefore one would expect a greater sensory firmness closer to the tail end of the fillet. The 'Norwegian quality Cut', considered to be the best part of the fillet in terms of sensory firmness and high Roche score, is located towards the caudal end of the fillet where one would expect a high muscle fibre density. No work has yet investigated the influence of muscle fibre density on instrumental measurement of texture within a single species or between sites on the same fillet.

Although texture is an important indicator of quality in salmon, both visual colour and fat level can have as important an influence on product variability and desirability (Koteng, 1993). Sheehan *et al.* (1996), found that high fat fish were more susceptible to gaping but had a firmer texture than fish fed a low fat diet while Bjerkeng *et al.* (1997) suggested that intestinal astaxanthin absorption was facilitated by dietary fat content. Colour and fat levels are also known to vary within a single fillet, Choubert *et al.* (1997) found that that fillet location in rainbow trout significantly influenced colour with significantly higher readings in the tail than in the dorsal region. Nickell and Bromage (1998) found significantly higher concentrations of astaxanthin and a* value scores in the tail region of rainbow trout fillet, compared with the dorsal and belly regions.

The aim of the present study was to investigate the relationship between texture as measured using the Warner-Bratzler shear test and the density of muscle fibres in the fillet. The relationship between muscle fibre density, fat level and visual colour score were also evaluated.

5.2 Materials and Methods

1000 mixed sex, two-sea winter Atlantic salmon (*Salmo salar* L.) (NLA strain, single family) reared under ambient photoperiod and temperature conditions, fed Skretting Royale dry pellets (3mm-9mm, 40% protein, 30% oil, 6% moisture) and covering a size range of 1-5kg were sampled from sea cages at Matre Aquaculture Research Station on 30th September 1998. The fish were killed by percussion stunning, bled on iced water and transported by road to Innstefjord processing plant (20km North of Matre). A sub-sample of forty fish were selected for flesh quality analysis and marked with floy tags (Hallprint Ltd. Australia); any fish with obvious deformities or low condition factor (<0.90) were discarded from the sample. All fish were weighed to the nearest 1g, measured for fork length, gutted and assessed for sexual maturity. Tagged fish were packed on ice in polystyrene boxes and transported to Matre aquaculture research station and stored at 4°C for four to five days until rigor was resolved.

All fish were filleted by hand and colour assessed at points 'A' and 'B' ('A' representing the 'Mowi' cut and 'B' representing the Norwegian quality cut, NQC) shown in Fig 5.1 using a Hunterlab Miniscan XE (Hunterlab Reston, VA, U.S.A.) (Skrede and Storebakken 1986; Choubert *et al.,* 1997). Three measurements were taken at each point and the mean value for each axis (L* lightness, a* redness and b* yellowness) was used for the final results. Two sections were taken from the fillet at the points shown in Fig. 5.1, sealed in plastic bags and stored at 4°C ready for instrumental texture analysis. The remaining fillet was skinned and identical areas to those taken from the first fillet were excised, homogenised with 1ml of antioxidant (acetone containing

100ppm of 2,[6]-di-tert-butyl-p-cresol, BHT) and placed in a plastic tub prior to freezing for later analysis of total lipid and dry matter.

5.2.1 Textural and histological analysis

Fillet sections were removed from the refrigerator and allowed to reach a temperature between 8-10°C before texture analysis was carried out. A 1.5cm diameter bore was used to remove a standard plug of muscle from the lower epaxial area shown in Fig. 5.1. The bore section was then divided in two and texture assessed on both pieces by the Warner-Brazler Shear test, measuring the maximum force required to shear the bore sample (MSF) (Veland *et al.*, 1997; Mittal *et al.*, 1992). Muscle samples were removed from each of the cut plugs and immersed in cryo-protectant (Tissue-Tek, Shandon U.S.A.) and frozen in 2-Methylbutane (iso-pentane) cooled to it's melting point in liquid nitrogen (-196°C). Frozen muscle blocks were wrapped in aluminium foil, sealed in plastic bags and stored at -80°C prior to sectioning. Transverse sections were cut at 10-12μm on a Bright Starlet Microtome set to -20°C and mounted on poly-L-lysine coated slides (Sigma, Poole, Dorset, UK). Muscle sections were air dried and then stained with carbaniline red (Scarba red) before mounting in glycerol gelatin (Sigma, Poole, Dorset, UK). Muscle fibre cross-sectional area was determined by digital planimetry using an Image Pro 3.0© analysis system (Caltech. Co. USA) attached to a light microscope set to X10 objective. Two hundred muscle fibres were measured from each bore section to ascertain the mean fibre cross-sectional area. Muscle fibre density (MFD, no. fibres/mm^2 muscle) was calculated by dividing the total cross-sectional area of muscle measured by the number of fibres counted.

5.2.2 Total Lipid and Dry Matter Analysis

Triplicates of 3g (± 0.0001g) from homogenised bore sections were added to 20ml pre-weighed glass tubes, dried at 105°C overnight, stored in an exicator at room room temperature and weighed again for determination of dry matter. Subsequently, 1.5ml of ethylacetate was added to the samples, shaken for 1 hour, and stored overnight at 4°C. An aliquot of 10ml was collected from each sample and transferred to new preweighed glass tubes, evaporated in a water bath (90°C) for 5 hours, dried at 105°C for 20 minutes, cooled to room temperature in an exicator and weighed (± 0.0001g) for determination of total lipid content.

5.2.33 Statistics

Differences in flesh quality and muscle characteristics between sexes were analysed by analysis of variance (ANOVA) with a significance level of $P<0.05$ applied to results. A paired t-test was used to test for differences between 'Mowi' and 'NQC' sample sites on the same fillet. To investigate the influence of muscle fibre density on flesh quality, data from different sexes and positions were combined. Regression analysis was then used to establish the relationship of muscle fibre density (MFD), with maximum shear force (MSF), colour and % lipid.

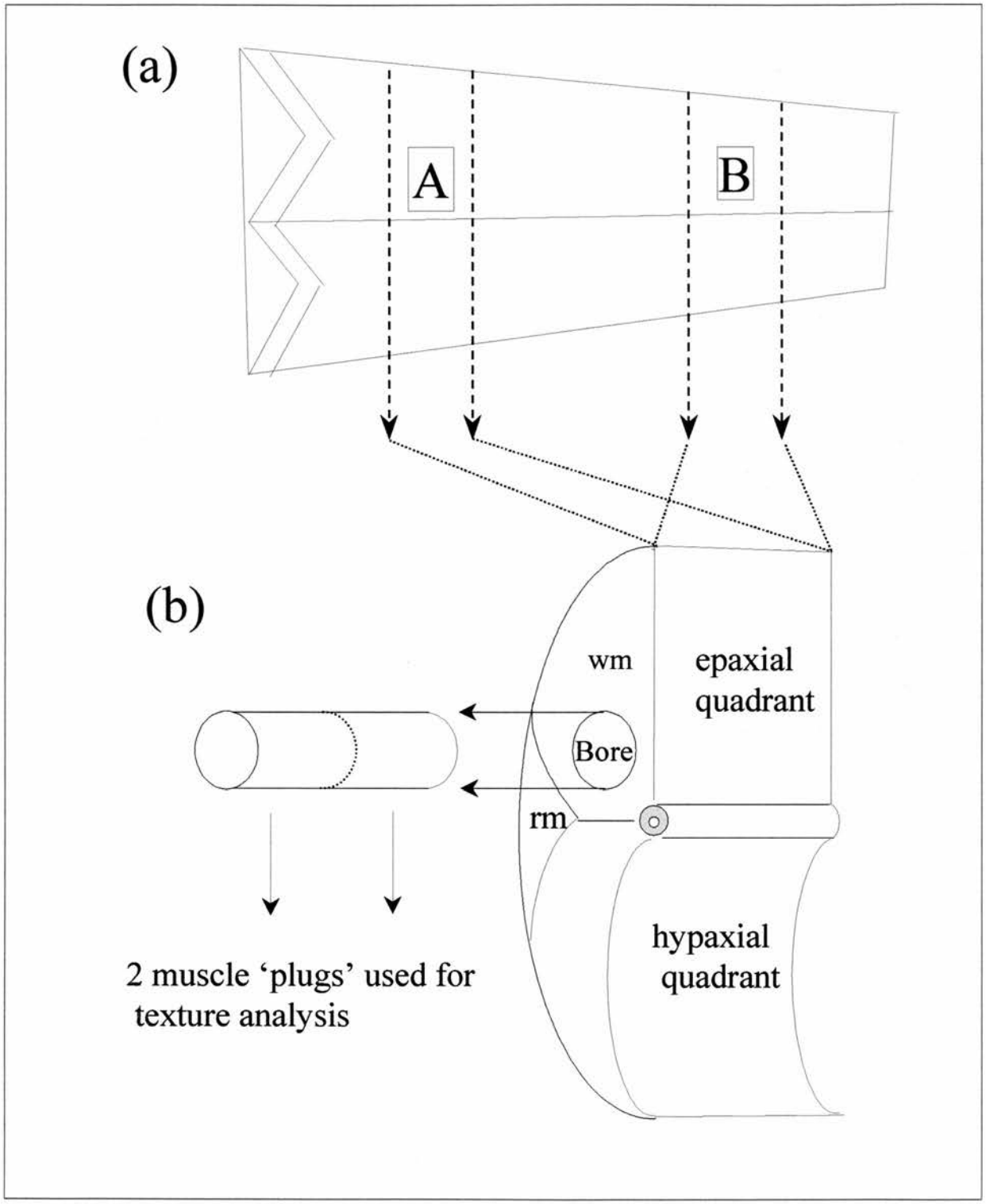


Figure. 5.1 (a) Diagram showing the two main sample sites A (Mowi) and B (NQC) on the epaxial quadrant of fillets taken from Atlantic salmon and (b) cutlet cross-section showing position of bore plug taken for analysis.

5.3 Results

5.3.1 Body mass and Flesh Quality

Fork length was linearly related to body mass in both males and females (Fig. 5.2, r^2 adj. =0.915, $F_{1,18}$=536.99, P<0.0001, r^2adj=0.951; $F_{1,18}$= 370.26, P<0.0001,) with no significant differences between sexes (ANCOVA $F_{1,37}$ =1.10, P>0.05). However, males were significantly heavier (3019g ± 174) than females (2338g ± 190) (mean ± SE, *n*=20) ($F_{1,38}$ =6.93, P<0.05). Body mass was weakly correlated with fibre density with larger fish having a lower fibre density than smaller fish (r^2 adj = 0.188, $F_{1,38}$=10.03, P<0.001, (MOWI), r^2 adj= 0.252, $F_{1,38}$ =14.19, P<0.001 (NQC) Fig. 5.3a). Figure 5.4 shows force/time curves generated by the Warner-Bratzler shear test in the Mowi and NQC regions, showing the point of maximum shear force. Body mass showed a weak but significant relationship with maximum shear force in the Mowi section (r^2 adj. = 0.130 $F_{1,38}$ =6.83 P<0.01) but no significant relationship in the NQC section (r^2 adj. = 0.03 $F_{1,38}$ =2.57 P>0.05) (Fig. 5.3b).

5.3..2 Sex differences in Flesh Quality

Colour, texture and muscle fibre density was not significantly different between sexes in the Mowi (Table 5.1) and NQC (Table 5.2) regions. Total percentage lipid was, however significantly higher in males (15.07±0.58) than females (13.60±0.51) ($F_{1,38}$, P<0.001) although this was not significant when analysed with body mass as a covariate ($F_{1,37}$ = 3.124 P>0.05 (Mowi); $F_{1,37}$ = 13.13, P>0.05 (NQC).

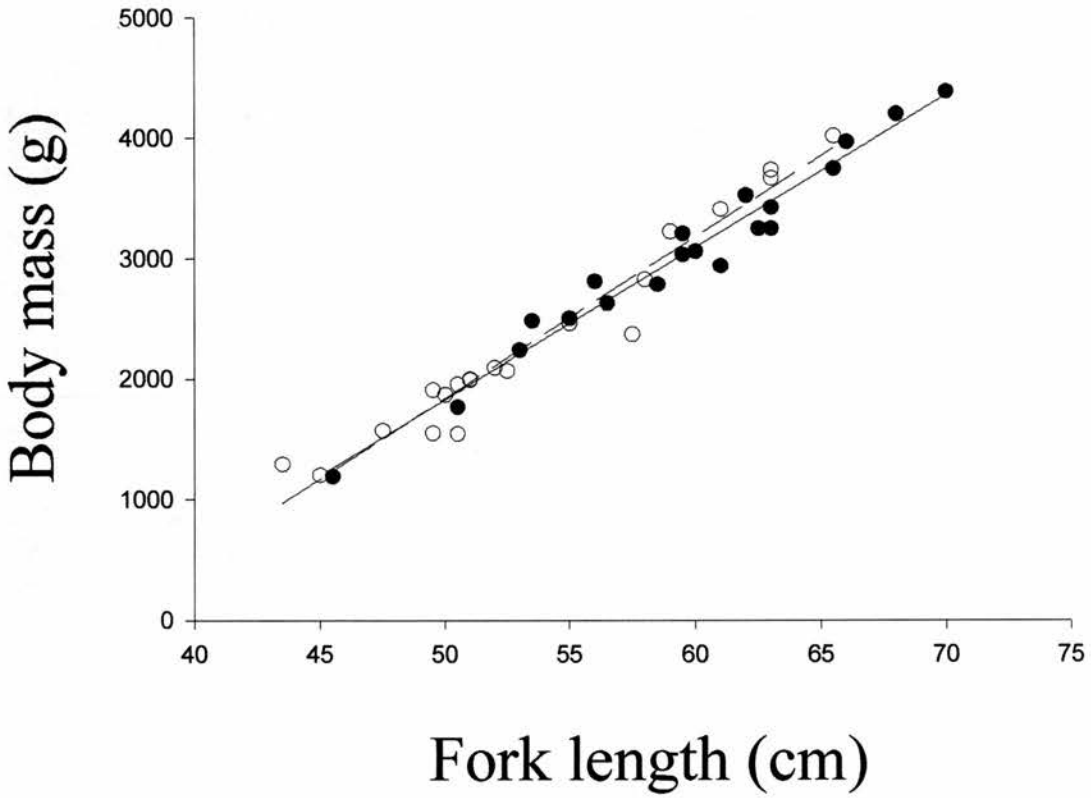


Fig. 5.2 The relationship between fork length and body mass in male and female Atlantic salmon from 1-5kg body weight. The lines were fitted by least-squares linear regression. Females (r^2 adj.=0.915) are represented by open circles while males (r^2 adj.= 0.951) are represented by filled circles.

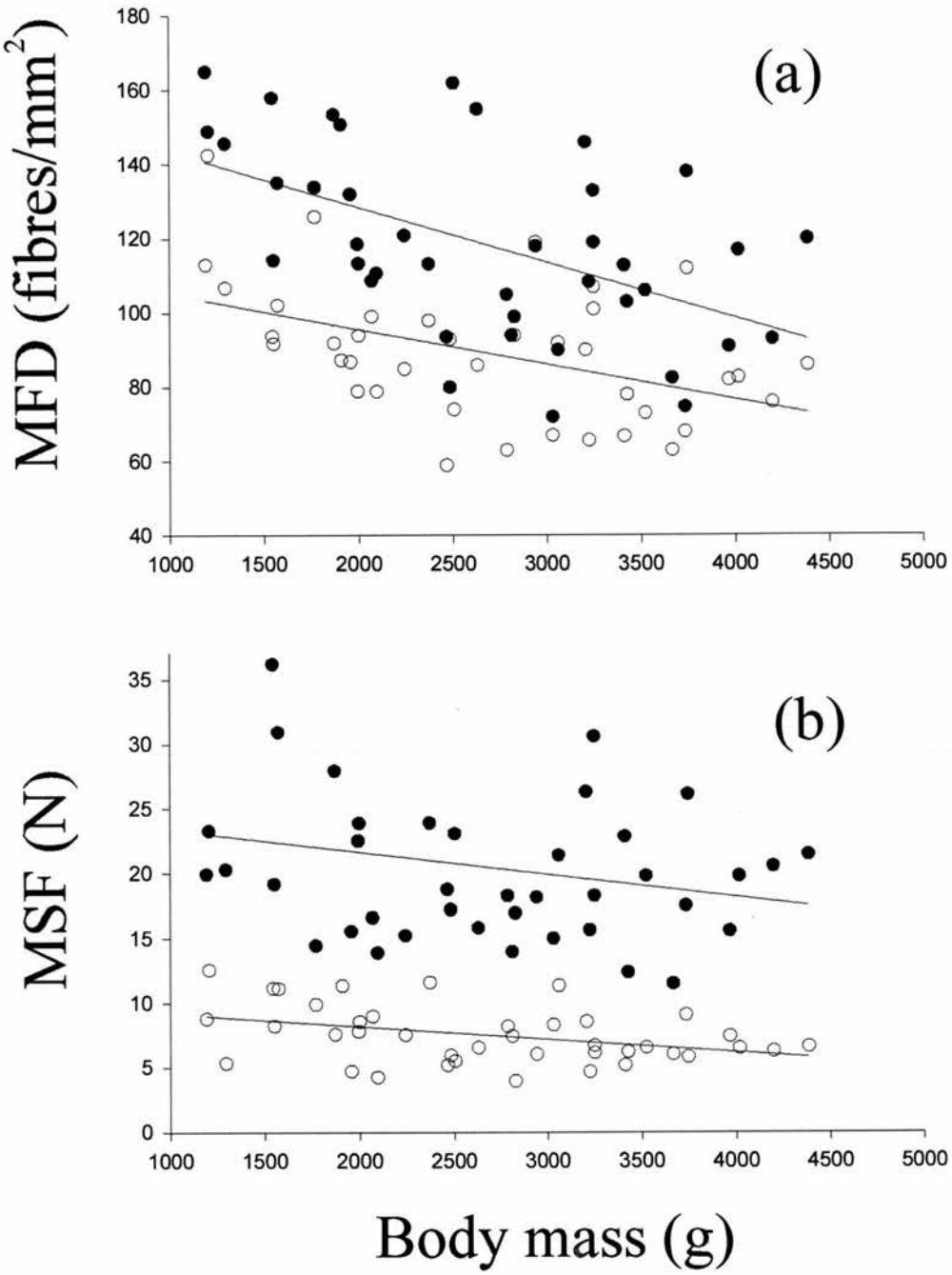


Fig. 5.3 The relationship between (a) body mass and muscle fibre density (MFD) and (b) body mass and maximum shear force (MSF) in the anterior (MOWI) and posterior (NQC) sections of Atlantic salmon from 1-5kg body weight. The lines were fitted by least-squares linear regression. The MOWI cut is represented by open circles while the NQC cut is represented by filled circles.

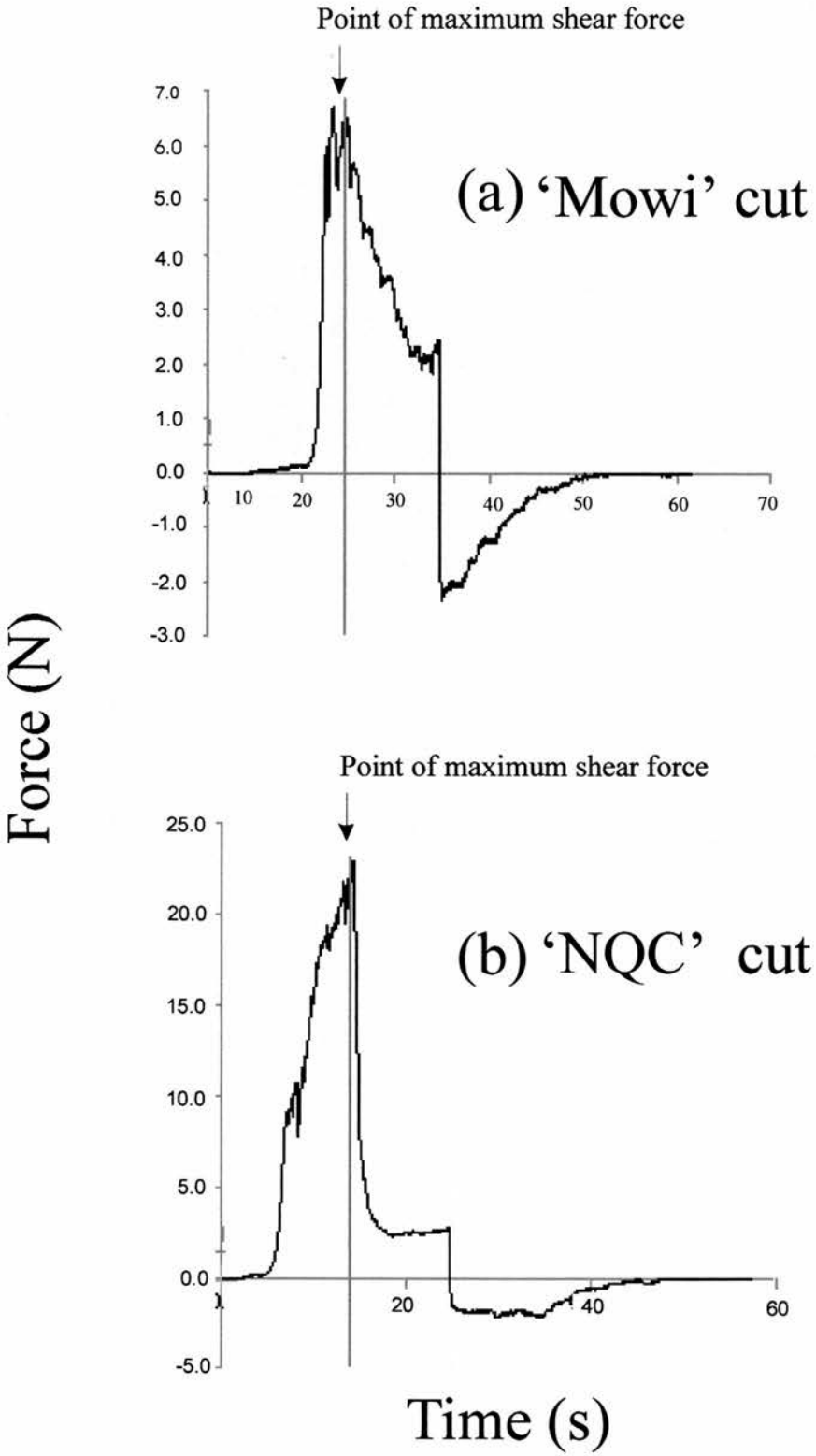


Fig. 5.4 Force-time graphs resulting from a Warner-Bratzler shear test on (a) anterior (Mowi) and (b) posterior (NQC) sections of an Atlantic salmon (fork length 59cm, body mass 3225g)

	Female	Male	ANOVA	
Parameter	Mean±SE(n=20)	Mean±SE(n=20)	$F_{(1,38)}$	P
Muscle fibre density (cells/mm^2)	87.56 ± 4.25	90.85 ± 3.91	0.32	0.573
Maximum shear force (N)	7.70 ± 0.62	7.30 ± 0.33	0.32	0.575
L*	50.85 ± 1.90	50.46 ± 0.44	0.11	0.738
a*	23.38 ± 0.58	24.40 ± 0.42	1.97	0.169
b*	22.71 ± 0.51	25.58 ± 0.39	0.699	0.410
% Lipid	13.60 ± 0.51	15.07 ± 0.58	3.58	0.006

Table 5.1 Results of Analysis of variance for flesh quality parameters measured anterior to the dorsal fin (MOWI) in immature female and male Atlantic salmon (significance level $P<0.05$).

	Female	Male	ANOVA	
Parameter	Mean±SE(n=20)	Mean±SE(n=20)	$F_{(1,78)}$	P
Muscle fibre density (cells/mm^2)	119.57 ± 5.26	117.25 ± 6.06	0.08	0.773
Maximum shear force (N)	21.74 ± 1.56	19.18 ± 1.04	1.85	0.180
L*	48.26 ± 0.51	47.89 ± 0.49	0.265	0.610
a*	25.47 ± 0.50	23.24 ± 0.39	0.03	0.855
b*	24.50 ± 0.38	24.20 ± 0.36	0.315	0.315
% Lipid	9.99 ± 0.37	11.37 ± 0.34	7.36	<0.001

Table 5.2 Results of Analysis of variance for flesh quality parameters measured in the tail region (NQC) in immature female and male Atlantic salmon (significance level $P<0.05$).

	MOWI	NQC	Paired t-test	
Parameter	Mean±SE(n=40)	Mean±SE(n=40)	$T_{(df\ 39)}$	P
Muscle fibre density (cells/mm^2)	89.21 ± 4.26	118.41 ± 5.01	8.22	<0.000
Maximum shear force (N)	7.50 ± 1.49	20.74 ± 2.45	15.39	<0.000
L*	50.66 ± 1.90	48.08 ± 1.50	5.04	<0.000
a*	24.06 ± 1.41	25.53 ± 1.42	7.00	<0.000
b*	22.97 ± 1.43	24.36 ± 1.28	5.77	<0.000
% Lipid	14.34 ± 1.59	10.85 ± 1.44	12.05	<0.000

Table 5.3 Results of paired t-test for flesh quality parameters (male and female grouped) measured anterior to the dorsal fin (MOWI) and in the tail region (NQC) (significance level $P<0.05$).

5.3.3 *Anterior -Posterior Muscle Cellularity and flesh quality*

Muscle fibre density (muscle fibres/mm^2) was significantly higher in the NQC region (118.41 ± 5.01) than the Mowi region (89.21 ± 4.26) (T_{39}=8.22 P<0.000). The NQC region was also significantly firmer than the Mowi region, with mean maximum shear force readings of 20.74N ± 2.45 and 7.50N ± 1.49 respectively (mean ± se, *n*=80) (Table 5.3). The NQC region scored higher than the Mowi region for 'redness' and 'yellowness' with a significantly higher a* value of 25.53 ± 1.42 compared to 24.06 ± 1.41 and b* values of 24.36 ± 1.28 compared to 22.97 ± 1.43. The Mowi region was significantly lighter with an L* value of 50.66 ± 1.90 compared to 48.08 ± 1.50 in the NQC cut The NQC region was also significantly leaner than the Mowi region with a % lipid of 10.85 ± 1.44., compared to 14.34 ± 1.59.

5.3.4 *Relationship between Muscle fibre density, % lipid and flesh quality parameters*

Maximum shear force was significantly correlated with muscle fibre density with higher fibre density giving a firmer texture, explaining almost 50% of the variation in firmness. (Fig.5.6). (r^2 adj. = 0.481, $F_{2,77}$= 37.54, P<0.0001,). Percentage lipid was not significantly related to maximum shear force in the Mowi (r^2adj. = 0.004, $F_{1,39}$ =0.846 P>0.05) or NQC section (r^2adj. =0.02, F1,39=0.436 P>0.05). Figure 5.7 shows the relationship between muscle fibre density and colour reading L* a* and b* taken from the Mowi and NQC sample sites. Lightness (L* value) and yellowness (b* value) showed no relationship with muscle fibre density. Redness (a* value) however, showed a significant negative relationship with muscle fibre density in the Mowi (r^2 adj. = 0.236, $F_{1,38}$ = 13.06, P<0.001,) and NQC (r^2 adj. = 0.237, $F_{1,38}$ = 13.16 P<0.001) regions indicating that within

the sample areas, lower cell density gives a 'redder' instrumental colour score. Percentage lipid was weakly correlated with body mass in the Mowi (r^2 adj.= 0.107, $F_{1,38}$ = 5.68 P<0.05) and NQC (r^2 adj. = 0.329, $F_{1,38}$= 20.19, P<0.0001) regions (Fig.5.8aa) Redness (a* value) increased significantly with body mass in both the Mowi (r^2adj. = 0.329, $F_{1,38}$ = 20.20, P<0.0001) and NQC regions (r^2adj.= 0.251, $F_{1,38}$ = 14.13, P<0.0001) (Fig.5.8bb).

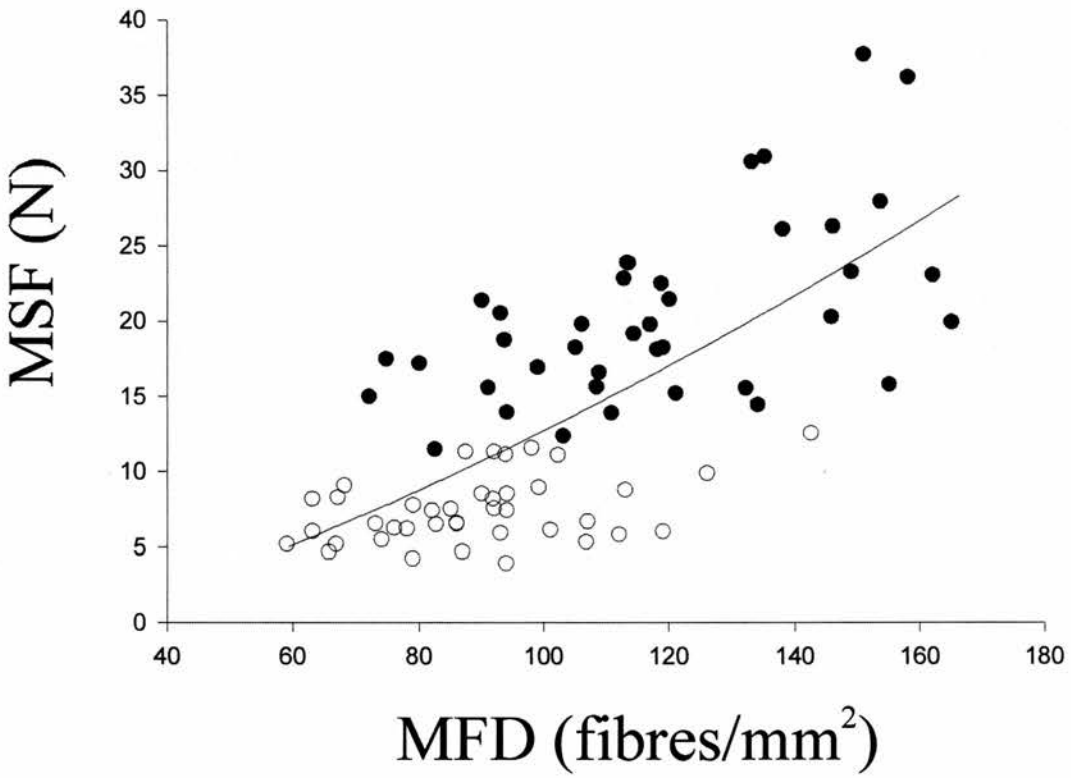


Fig. 5.6 The relationship between muscle fibre density (MFD) and maximum shear force (MSF) in sections of Atlantic salmon from 1-5kg body weight (r^2 adj.=0.481, P<0.0001). The line was fitted by exponential (Stirling model) regression. Data from the anterior (MOWI) cut is represented by open circles while the (NQC) cut is represented by filled circles.

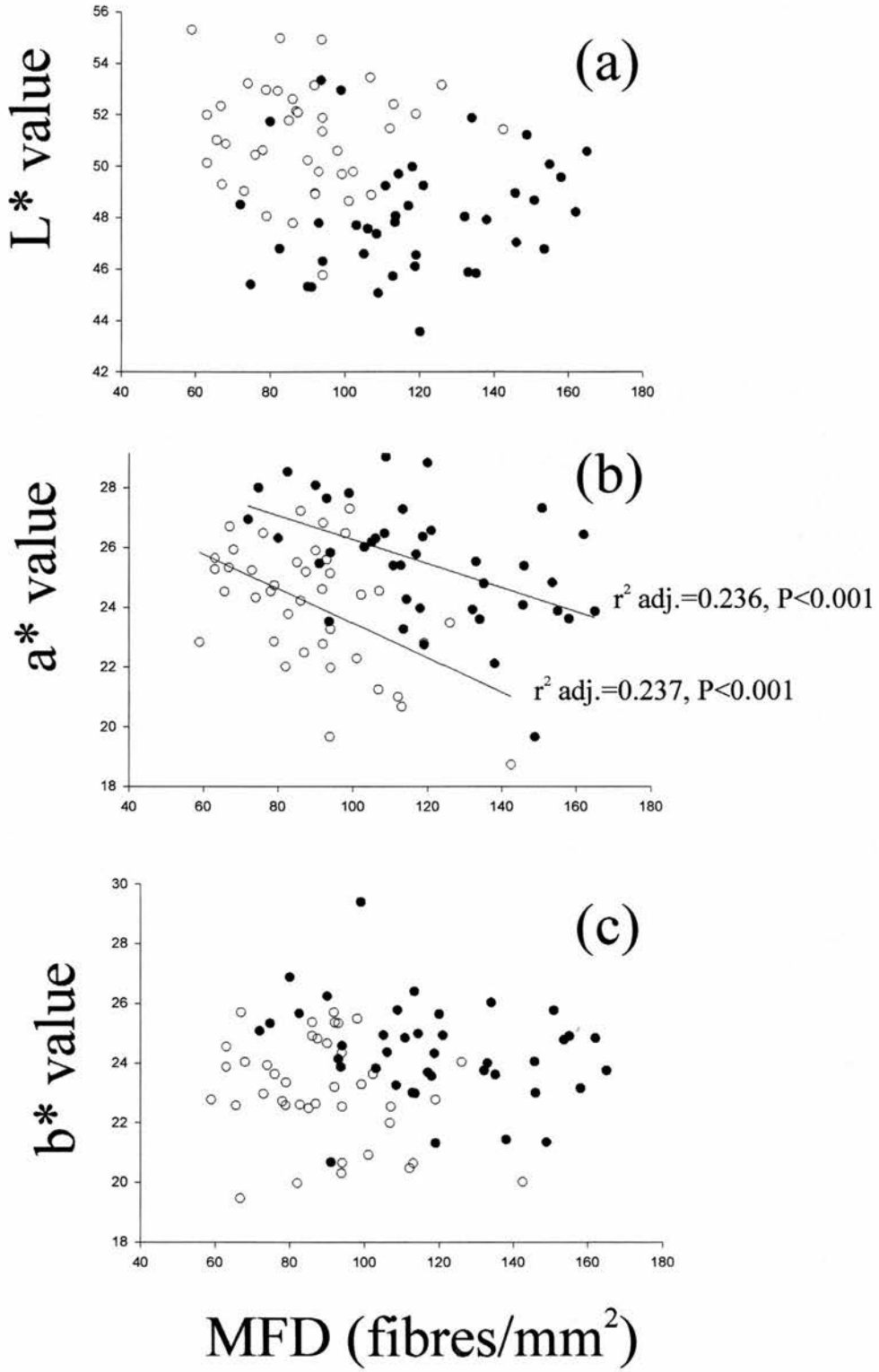


Fig.5.7 The relationship between muscle fibre density (cells/mm^2, MCD) and colour readings L*, a* and b* in the anterior (MOWI) and posterior (NQC) sections of Atlantic salmon from 1-5kg body weight. The lines were fitted by least-squares regression; data from the anterior (MOWI) cut are represented by open circles while data from the posterior (NQC) cut are represented by filled circles.

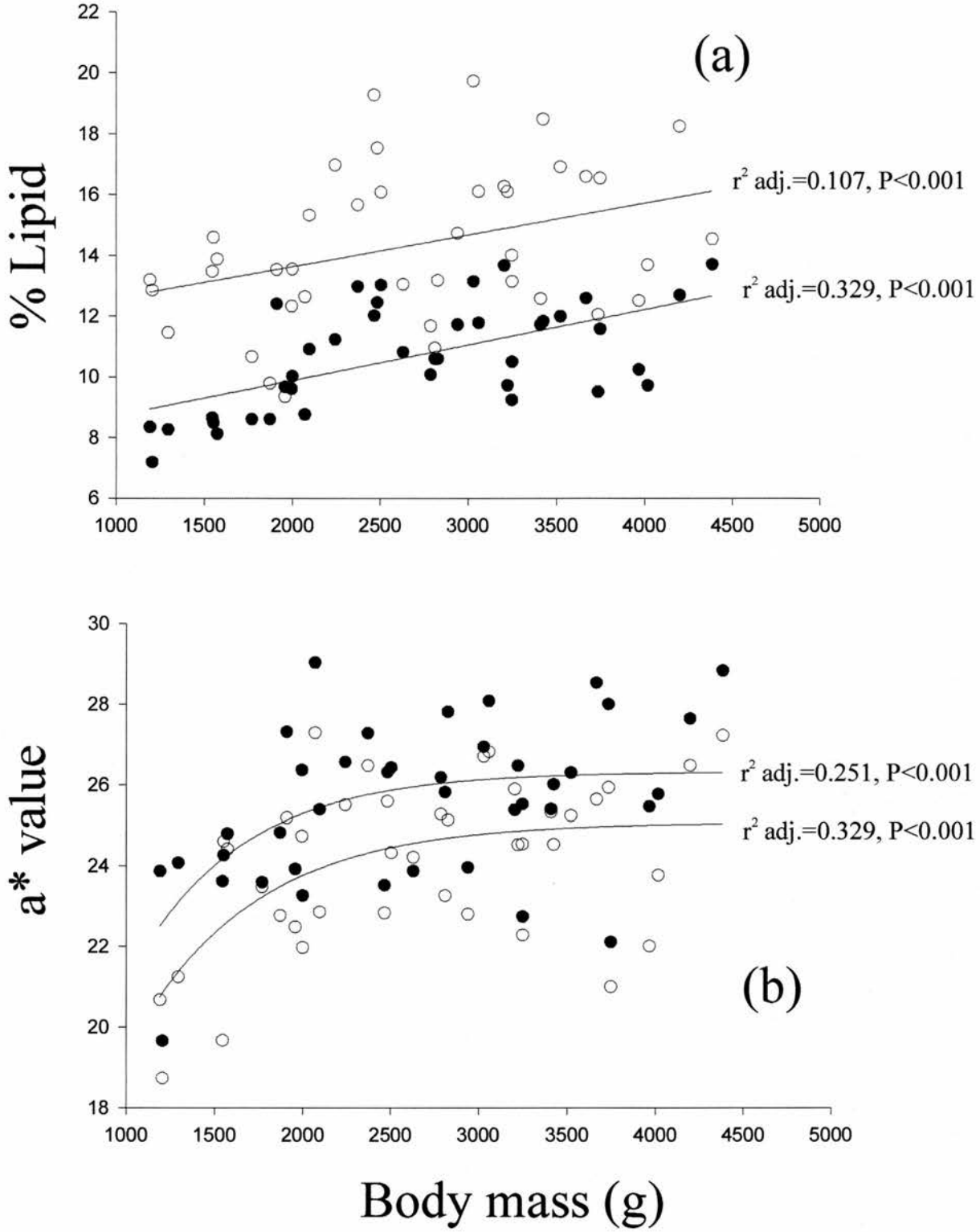


Fig. 5.8 The relationship between (a) body mass and % lipid and (b) body mass and colour reading a* (redness) in the anterior (MOWI) and posterior (NQC) sections of Atlantic salmon from 1-5kg body weight. Lines were fitted by (a) least-squares and (b) exponential regression. Data from the anterior (MOWI) cut are represented by open circles while data from the posterior (NQC) cut are represented by filled circles.

5.4 Discussion

Flesh quality is usually defined in terms of appearance, taste, smell, firmness juciness and process characteristics. Desired flesh characteristics vary widely between markets and may differ significantly for raw and processed products. The current experiment analysed the influence of muscle cell density on instrumental texture, flesh colour and lipid content in Atlantic salmon fillets. Sensoric analysis is open to subjective interpretation and instrumental test methods can deal with only single parameters. The use of instrumental methods, such as the Warner-Bratzler shear test, can explain or describe most of the sensory textural attributes of meat (Beilken *et al.,* 1991, Andersen 1995).

Recent work on flesh quality in fish has related muscle cellularity to different flesh quality traits. Johnston *et al.* (2000b) found that muscle fibre density explained 40% of the variation of 'firmness' in smoked Atlantic salmon. Hurling *et al.*, (1996) showed that muscle fibre cross sectional area was related to sensory firmness in a number of different species, fish with smaller fibres having a greater sensory firmness. Firmer fillet texture has also been shown to give less weight loss during smoking (Røra *et al.*, 1999).

The textural properties of fish have been related to the basic structural properties of muscle by Hatae (1986) and Johnston *et al.* (2000a). Hatae, (1986) proposed a 'lattice' theory of sensory firmness where smaller, more closely packed muscle fibres provide a denser, firmer structure and higher intrinsic strength than larger, more loosely packed

fibres, which give a softer texture. In the current experiment, muscle fibre density correlated with texture, and explained almost 50% of the variation in texture in the 40 fish sampled. This confirms the hypothesis of Hatae *et al.,* 1990 and the findings of Johnston *et al.* (2000b) that high fibre density gives a 'firmer texture'. The significant negative relationship between muscle fibre density and Hunterlab a* value found in the current experiment is in contrast to the findings of Johnston *et al.* (2000b) who discovered a positive relationship between muscle fibre density and fillet redness. These findings are difficult to explain since salmon fillets are generally 'redder' in the tail section where muscle fibre density is highest. The negative relationship found here may be due to redness being 'masked' by connective tissue overlying the high density muscle areas.

In salmon farming, mixed sex (male and female) groups are commonly reared to harvest size. Dunajski (1979) suggested that sex could affect the quality of fish meat although no differences in muscle fibre density, texture or colour could be found in the current experiment. This is in agreement with Choubert *et al.* (1997) who found that sex had no significant impact on fillet colour in the rainbow trout (*Oncorhyncus mykiss*). Immature male fish did, however have higher lipid levels than females but this could be explained by a higher body mass in male fish (Shearer, 1994).

Dunajski (1979) also suggested that variations in the quality of the fillet along the length of the body are largely due to variations in the size distributions of muscle fibres and are correlated with the amount of collagen present (Bordeiras and Montero, 1990).

Several authors have investigated the 'gradient' of flesh quality along the length of the fillet. Nickell and Bromage (1998) and Choubert *et al,* (1997) found that a* colour values ('redness') and astaxanthin concentration were higher in the tail section of rainbow trout compared to the anterior section. Bell *et al.* (1998) found that lipid content analysis of different body regions of farmed Scottish Atlantic salmon showed that the highest levels were found immediately in front of the dorsal fin and that the lowest lipid levels were in the tail region. Analysis of muscle from the NQC section revealed significantly lower lipid levels compared to the anterior 'Mowi' section. Sensory attributes of fish flesh including firmness, flavour and intensity can be affected by dietary fat level (Waagbø *et al.*, 1993; Nortvedt and Tuene, 1998). Accumulation of fat has either positive or negative consequences for sensory evaluation depending on the source and composition of fat (Fauconneau *et al.,* 1995). Andersen *et al.* (1997) found a reduction in resistance to compression in fish fed on a high fat diet. In contrast, Sheehan *et al.* (1996) found that high fat fish were more susceptible to gaping, but had a firmer texture than fish fed a low fat diet. In the current study, the NQC cut was significantly leaner, had a higher muscle fibre density and was firmer than the 'Mowi' cut. However, no direct relationship between % lipid and maximum shear force was found in the current study.

In addition to muscle fibre density and lipid level, it is also important to consider the contribution of connective tissue towards textural properties. This was not assessed in the current study, although the effects of collagen on flesh quality are somewhat controversial. (Dunajski 1979). Sato *et al.* (1986) suggested that the point of main flexion on the body of fish comprise a high proportion of collagen. His results also indicate that

muscle collagen contributes to the toughness of sliced meat. The tail region of the Atlantic salmon is subjected to the greatest flexure and it is therefore not unreasonable to surmise that this has a positive effect on firmness in this region of the fillet. Collagen may, therefore, play a part in the texture of raw and smoked salmon but the interpenetrating collagenous fibres and sheets are converted to gelatinous solutions at comparatively low temperatures and therefore play no measurable part in influencing the texture of cooked fish (Love 1983). The heat-coagulating material does however seem to obstruct the displacement of muscle fibres during compression tests, contributing towards increased sensory firmness. The density of muscle tissue will influence the surrounding collagenous tissue and will therefore have a bearing on the firmness of fish muscle tissue following cooking (Hatae *et al.*, 1990).

White Muscle accounts for approximately 65% of the total body mass of an Atlantic salmon and over 90% of the final product. A greater understanding of the structure of this tissue and the effect it has on product quality is therefore, of great importance to salmon farming and the wider aquaculture industry. Several studies have shown that environmental factors including temperature (Beattie *et al.,* 2000a), exercise (Totland *et al.*, 1987), light (Beattie *et al.*, 2000b) and diet (Kiessling *et al.*, 1991 influence muscle growth patterns. Johnston *et al.* (2000a) found that genetic strain can influence muscle fibre cellularity and quality of the smoked product. Additionally, significant seasonal changes in flesh quality such as the marked reduction in pigmentation in some salmon stocks during Spring, may be related to changes in muscle cellularity. The manipulation of these variables by the fish farmer, coupled with new criteria for selective breeding, should therefore facilitate improvement of the final

product. The results of the present study suggest that these changes will have significant impacts on flesh quality and provides an important foundation for future study.

Chapter 6

General Discussion

6.1 Summary of results and conclusions

In the wild, salmonid fish occupy uncertain environments (fresh water) for at least part of their lives, and flexibility is a prime characteristic of their life history patterns (Thorpe, 1989). The results of this thesis suggest that this flexibility extends to the growth of the white muscle at all stages of the cycle. The manipulation of various environmental factors to reduce time to hatching and promote growth and feeding is now widespread in salmon farming. The majority of studies on the effects of temperature on muscle development in salmonids have focused on the early life stages since they are relatively easy to rear, sample and analyse (Stickland *et al.*, 1988, Matschak *et al.*, 1995, Nathanailides *et al.*, 1996). The long-term effects of temperature have not, therefore, been investigated.

In Chapter two, it was found that fish reared at the temperature of normal ambient river water recruited significantly more white muscle fibres per myotome than fish reared at higher temperatures. Incubation temperature had significantly affected muscle cellularity up to 26 months following hatch. Percentile plots of fibre diameter also revealed a close relationship between water temperature and the balance of hypertrophy and hyperplasia, with hypertrophy being the preferred strategy at higher water temperatures (Chapter 2). In agreement with Kiessling *et al.* (1991) periods of increased muscle fibre hypertrophy were associated with periods of increased growth. To say that hypertrophy is linked with high growth rate is, however slightly misleading. Evidence of

distinct periods of fibre recruitment were seen in both ambient and heated temperature groups prior to smoltification (Chapter 2) and a subsequent increase in body mass. An increase in fibre recruitment was also seen in fish given a simulated short winter (Chapter 3), prior to a significant increase in body mass, suggesting that hyperplasia lays the foundation for a fast increase in body mass by hypertrophy. A distinct lengthening of the skeleton was seen in both Chapter 2 and Chapter 3 prior to smoltification and was associated with fibre recruitment. This suggests a well timed co-ordination of somatic growth where the skeletal system is extended first to support an increase in body mass resulting from rapid hypertrophy of newly formed fibres. Fauconneau *et al.* (1997) concluded that the lack of distinct separation between new and old muscle fibres in the frequency distribution of rainbow trout (*Oncorhyncus mykiss*) suggests an almost continuous recruitment process. There was evidence in Chapters 2 and 3 for constant recruitment of muscle fibres throughout the year although probability density plots suggested increased recruitment prior to smoltification and following seawater transfer. In larger fish, the separation between large and small fibres is greater, producing distinctly bimodal distributions (Johnston *et al.*, 2000a). However in smaller fish, the lack of clearly bimodal distributions in the frequencies of muscle analysed in Chapters 2 and 3 suggested that once recruited, the smaller fibres were quickly absorbed into the bulk of the frequency distribution. The timing of samples is therefore crucial if distinct phases of muscle fibre recruitment are to be identified.

As a migratory species, the biology of the Atlantic salmon is governed by the appearance and disappearance of seasonal cues co-ordinating movements in and out of

the natal stream (Mills, 1992; Huntingford and Thorpe, 1992). Each process is circannual, endogenously rhythmic but synchronised by photoperiod and involves a choice of developmental route (Thorpe, 1989). Evidence of increased fibre recruitment was seen in both ambient and heated temperature groups prior to smoltification and a subsequent increase in body mass in agreement with Higgins and Thorpe (1990). Similar patterns of muscle frequency distribution were seen in February 1998 in the heated group and May 1998 in the ambient group. Hoar (1988) concluded that, in general, temperature controls the rate of the physiological response to photoperiod but smoltification occurs sooner at higher temperatures. The exact mechanism for this advancement by temperature alone is unclear but may be linked to an increased feeding opportunity and, therefore, higher total feed intake from hatch in the heated group. Kiessling *et al.* (1991) found that by reducing or increasing feed rations, the muscle growth program of rainbow trout could be respectively delayed or advanced. Growth patterns found earlier in fish fed 100% ration were seen months later in fish fed 50% ration. This suggests that high temperature incubation advanced the normal muscle growth program through better yolk-sac conversion and increased feeding opportunity, leading to earlier recruitment of muscle fibres prior to smoltification.

Evidence for photoperiod influencing developmental timing was also seen in Chapter 4, where a number of light-manipulated fish showed increased variability in their fibre distributions concomitant with a seasonal recruitment of new fibres. Johnston *et al.* (1999b) found distinct bimodal frequency distributions in seawater stage diploid and triploid Atlantic salmon. In Chapter 4 however, the apparent recruitment of new fibres

was not linked to a significant increase in body mass in the closed pen group, which experienced lower water temperatures than the open cage group. This probably reflects the influence of photoperiod as the main cue determining developmental change in the salmon, but also the restrictive effect of low temperatures and low feed intake on fibre hypertrophy. The recruitment of muscle in salmon may change as part of a endogenous cycle entrained by photoperiod acting as a seasonal cue. The rate of that response is, however, governed by temperature and food availability.

The current study suggests the following model of muscle growth in salmon. Two mechanisms are, therefore, at work in the growth and development of muscle in the Atlantic salmon. Firstly short term changes linked to increased food intake and somatic growth such as those seen in the constant light group (Chapter 3), may lead to increased hypertrophic growth which then stimulates hyperplasia as the critical myonuclear to cytoplasm ratio is exceeded. Secondly, the endogenous muscle growth program linked to the life cycle of the fish (Chapter 3,4,5), which is controlled by seasonal cues including temperature and light. Muscle cellularity can be altered by compressing or advancing the natural seasonal growth cycles of the fish (Chapter 2, Chapter 3, Chapter 4). The most significant finding of the current study is that high temperature incubation alters the muscle characteristics of the fish even after seawater transfer. Weatherley (1990) concluded that a high degree of growth control exists in fishes, which enables them to conserve the size of organs and tissues relative to the whole body size. The direct stimulation of muscle growth by short-term changes in environmental variables such as temperature would result in uncontrolled growth and would therefore be selected against

in an evolutionary sense. It is possible however, that salmon have specific 'ontogenic windows' where changes in incubation temperature can alter muscle related characteristics such as the number of muscle stem cells. Additionally, the post-hatch development stages of salmon (smoltification and maturation) may provide another 'window' where muscle development can be influenced.

In Chapter 5, a direct link between muscle fibre density and the firmness of the raw muscle was found. The evidence of changes in the cellularity of muscle induced by environmental factors in Chapters 3, 4 and 5 may therefore impact on final product quality. Since muscle is the fundamental component of the salmon product, its structure not only affects firmness but also gaping (Lavéty *et al.,* 1988) and colour (Johnston *et al.* 2000b). Flesh quality studies often sample from different areas of the fillet. In Chapter 4 and Chapter 5, significant vertical and horizontal differences were found in muscle fibre diameters producing muscle fibre densities, which generally decreased dorso-ventrally and increased rostro-caudally. Within fillet differences in flesh quality including lipid and colour have been well documented (Aursand *et al.,* 1994; Choubert et al 1997, Nickell and Bromage, 1998). Due to this variability, flesh quality studies can produce highly variable and incomparable results. Future studies should take into account the gradation in fibre size found in Chapters 4 and 5 when planning experiments. Few studies have addressed the influence of sex on the flesh quality of salmon which is surprising since Scottish farmed stocks are usually 50:50 male:female. In chapter five, no differences were found in flesh quality parameters between sexes although significant within fillet differences were found in both male and female fish.

The results of this study indicate that muscle growth and cellularity in Atlantic salmon respond to environmental influences and the demands of somatic growth rate but that the nature of the response is somewhat plastic. Significant links between muscle fibre cellularity and flesh quality parameters make this plasticity commercially important. The environmental manipulation of these fish should take into account the final influence on flesh quality.

6.2 Future studies

The recent characterisation of several transcriptional regulators in the myogenic pathway has led to an understanding of the molecular mechanisms, which are responsible for the establishment of muscle specific gene expression (Molkentin and Olson, 1996). These muscle transcriptional factors belong to a family of basic helix-loop-helix (bHLH) DNA binding proteins, and include myogenin, MyoD, myf-5 and MRF4 (for review see Buckingham, 1994; for fish see Rescan *et al.*, 1995). A better understanding of the molecular mechanisms behind muscle growth will allow future studies to define more closely, the relationship between the influence of temperature, light, exercise and diet on the plasticity and growth of fish muscle. One future avenue of research may be to analyse the dietary requirements of hypertrophy and hyperplasia in fish, based on the seasonal shift in muscle growth mechanisms revealed in Chapter 2. Hyperplasia has a higher energy requirement than hypertrophy (Cheek and Hill, 1970) and it may therefore be possible to manipulate diet to maximise a specific muscle growth process.

The intensive farming of fish is still in its infancy compared to the well established breeding lines and techniques of terrestrial farming (Gjedrem, 1997). Great potential lies in the application of muscle research to future breeding programs, which may select for specific muscle growth and harvest quality traits. The development of molecular markers for the satellite cell population (c-met) (Cornelison and Wold, 1997) and for differentiating satellite cells (mnf-β) (Yang *et. al.*, 1997), together with the development of molecular markers for muscle regulatory factors will allow the investigation of the earlier stages of myogenesis to be studied. It may also shed some light on the hypothesis that a high density of satellite stem cells is a good predictor of future fibre recruitment. A study of the effect of genetic and environmental factors on the number and behaviour of myosatellite cells and their relationship to adult somatic growth rate will also help to improve breeding programs which seek to maximise production. The use of molecular markers and biopsy techniques will allow non-destructive longitudinal studies which will give a better understanding of the mechanisms of muscle growth in fish and its response to changing environmental conditions.

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Matre – '*Somewhere Under the Rainbow*'