MUSCLE GROWTH AND FLESH QUALITY OF FARMED ATLANTIC HALIBUT (HIPPOGLOSSUS HIPPOGLOSSUS) IN RELATION TO SEASON OF HARVEST

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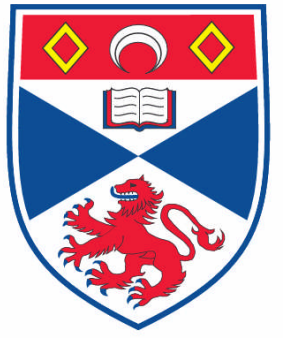
A Thesis Submitted for the Degree of PhD
at the
University of St. Andrews



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St Andrews, July 2008

This thesis is dedicated to my family. I could not have managed without their loving support.

Thank you so much!

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Publications

Publiched papers:

Hagen, Ø., Solberg, C. and Johnston, I. A. (2006). Sexual dimorphism of fast muscle fibre recruitment in farmed Atlantic halibut (*Hippoglossus hippoglossus* L.). *Aquaculture* **261**: 122-1229.

Hagen, Ø., Solberg, C., Sirnes, E. and Johnston, I. A. (2007)**.** Biochemical and structural factors contributing to seasonal variation in the texture of farmed Atlantic halibut (*Hippoglossus hippoglossus* L.) flesh. *J. Agric. Food Chem*. **55**: 5803-5808.

Hagen, Ø., Vieira, V. L. A., Solberg, S. and Johnston, I. A. (2008). Myotube production in fast myotomal muscle is switched-off at shorter body lengths in male than female Atlantic halibut (*Hippoglossus hippoglossus* L) resulting in a lower final fibre number. *J. Fish Biol.* **73**: 139-152.

Hagen, Ø., Solberg, S. and Johnston, I. A. (2008). Activity of aspargate (cathepsin D), cysteine proteases (cathepsin B, B + L, H) and matrix metallopeptidase (collagenase) and their influence on the water holding capacity of muscle in commercially farmed Atlantic halibut (*Hippoglossus hippoglossus* L.). *J. Agric. Food Chem.* Doi: 10.1021/jf801215b.

Hagen, Ø., Fernandes, J. M. O., Solberg, C. and Johnston, I. A. (2008). Muscle expression of growth-related genes during fasting and refeeding in juvenile Atlantic halibut, *Hippoglossus hippoglossus* L. *Comp. Biochem. Physiol* (B). In press.

Other publications during the PhD student period:

Fernandes, J. M. O., Mommens, M., **Hagen, Ø**., Babiak, I. and Solberg, C. (2008). Selection of suitable reference genes for real-time PCR studies of Atlantic halibut development. *Comp. Biochem. Physiol*. **150**(B): 23-32.

Declarations

I, Ørjan Hagen, hereby certify that this thesis, which is approximately 65000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

I was admitted as a part time research student in October, 2003 and as a candidate for the degree of PhD in October, 2004 the higher study for which this is a record was carried out in the University of St Andrews between 2004 and 2008.

Date …… Signature of candidate ……

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of PhD at the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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List of abbreviations

µg	Micrograms
µl	Microlitres
µm	Micrometres
µM	Micromolar
µmol	Micromol
18S rRNA	18s Ribosomal RNA
Actb	β-Actin
a-i	alkaline insoluble
AMC	7-Amino-4-methylcoumarin
a-s	alkaline soluble
ATP	Adenosine triphoshpate
bp	base pairs
BSA	Bovine Serum Albumin
BCA	Bicinchoninic acid
cDNA	complementary Deoxyribonucleic Acid
C-factor	Condition Factor
cm	centimetre
C_T	Cycle Threshold
ctsb	Cathepsin B transcript
Ctsb	Cathepsin B protein
ctsd	Cathepsin D transcript
Ctsd	Cathepsin D protein
d	days
DNA	Deoxyribonucleic Acid
DHA	Docosahexaenoic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreiterol
EDTA	Ethylenediaminetetraacetic acid
Eef2	Eukaryotic translation elongation factor 2
ECM	Extra Cellular Matrix
e.g.	exempli gratia (for example)
EPA	Eicosapentaenic acid
EST	Expressed Sequence Tags
Fau	40S Ribosomal protein S30
FA	cumulative cross-sectional area
FD	Fibre density
Fig	Figure
FMOC	Fluorenylmethoxycarbonyl
FN	Fibre Number
FN_{max}	Fibre Number maximum
g	gram
gDNA	genomic Deoxyribonucleic Acid
GAM	General Additive Model
GH	Growth Hormone
GLM	General Linear Model
GSI	Gonad Somatic Index
h	hour
HPLC	High Performance Liquid Chromatography

HYP	Hydroxyproline
IGF-I	Insulin-like Growth Factor-I
IGF-IRa	Insulin-like Growth Factor-I receptor a
IGF-IRb	Insulin-like Growth Factor-I receptor b
IGF-II	Insulin-like Growth Factor-II
Int. std	Internal standard
kg	kilogram
l	litre
L_F	Fork length
LS	Standard Length
m	metre
mATPase	myosin Adenosine triphoshpatease
mm	millimetre
M	Molar
Mb	Body mass
mg	milligrams
MGF	Menchano Growth Factor
mJ	milliJoule
ml	millilitre
MLR	Multiple linear regression
mM	millimolar
MMPs	Materix Metalloproteinases
mRNA	messenger Ribonucleic Acid
MRF	Myogenic Regulatory Factors
mTOR	mammalian Target Of Rapamycin
Myf	Myogenic factor
MyoD	Myogenic determination factor
nmol	nanomol
Pax	Paired box
PBS	Phosphate Buffer Saline
PC	Principal component
PCR	Polymerase Chain Reaction
PCr	Creatin phosphate
pdfs	probability density function
pf	post fertilisation
PIT-tags	Passive Integrated Transponder-tags
pmol	picomol
PLS	Partial Least Squares
PYD	Hydroxylysyl pyridinoline
qPCR	quantitative Polymerase Chain Reaction
s	second
S-58	Antibody for slow muscle myosin
SDA	Specific Dynamic Action
SDHase	succinic dehydrogenase
Shh	Sonic hedgehog
TCA	Total Cross-section Area
TFC	cumulative number of fibres
T-SR junction	T system-sarcoplasmatic reticulum
Tubb2	β2-Tubulin
WHC	Water Holding Capacity

Thesis abstract

In the present study, muscle growth and flesh quality have been investigated from both commercially farmed Atlantic halibut (*Hippoglossus hippoglossus*) (Aga marine AS, Norway) and halibut obtained from small-scale trials at Mørkvedbukta Research Station (Bodø University College, Norway).

Morphometric techniques have been utilized to investigate fast muscle growth in halibut ranging from *circa* 2 g to 100 kg, and it was established that fast muscle fibre recruitment ceases when the fish attain approximately 81 and 177 cm, in the case of males and females, respectively. Different muscle fibre types were distinguished using histochemical (myosin ATPase and succinic dehydrogenase) and immunohistochemical (*S-58*, an antibody against slow muscle myosin) staining techniques. Females recruit twice as many fast muscle fibres compared to males, which allows them to reach a larger final size. Furthermore, the seasonal growth patterns during a one year production cycle in commercial farmed halibut revealed a winter depression in growth leading to loss of biomass, which was attributed to the maturation of males. Commercial farmed fish of equal size (~1.5 kg) showed sexual dimorphism of fast muscle fibre number, caused by a significantly higher rate of fast muscle fibre recruitment in females. During the winter season fast muscle fibres shrunk significantly, especially in male fish, as a consequence of loss of appetite, low water temperatures and sexual maturation. None of the female fish matured during the trial.

Flesh quality of halibut deteriorated during winter and spring, since it had a softer appearance and significantly lower myotomal protein content, particularly in males. Cathepsin activity was measured using spectroscopy and showed a strong negative correlation to protein content,

displaying a seasonal variation. The proteolytic depletion of fast muscle proteins affected the water holding capacity of the muscle (determined by centrifugation), which showed concomitant changes with the increase in cathepsin activity and drop in protein content.

Despite the soft appearance, the firmness (shear force) of the flesh increased during the winter. The hydroxylysyl pyridinoline cross-link content of the collagen matrix, determined by HPLC, showed a strong correlation to the fillet texture. The increased firmness during the winter, a period of little (female) or negative growth (males), was probably due to an increased cross-linking of the collagen compartment.

Partial sequences of *IGF-I* and *IGF-II* were cloned from fast muscle of Atlantic halibut, and their relative gene expression levels were determined along with those of *cathepsin B*, *cathepsin D* and *IGF-IRa* in male halibut using qPCR during a fasting and refeeding trial. Transcript levels of *cathepsin B* and to some extent *cathepsin D* were significantly higher during fasting than refeeding, suggesting an increased enzyme production during periods of food deprivation. A temporary increase in *IGF-I* transcripts was observed after 7 days refeeding suggesting that this growth factor is involved in muscle growth control. Both *IGF-IRa* and *IGF-II* were down-regulated during refeeding.

Chapter 1

General introduction

1.1 Biology of the Atlantic halibut

Atlantic halibut (*Hippoglossus hippoglossus* L.) belongs to the subfamily Pleuronectinae within the family Pleuronectiformes, and is the largest of the right-eyed flounders (Fig. 1.1). The species are notorious for their large size, achieving a body mass of more than 300 kg and reaching an age of more than 50 years (Bromage et al., 2000). Atlantic halibut are distributed in parts of the Artic Ocean and in the northern part of the Atlantic Ocean. The western and eastern most extents of their range is New York and the Bay of Biscay respectively (Haug, 1990). Halibut is more abundant along the coast of Norway, Faroes, Iceland and southern part of Greenland (Haug, 1990), but the wild catches have declined in the last decade. Large adult fish lives in deep water from 300 – 2000 meters and are annual batch spawners (Haug, 1990). The spawning takes place between December and April at defined spawning grounds from 300 – 700 meters depth at temperatures of 5 – 7 ºC (Kjørsvik et al., 1987; Jakupsstovu and Haug, 1988). The eggs (~3.0-3.5 mm) are transparent and *post* fertilisation (pf) they are found floating at intermediate depth (~200 m) at temperatures of 4.5-7 ºC (Haug et al., 1984). At hatching (18 days pf at 5 ºC, Blaxter et al., 1983) the larvae are about 6-7 mm and poorly developed (e.g. large yolk sac, eyes and mouth are missing). The yolk-sac stage lasts for approximately 50 days (~300 degree days) when the larva has adapted for exogenous feed intake, having developed functional eyes, jaws, fins and intestines (Pittman et al., 1990). At the end of the larvae stage, before the fish becomes a juvenile, the larvae has to go through a comprehensive metamorphosis compared to that of other non-flatfish species. The metamorphosis is initiated around 70-80 days pf and involves several morphological changes such as right hand side eye migration, development of functional pelvic fins, pigmentation of the body and a dorsal-ventral flattening of the body (Sæle et al., 2004). During this period the pelagic larvae is transformed into a benthic juvenile.

Juveniles live in shallow costal waters at 20–60 metre depth, where they spend most of their time until maturation (4–6 years for males), in areas referred to as “nursing grounds” (Haug, 1990). Males and females matures at a size of approximately 55 cm (~1.7 kg) and 110-115 cm (~18 kg) (Jakupsstovu and Haug, 1988) respectively, but geographical differences in age at first maturity are known (Bowering, 1986; Haug and Tjemsland, 1986). The timing of maturation in halibut seams to be a function of growth rate rather than age and size (Roff 1982). In respect of growth males and females have a uniform growth until ~6 years of age when males starts to mature (Jakupsstovu and Haug, 1988). From this point females out-grow males which seldom achieve a body size of more than 50 kg (Jakupsstovu and Haug, 1988; Björnsson, 1994; Moen and Svensen, 1999).

Compared to other commercially fish species in the North Atlantic Ocean, halibut are considered to be an exclusive fish with a higher marked value. Most of the fish caught in Norway are exported to neighbour Scandinavian countries, Western Europe and the United States.

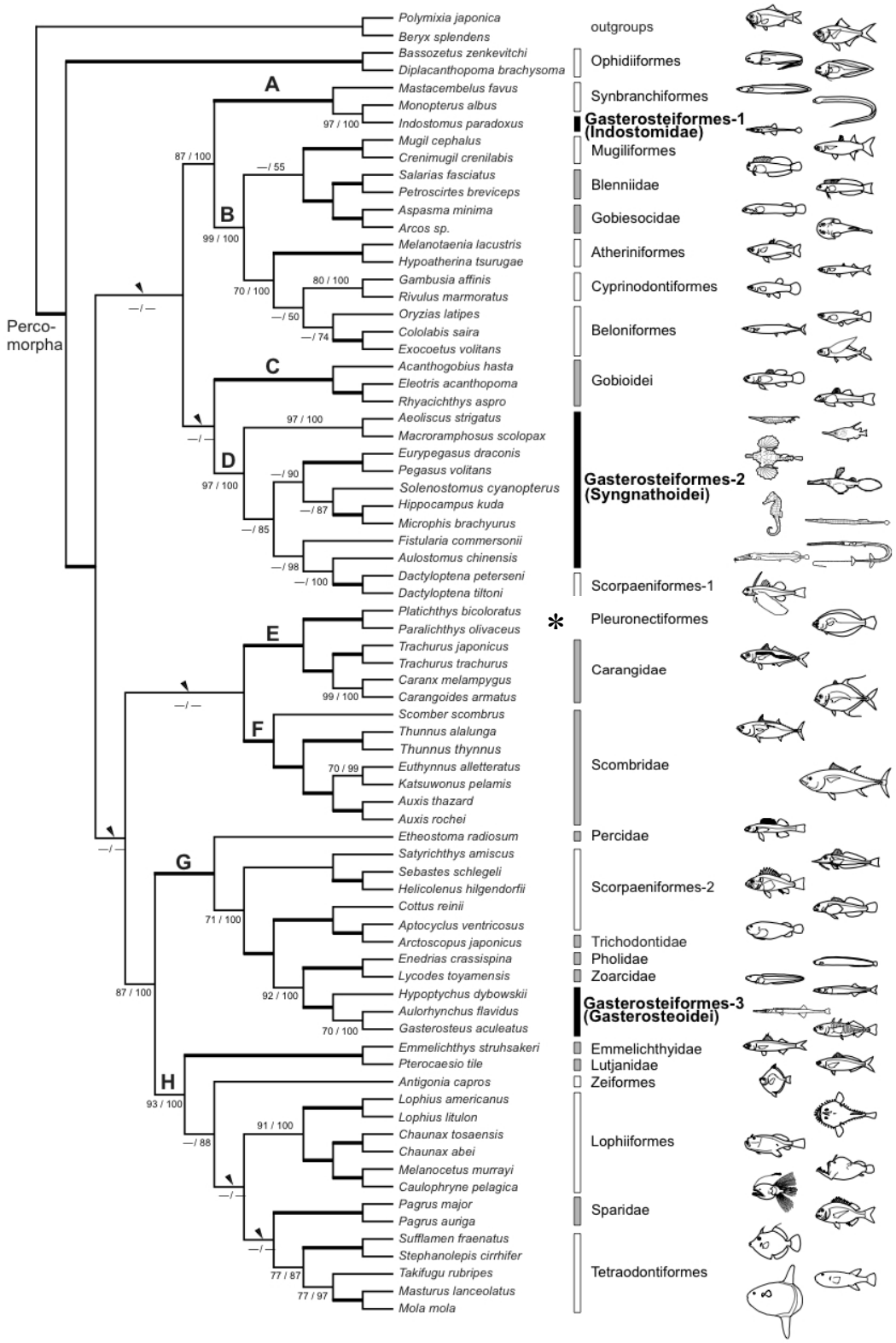

Figure 1.1. Maximum likehood tree. The Pleuronectiformes, the family of Atlantic halibut are indicated with a * (Kawahara et al., 2008).

1.2 Myotomal and connective tissue architecture

1.2.1 Myotomal architecture

The striated muscle of fish has two main purposes, namely a locamotory role and as energy storage during periods of food deprivation (Love, 1988). In most fish species the striated muscle is the largest organ in the body, often making ~60% of the total body mass (Sänger and Stroiber, 2001). The myotomal muscle has a unique structure, and when the skin is removed exposing the muscle, "W-shaped" white lines (myocommata) running across the muscle are apparent. The myocommata is located between the muscle segments called myotomes and separates them (Fig. 1.2 A). The myotomal muscle comprises of repeated myotomes, and the shape of the individual myotomes changes in an anterior-posterior direction as the body gets slimmer towards the tail region (Leeuwen, 1999). A cross-section of the trunk reveals a major horizontal myoseptum, which separates the myotomes into a dorsal and ventral compartment (both sides of the body axis). The lateral line is located in the superficial layer of the fish muscle running parallel with the body axis (horizontal myoseptum) and is an important sensory organ used to detect movements and vibrations in the surrounding water (Ghysen and Dambly-Chaudière, 2004) (dotted line Fig. 1.2 A). In addition to the shape of the myotomes, the width changes as well (Love, 1958). The total number of myomeres varies between species, but is usually fixed by the time of hatching. During a life cycle (e.g. Atlantic halibut) the fish increases several thousand fold in size. To be able to do so the width of the individual myomeres increases with age (Love, 1958). For example, the cod (*Gadus morhua*) trunk comprises of ~50 myotomes and in a posterior-anterior direction the width of the myotomes increases until the 12^{th} myotome after which it decreases towards the tail (Love, 1958). As a consequence the posterior part of the trunk has a

greater density of myomeres than the anterior part. Environmental fluctuations during embryogenesis such as a rise in temperature and salinity decreases and increases the total number of myomeres in herring at hatching respectively (Hempel and Blaxter, 1961). The origin of the individual myotome is determined at an early life stage when the segmental plate divides, forming the somites which are the precursors of the myotome (Mullins, 1999).

On the surface, the structure of the myotomes does not seem particularly complicated, but the deeper zones of the trunk reveal its complexity (Fig. 1.2 B). The individual muscle fibres varies from 2 – 17 mm in length (Leeuwen, 1999) and are arranged into bundles, representing the macro structures of the myotome, varying both in shape and size (illustrated in Fig. 1.2 C). Directly underneath the skin the muscle fibres run parallel with the body axis, whereas in the deeper layer the muscle fibres are arranged differently, forming angles up to 40º (Alexander, 1969; Leeuwen, 1999). The structural elements of the fish muscle fibres are numerous, and are divided into different groups based on their function such as contractile proteins (e.g. actin, myosin), regulatory proteins (e.g. tropomyosin, troponin) and cytoskeletal proteins (e.g. titin, myomesin) (Pearson and Young, 1989). I will therefore only focus on a few of the major constituents. The muscle fibres contain several hundred myofibrils that occupies between 75-95% of the fibre volume (Johnston, 1980) (Fig. 1.2 D). The myofibrils are made up of repeated filaments arranged in units called sarcomeres, which are divided into different zones (Z-band, I-band and the A-band) according to their characteristic banding pattern (Fig. 1.2 E). The I-band and A-band contains a variety of different proteins, but it is the thin actin and thick myosin filaments that gives them the characteristic appearance. Located in the sarcomere boundaries are the zigzag structures called Z-bands (also called Z-lines or Z-disk) which composes of many different proteins including, desmin, filamin, synemin and α-actinin (Greaser at al., 1981). α-actinin filaments are believed to be important for the connection of the actin filaments between adjacent sarcomeres (Goll et al., 1972; Suzuki et al., 1976). The

width of the Z-band is known to vary with different types of muscle tissue in both mammals (Eisenberg, 1983) and fish (Luther, 1991), with fast striated muscle having a smaller Z-band width compared to slow muscle. When comparing fish and mammals, fish have a very simple and narrow Z-band structure (~38 nm) compared to that of canine muscle (~152 nm), which comprises of several repeated structural units (Goldstein et al., 1979). The Z-band constitutes an anchors point for the thin actin filaments (often refereed to as the thin filaments) which project into the sarcomere on both sides. Actin filaments are ~6-8 nm in diameter and 1 μm in length, being composed of polymerized G-actin monomers (Person and Young, 1989), and its main function is to provide binding sites for the myosin molecules. The G-actin monomers are arranged into two helical chains that are twisted around each other. The most important protein associated with actin is tropomyosin and troponin, which both have a key regulatory role in muscle contraction. Muscle contraction is initiated by a neural signal releasing Ca^{2+} from the sarcoplasmic reticulum which binds to the troponin complex (troponin C, I and T). The binding of Ca^{2+} to troponin C in the troponin complex causes a movement in the tropomyosin exposing the myosin binding sites, which makes binding of the myosin motor region to actin possible. The contraction mechanism of the muscle relies on the sliding of actin and myosin between one another and was first described in the 1950's (Huxley and Hanson, 1954) (Fig. 1.2 F). Since the volume of the muscle remains constant during contraction, the diameter increases during contraction. An X-ray diffraction study showed that the distance between thin and thick filaments remained constant during contraction (Millman, 1998). The thickening of the contracting muscle fibres is probably achieved by dislocation of cytoplasm (Agarkova and Perriard, 2005).

The myosin referred to as the thick filaments are ~14-16 nm in diameter and 1.5 μm in length (Person and Young, 1989). In comparison to the actin, myosin is more diverse and compromise of a large super family, with more than 17 distinct classes (Sellers, 2000).

Myosin class II is the largest, the first to be discovered and is the type of myosin found in striated muscle (Sellers, 2000). The myosin molecule consists of three structural units, the myosin head (motor domain) which binds to actin, the neck region (flexible part) and the tail region which acts as an anchor point to sustain the motor regions position to actin assuring that the binding takes place (Sellers, 2000). In the centre of the sarcomere a structural unit called the M-band has the unique feature of connecting, aligning and stabilizing the myosin filaments. Each myosin filament is cross-linked to six of its closest myosin neighbours at the M-band (Person and Young, 1989). The components of the M-band are the myomesins (Agarkova and Perriard, 2005; Schoenauer et al., 2008), M-protein (Masaki and Takaiti, 1974) and creatine kinase (Wallimann et al., 1977). Creatine kinase has an important role in periods of extensive fatigue, being involved in energy transformation. However, there is evidence that creatine kinase also serves as a structural unit (M-bridges) as well as having an enzymatic function (Wallimann et al., 1977; Hornemann et al., 2003).

Titin (also called connectin) is the largest protein in mammals (LeWinter et al., 2007), the third most abundant protein of the sarcomere (Trinick et al., 1984), being anchored in the Z-band and stretches to the M-band connecting the two longitudinally (Agarkova and Pirriard, 2005). Titin has an elastic characteristic which generates a passive force pulling the sarcomere back to the unstretched length after contraction (Smith 2006). Titin is known to have other functions as well, including the sarcomere maintenance stability (Trinick, 1984), and cell signal transduction and sensing (se review of LeWinter et al., 2007).

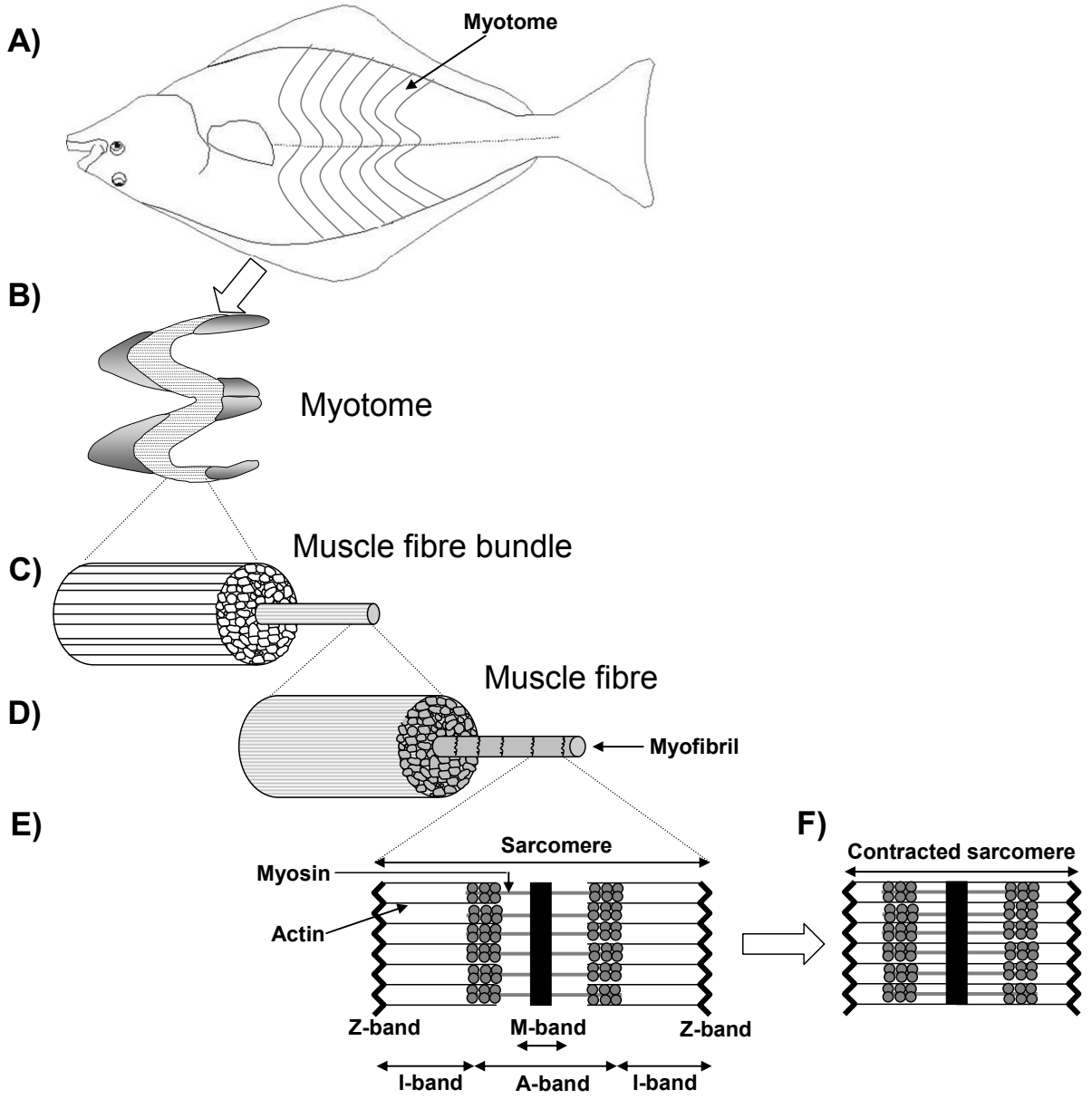

Figure 1.2. A simplified cartoon illustrates macro and micro structure of the fish muscle structure. (A) In a partly skinned Atlantic halibut the contractile elements of the muscle are located in the myotomes of the muscle (indicated with arrow), separated by connective tissue sheets (myocommata). (B) A sketch of an individual myotome of the muscle. (C) One of many bundles of muscle fibres in a myotome. (D) Individual muscle fibre. (E) Structure of the main contractive elements (named with illustrative arrows) of the muscle fibre within a sarcomere, including Z, I, A and M-bands, actin and myosin. (F) Sarcomere in a contracted state.

1.2.1.1 Myotendious junctions (MJ) in teleosts

Desmin plays an important structural role and occupies a strategic position connecting the individual myofibrils laterally at the Z-band and interconnecting sarcomeres to the sarcolemma system (Greaser, 1991, Small et al., 1992). Further, the fine structure of the connective tissue in king salmon (*Oncorhynchus tshawytscha*) (Fletcher et al., 1997) and hoki (*Macruronus novaezelandiae*) (Hallett and Bremner, 1988) are connected to the muscle fibre base with membrane-lined groove-like invaginations containing collagen fibres. In addition to membrane-linked groove-like invaginations, secondary invaginations are also present. These secondary invaginations contain collagen fibres that stretch into the sarcoplasma, having a tube/finger-like shape. In hoki, branched like structures of 1-10 μm in length penetrates longitudinally in-between the muscle fibres (Hallett and Bremner, 1988). The same observations were made for lamprey (*Lampeetra japonica*) (Nakao, 1975) and blue grenadier (*Macruronus novaezelandiae*) (Bremner and Hallett, 1985), suggesting uniform MJ across different fish species. A scanning electron microscope study showed that socket-like holes appeared in the myocommata when the myomeres were removed (Bremner and Hallett, 1985). These sockets are equivalent to those in king salmon (Fletscher et al., 1997) and Atlantic salmon (Li et al., 2005). In addition, the interfacial ratio (ratio between the area of the junctional sarcolemma and the cross-sectional fibre area) of the MJ is known to differ between muscle type and sample location (Spierts et al., 1996). The load/force on the MJ is higher in posterior than anterior myotomal muscle (Davies et al., 1995). Spirers et al., (1996) hypothesised that the magnitude of the load would be reflected in the structure of the MJ. The results showed that the MJ in caudal muscle had a larger interfacial ratio than rostal myotomal muscle, being related to the larger work load on the MJ. The same study also found that slow muscle had a larger interfacial ratio compared to fast muscle, possibly caused by; 1) a higher

contraction velocity, 2) a larger strain and 3) a longer duration of the load on the MJ (Spirers et al., 1996). Thus, a scanning electron micrograph of slow and fast muscle showed that fast muscle had smaller and more numerous finger-like extinctions than slow muscle (Spirers et al., 1996). The connective tissue junctions in fish have a very similar structure to that of mammals (Korneliussen, 1973; Ajiri et al., 1978; Maruyama and Shimada, 1978), but fish have a lower interfacial ratio (Trotter, 1993).

1.2.2 Connective tissue architecture

The connective tissue which has a supportive and connective role for the different tissues and organs in the fish consists of a number of different cells and the extracellular matrix (ECM). The cells frequently present in or associated with the ECM are fibroblasts (involved in the synthesis of collagen, see below), macrophage, mast cells and plasma cells (Kierszenbaum, 2002). Apart from the different types of cells, the ECM is composed of variety of different collagen types, noncollagenous glycoproteins (elastin) and ground substance which is a common noun for different types of proteoglycans. Ground substance is a large group which often fills the "gap" between the collagen fibres and their functions are often to connect or bind cells and/or fibres (Kierszenbaum, 2002). Elastin is the last major group and their function is as their name implies, to give elasticity to tissue or organs and to restrain excessive stretching. Elastin is an important structural unit in blood vessels, especially in the large veins. Some of the constituents of the ECM have been studied in more detail in fish (e.g. collagen, see next section) while others are less studied and understood (Ofstad et al., 2006). In addition to the supportive role, the ECM in mammals are also involved in cell to cell signaling and signaling provided by growth factors, affecting gene expression and influencing

cell function (Huhtala, 1995; Werb et al., 1989). However it is not unlikely that the ECM plays a key role in several signaling pathways in fish as well.

In mammals, the collagen compartments of meat are divided into epimysium, perimysium and endomysium (Purslow, 2002). The epimysium surrounds a whole muscle group, the perimysium surrounds a muscle bundle, while the endomysium surrounds each individual muscle fibre. The connective tissue in fish is distinctly different from that of mammals and the most striking difference is that myotomal muscle in fish is not divided into groups separated by the epimysium, but is considered as one unit referred to as the fillet. In fish the term epimysium is not used, but is replaced by a term called myocommata (Fig. 1.3 A). This is the largest connective tissue compartment in fish muscle, and in a skinned fish the myocommata can be seen as curved (W-shape) strips running across the fillet (Fig. 1.3 A). However, the terms perimysium and endomysium (Fig. 1.3 B) are also used to describe the pericellular connective tissue in fish (Bremner and Hallet, 1985). The skin and skeletal muscle are internally connected to the myocommata, central vertical septum and the membrane lying between the epaxial and hypaxial planes (Nursall, 1956).

In respect of collagen concentration, total mammalian proteins consist of approximately 10% collagen, while the values in fish normally vary between 2–5% (Dunajaski, 1979). The distance between the myocommata decreases towards the tail as the myotomal muscle segments gets narrower (Love 1958). For example, in hake the collagen content is about 0.2% close to the anterior part and 0.9% in the posterior region of the fillet (Montero and Borderia, 1989).

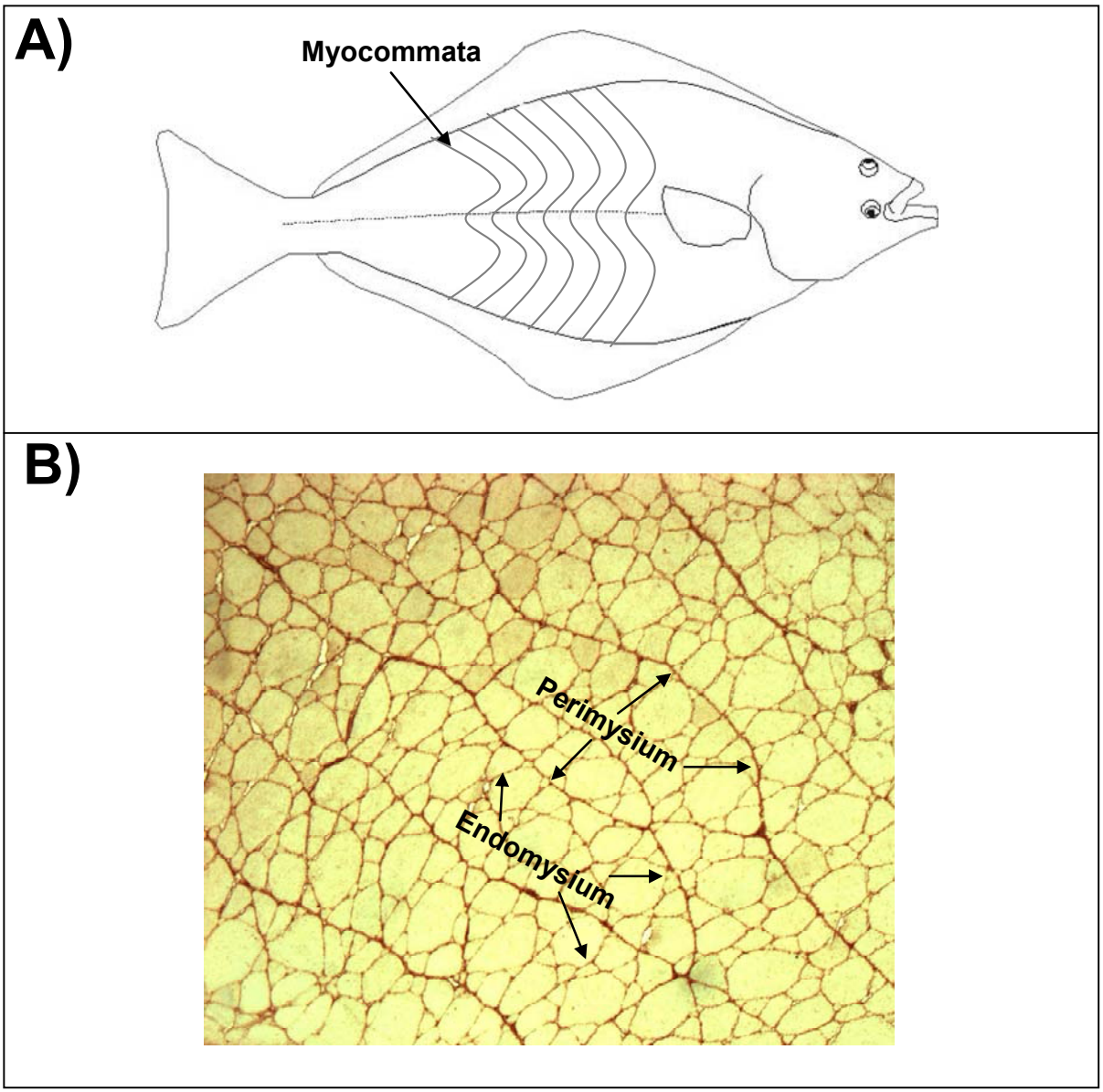

Figure 1.3. The three different extracellular matrix compartments of Atlantic halibut muscle. (A) The sketch illustrates an Atlantic halibut with the mid-section partly skinned, exposing the myocommata which can be seen as w-shaped lines running across the muscle, separating the individual muscle segments. (B) Picture shows a cross-section of an Atlantic halibut stained with a collagen type I antibody. The arrows in the picture show the perimysium and endomysium surrounding bundles of, and individual -muscle fibres, respectively.

Of the ECM constituent collagen is the largest and 27 distinct collagen types have so far been identified (Myllyharju and Kivirikko, 2004; Pace et al., 2003). For example, in cod (*Gadus morhua* L.) it was calculated using transmission electron microscope that the collagen compartment of the ECM comprised ~85% of the relative cross-sectional area (Ofstad et al., 2006). Collagen has been studied and characterized from a whole range of different fish species including Alaska Pollack (*Theragra chalcogramma*) (Kimura and Ohno, 1987), backed toadfish (*Lagocephalus gloveri*) (Senaratne et al., 2006), skate (*Raja kenojei*) (Mizuta et al., 2002), Nile perch (*Lates niloticus*) (Muyonga et al., 2004), Skipjack tuna (*Katsuwonus pelamis*), Japanese sea-bass (*Lateolabrax japonicus*), ayu (*Plecoglossus altivelis*), yellow seabream (*Dentex tumifrons*), chub mackerel (*Scomber japonicus*), horse mackerel (*Trachurus japonicus*) (Nagai and Suzuki, 2000), Japanese amberjack (*Seriola quinqueradiata*) (Nishimoto et al., 2004), carp (*Cyprinus carpio*) (Sato et al., 1989b), lamprey (*Entophenus japonicus*), Japanese eel (*Anguilla japonica*), lizardfish (*Squirida elongate*) (Sato et al., 1989a) and cod (*Gadus morhua*) (Brüggemann and Lawson, 2005).

In fish the major and minor collagen types are collagen type I and V respectively (Sato et al. 1988, 1989a, 1989b, 1991). Collagen type III was for a time not identified in fish (Sato et al., 1988; Sato et al., 1989a), but by the use of confocal microscopy and immunohistochemical staining techniques a more recent study confirmed the presence of collagen type III in cod (Brüggemann and Lawson 2005). The collagen superfamily was first divided into two groups; 1) the fibril forming and 2) non fibril forming collagens, but the continuous discovery of new collagen types made this grouping inappropriate and the collagens have been further divided into a total of eight sub-families based on their structure (see review of Myllyharju and Kivirikko 2004). The different sub-families are: 1) fibril forming collagens (e.g. I and V), 2) fibril-associated collagens with interrupted triple helices (FASIT) and related collagens, 3) collagens forming hexagonal network, 4) the family of type IV collagens, 5) type VI collagen

forming beaded filaments, 6) type VII collagen forming anchoring fibrils, 7) collagen with transmembrane domains and 8) type XV and XVIII collagens. In addition, a group of proteins containing triple-helical collagenous domains is grouped with the collagens, but not classified as one of them (Myllyharju and Kivirikko, 2004).

Fibrous collagen type I is the most abundant and consists of three collagen fibres tightly packed into a triple stranded helical macromolecule. Every third amino acid of the triple helix is Glycine (Gly-X-Y) and is essential for the packing of the coiled structure (Prockop *et al.,* 1979). The X and Y position can be any random amino acid, but are frequently occupied by the imino acid proline and hydroxyproline respectively. The imino acid hydroxyproline is characteristic for collagen and is often used for quantification of collagen (Love et al., 1976; Bateman et al., 1996; Li et al., 2005). Hydroxyproline has an important role in the stabilisation of the collagen triple helix. In fibril forming collagens, the three α chains can be identical, but the triple helix can also be composed of two or three different α chains (Myllyharju and Kivirikko, 2004). Collagen type I is present in most tissue and is a heteropolymer consisting of two α1 chains and one α2 chain.

Collagen synthesis takes place in the fibroblast (Fig. 1.4 A) and osteoblast located in close relation to or inside the connective tissue. The first building blocks of collagen type I is called procollagen and is synthesised as soluble precursors inside the Rough endoplasmic reticulum (RER). From the RER the procollagen is transported to the Golgi apparatures where it is packed and secreted to the extra cellular space (Kielty et al., 1993) (Fig. 1.4 A-C). The procollagen molecule requires the action of an enzyme (procollagen metalloproteases) to remove the extended N and C-terminal propeptide (non helical region) to become the mature and fully functional tropocollagen molecule (Kadler et al., 1996; Keirszenbaum, 2002; Ricard-Blum and Ruggiero, 2005) (Fig. 1.4 D). Without the action of procollagen metalloproteases the collagen fibril formation would not take place (Kadler et al., 1996). Once

the tropocollagen molecules are formed in the extracellular space they spontaneously self assemble into a collagen fibril (Kadler et al., 1996) (Fig. 1.4 E). After the cleavage of the propeptide, this region is then called a telopeptide and is a very important site for the cross-linking of the tropocollagen molecules. The cross-linking process is the final step in the collagen fibril formation, and the copper dependent enzyme lysyl oxidase is the only enzyme known to be directly involved and plays a crucial role in the side-by-side cross-linking of tropocollagens (Bailey et al., 1989, 2001; Reiser et al., 1992) (Fig. 1.4 E). However, the number of cross-links per tropocollagen is limited by the number of binding sites. In collagen type I four cross-link binding sites have been identified, one of which is located in each of the N-terminal and C-terminal telopeptide and two within the helical region (McCormick, 1999). The functionality and tensile strength of the collagen fibril is primary due to the intermolecular cross-link formation (McCormick, 1999).

Cross-link formation can take place either via an enzymatic step or a step called glycation. The enzyme mediated lysine-aldehyde cross-link in collagen type I has two major pathways:

1) The allysine pathway which produces aldimine cross-links formed from aldehydes.
2) The hydroxyallysine pathway which produces ketoamine cross-links formed from hydroxyaldehydes.

The aldimine and ketoamine –cross-links in muscle tissue are both divalent reducible cross-links capable of binding two tropocollagens together and takes place between the telopeptide and the helical region. In time these intermediate products are to a large extent replaced by mature none reducible trivalent cross-links (Bailey et al., 1998; Bailey, 2001). One example of a mature cross-link that accumulates in muscle tissue with age is the mature divalent hydroxylysyl pyridinoline (PYD) (Montero and Bordrías, 1990; Bailey et al., 1998; Bailey, 2001). The formation of mature cross-links increases the strength of the collagen fibres significantly, since PYD is capable of binding three tropocollagen molecules together

(McCormick, 1999). The PYD crosslink is formed via the hydroxyllysine pathway together with another mature cross-link, lysyl pyridinoline, a dominant cross-link in bone. The formation of PYD takes place via a condensation reaction of two ketoamine cross-links (McCormick, 1989, 1999). The final divalent cross-linking formation is mediated by fibre associated collagens and proteoglycans (Keirszenbaum, 2002), in which proteoglycans can bind to collagens via their glycosaminoglycan chains (Heino, 2007) (Fig. 1.4 F).

The collagen matrix has a low turnover rate and is therefore capable of undergoing age related changes, and this is where the second type of cross-link formation becomes important through a non enzymatic process called glycation (Bailey, 2001). The glycation process involves the reaction of sugars, especially glucose since it is normally the most abundant type of sugar in tissues (Bailey, 2001). The glycation process is very complex, not fully elucidated and will not be mentioned in detail (see reviews of Bailey et al., 1998, Bailey, 2001 and Reiser et al., 1992 for more details). Fig. 1.4 A-F is a simplified illustration of the main events during the collagen synthesis and cross-linking process. However, the cross-links illustrated in the Fig. 1.4 E are the mature PYD cross-links and not mediated lysine-aldehyde cross-links that are the first cross-links to form.

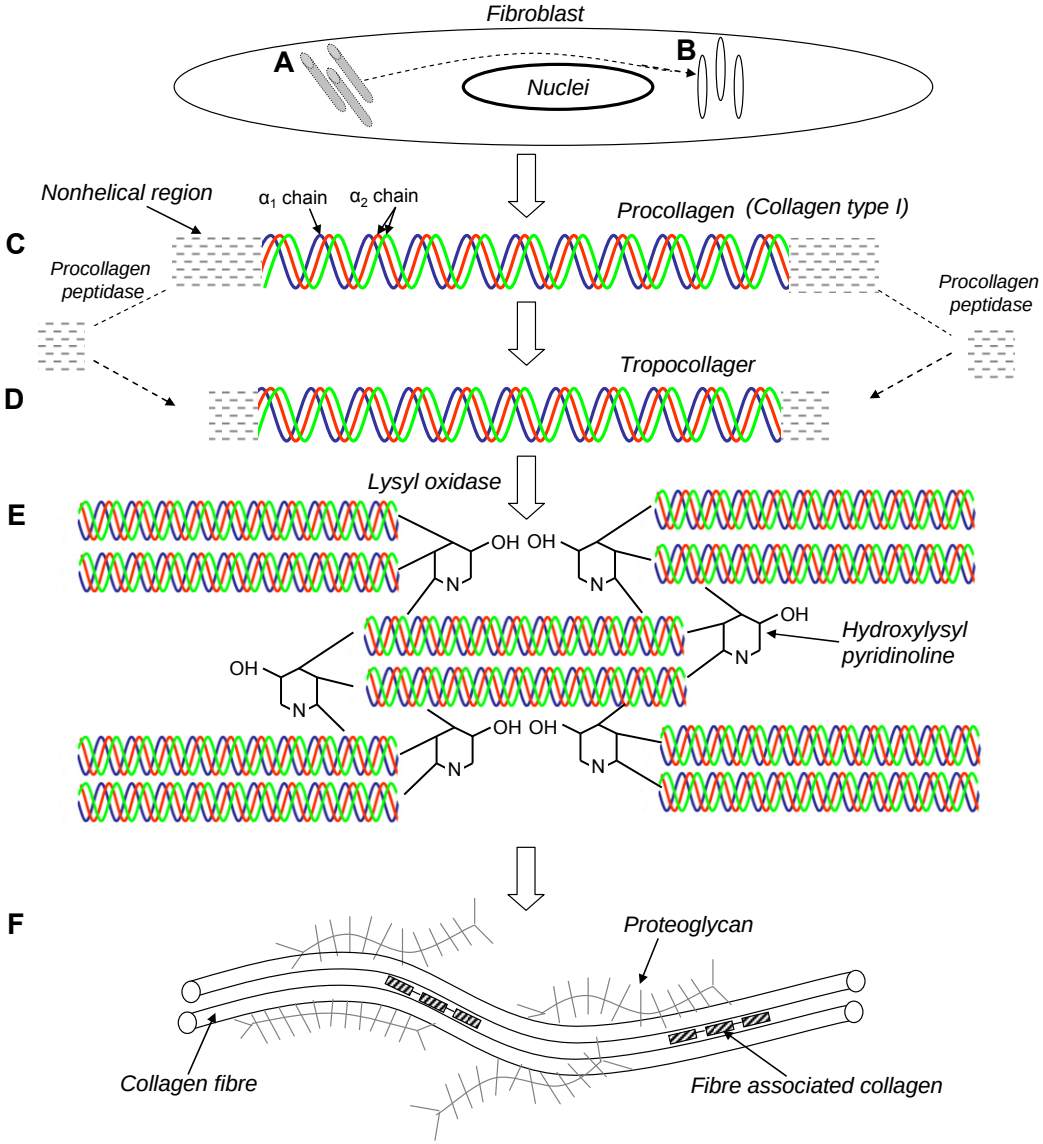

Figure 1.4. Synthesis and structure of collagen type I. (A) Procollagen is produced in the rough endoplasmic reticulum in the fibroblast associated within or in close contact with the ECM. (B) The Golgi apparatus of the fibroblast is essential for the packing and secretion of procollagen molecules. (C) *Post* secretion, the procollagen molecule has a triple helix structure (one α_1 chain and two α_2 chain) containing none helical regions at each C and N-terminal propeptide. (D) By procollagen peptidase part of the propeptide regions are removed and the tropocollagen molecule takes its final form. (E) Spontaneous self assembly of tropocollagen molecules into a collagen type I fibre. The enzyme lysyl oxidase is essential in the cross-linking process of the tropocollagen molecules. The first cross-links that form are immature reversible divalent cross-links. The cross-links in the figure represent the mature irreversible cross-link hydroxylysyl pyridinoline, which is capable of binding three tropocollagen molecules. (F) The structure and alignment of collagen type I fibres in the ECM involves proteoglycans as well as other collagens, such as fibril-associated collagens with interrupted triple helices (FASIT). Adapted, modified and redrawn from Kierszenbaum 2002.

1.3 Muscle fibre types

The three main muscle fibre types in fish are identified as fast anaerobic (white), slow aerobic (red) and intermediate (pink) muscle fibres, and they are arranged into distinct anatomical zones (Johnston et al., 1977; Bone, 1978) (Fig. 1.5 A&B). Intermediate fibres are located in-between the superficial slow layer and the deeper fast muscle compartment (Fig 1.5 B). The intermediate muscle fibres are not mixed with the fast fibres, but to a certain degree mingled with slow fibres (see review of Sänger and Stroiber, 2001). The slow muscle compartment is arranged directly beneath the skin in a few cell layer thickness, but the majority of slow muscles are concentrated around the horizontal myosepta. Thus, the distribution and proportion of slow muscle are known to vary between species (Fig. 1.6 A-D). The fast muscle represents the bulk of the striated muscle, often comprising more than 90% of the total muscle mass (Stickland, 1983; Hagen et al., 2006). Thus the ratio between slow and fast muscle differs considerably between species (Love, 1988). Slow muscle normally comprises less than 10% and never more than 30% (Green-Walker and Pull, 1975), a difference that is linked to the difference in swimming models between species (Love, 1988). The ratio of slow and fast muscle varies along the body length, and the proportion of slow muscle is higher in caudal than rostral myotomes (Johnston and Lucking, 1978; Johnston, 1982; Gill et al., 1989). In anchovy (*Anchoviella alleni*) the proportion of red muscle in the posterior part found to be six times that of the anterior parts (Johnston, 1982) (Fig. 1.7). In addition, the red muscle of skipjack tuna (*Katsuwonus pelamis*) is known to vary along the body in irregular ways (Johnston and Brill, 1984) (see Fig 1.6 A.). The main muscle groups have different characteristics, and show a distinct division of labour during swimming. Sustained locomotion at low swimming speed (tailbeat frequencies) is driven by the slow aerobic muscle (Johnston et al., 1977, Bone, 1966; Coughlin and Rome, 1999).

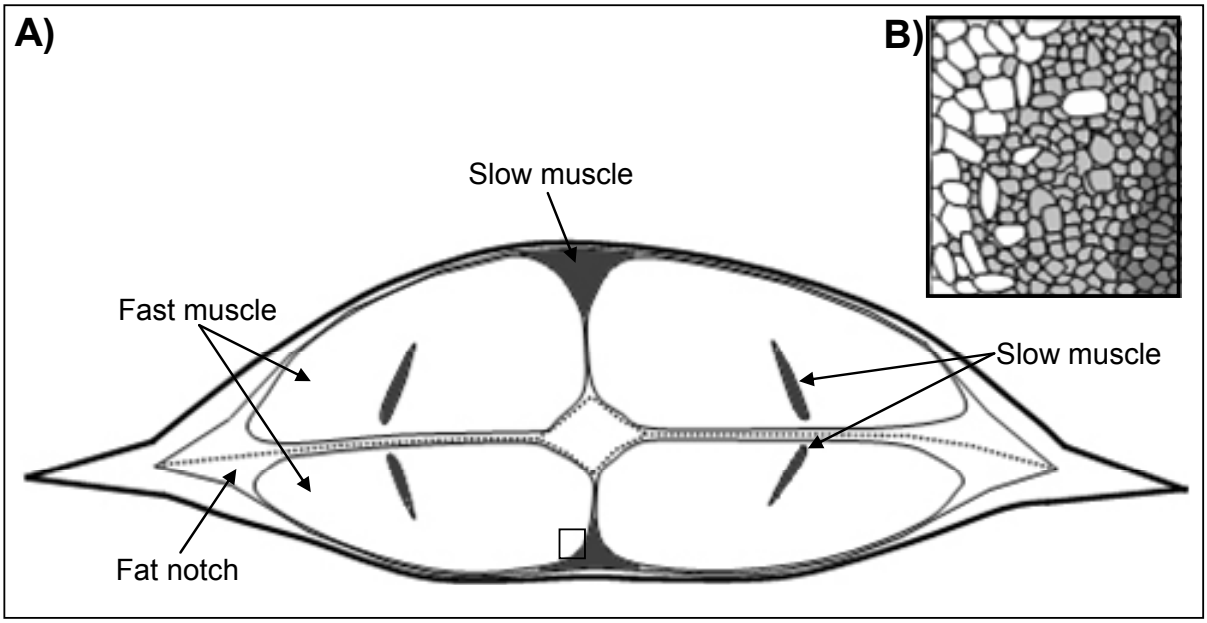

Figure 1.5. Drawing of a halibut steak (A). Grey areas dorsal and ventral side and internal strips illustrates the location of the slow (red) muscle. Rectangle illustrates the location of the insert (B) and dotted lines illustrate the bone structure of the halibut steak. B: Intermediate muscle (light grey) fibres are found in the inter-phase between slow (grey) and fast muscle (white). Modified from Hagen et al. (2008a).

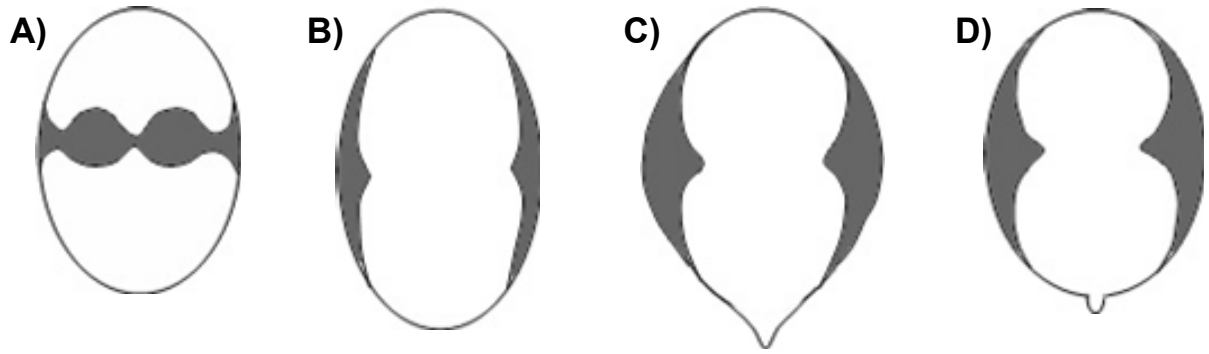

Figure 1.6. The figure illustrates how the distribution of slow (red) muscle varies between four different salt water species, tuna (A), cod (B), herring (C) and mackerel (D). Reproduced and modified from Love (1988).

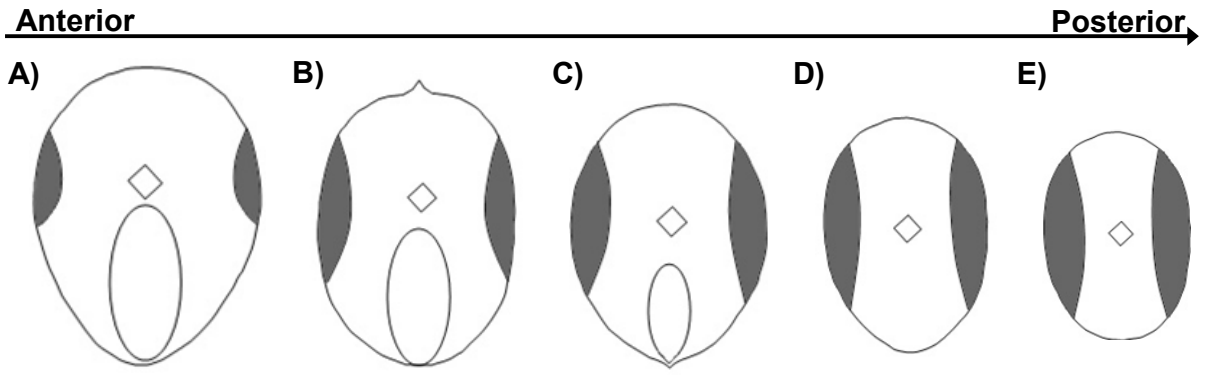

Figure 1.7. Illustration of the distribution of slow (red) muscle along the body length in anchovy (*Engraulis encrasicolus*). The percentage of total cross-sectional area occupied by slow muscle increased in an anterior-posterior direction. Adapted and modified from Johnston 1982.

, while rapid movements and burst swimming are powered by the bulk of fast anaerobic muscle fibres (Bone, 1966; Johnston et al., 1977; Gilly and Aladjem, 1987). Intermediate muscle fibres are recruited for activity levels in between (Johnston et al., 1977). For example, in mirror carp (*Cyprinus carpio*) at tailbeat frequencies below 0.5 body lengths (B/L) per s, slow muscle fibres are active alone, while intermediate and fast muscle is used for tailbeat frequencies around 1.1-1.5 and above 2.0-2.5 B/L per s respectively (Johnston et al., 1977). However, a few fish species, such as the European eel (*Anguilla anguilla*) lack intermediate muscle fibres intirely (Egginton and Johnston 1982).

Most fish are not capable of maintaining high speeds for prolonged periods of time since burst swimming will result in a rapid drainage of muscle energy stores and accumulation of metabolic end products. To maintain high speed for a longer period, fish fast muscle utilizes the creatine phosphate (PCr) and especially glycogen stores (Black et al., 1962; Johnston et al., 1977; Weber and Haman, 1996). Fast muscle of fish can be considered a closed system and the energy requirements for prolonged burst movements are almost entirely dependent on anaerobic glycolysis (Weber and Haman, 1996). PCr are mobilized in the initiating phases of burst swimming, but when a high activity endures, energy metabolism shifts from aerobic to anaerobic glycolysis which causes a rapid increase in lactate levels. In contrast to slow muscle, the removal of lactate from fast muscle takes a considerable longer time (Black et al., 1962), a process controlled by enzymatic breakdown and transportation of metabolic end products (e.g. lactate) (Johnston, 1977; Johnston et al., 1977). The fast muscle mitochondria also have a central role in lactate removal, converting lactate into glycogen (Arthur et al., 1992).

One of the reasons for the large difference between muscle fibre types is the well developed capillaries and good blood support to the slow muscle. During sustained swimming slow muscle is recruited and carbohydrates (e.g. glycogen) only plays a minor role compared to

burst swimming (Weber and Haman, 1996). In addition to the utilisation of carbohydrates the mitochondria of slow muscle are capable of using lipids and amino acids as fuel. As swimming speed increases lipids are considered the most important energy stores, followed by carbohydrates and proteins makeing the smallest overall contribution (Sänger and Stoiber, 2001). The dark red colour of the slow muscle is due to the myoglobins located within the muscle cells, but blood pigments (haemoglobin) contribute as well. The muscle fibre types have a significantly different ratio in myoglobin content, and the ratio of slow, intermediate and fast muscle of Mirror carp (*Cyprinus carpio*) has been estimated to 5:2:1 respectively (Johnston et al., 1977). Apart from having a low aerobic capacity due to low number of myoglobins and poorly developed capillaries, the differences are substantial. Some of the most striking differences are summarised in Table 1.1.

Table 1.1. Some of the structural and biochemical differences between red and white muscle.

+ = More and - = Less.

Structural component	Example species	Slow muscle	Fast muscle	Comment	Reference
Mitochondria	*Pleuronectes platessa*	24.6 ±0.8 Volume %	2.0 ±0.3 Volume %	Higher density in slow muscle. Large variation between species.	Galloway et al., 1999b; Luther et al., 1995; Johnston and Moon 1981
Myonuclei content	*Salvelinus alpinus*	2264 In a ~60 µm fibre	1126 In a ~60 µm fibre		Johnston et al., 2004a
ATPase activity	Carp *(Cyprinus carpio)*	0.25 ±0.03 P*i* released/mg myofibrillar protein/min	1.09 ±0.14 P*i* released/mg myofibrillar protein/min	Fast muscle contains acid labile mATPase, while slow contain alkaline labile mATPases. Differences between species. mATPase is linked to the rate of contraction.	Carpenè et al., 1982; Gill et al., 1989; Guth and Samaha, 1970; Johnston et al., 1974, 1977; Pai-Silva et al., 2003
SDHase activity	*Cyprinus carpio*	1332 ±101 Substrate utilised/g dry wt/min	199 ±60 Substrate utilised/g dry wt/min	Fast muscle have a very low SDHase activity.	Carpenè et al., 1982; Johnston et al., 1977; Nachlas et al., 1957; Sänger and Stroiber 2001
Creatine phosphokinase	*Cyprinus carpio*	1968 ±241 Substrate utilised/g dry wt/min	4242 ±659 Substrate utilised/g dry wt/min	Creatine phosphate is one of major metabolites in fast muscle.	Johnston et al., 1977; Sänger and Stroiber, 2001
Lipid content	*Scomber Scombrus*	13.1%	2.3%	Lipids are stored in muscle, liver or both and as lipid droplets located between the fibres. Large variation between species.	Carpenè et al., 1982; Love et al., 1988; Luther et al., 1995; Johnston et al., 1977
Collagen content	In general	+	-	Higher presents of proline and glycine in slow muscle, due to a higher fibre density, e.g. increased amount of endomysium.	Love et al., 1988
Glycogen content	*Cyprinus carpio*	5.6 ±1.2 mg/g dry wt	0.66±0.15 mg/g dry wt	Active fishes like tuna stores up to 8 and 4 times more glycogen in fast muscle than carp and trout respectively.	Arthur et al., 1993; Carpenè et al., 1982; Johnston et al., 1977; Luther et al., 1995.
Maximum fibre diameter	*Salmo salar*	< 50 µm	< 240 µm	Large differences between species. Nothoenoides (500-650 µm) and halibut (>350 µm) are capable of growing unusually large fast muscle fibres.	Hagen et al., 2008a Johnston et al., 2000a, 2003b; Weatherley et al., 1988
Blood supply	*Salmo trutta*	0.85 ±0.06 Numerical capillary to fibre ratio	0.39 ±0.05 Numerical capillary to fibre ratio	Poorly developed capillaries in fast muscle. Large variation between species depending on activity level.	Akster, 1985; Luther et al., 1995; Sänger and Stroiber, 2001
Myoglobin content	*Cyprinus carpio*	12.7 ±1.1 mg/g dry wt	2.8 ± 0.4 mg/g dry wt	Ratio of 5:2:1 for slow:intermediate:fast muscle of carp respectively.	Johnston et al., 1977; Luther et al., 1995
Z-band width	*Cyprinus carpio*	+	-	Thicker Z-bands in slow than fast muscle of carp.	Akster, 1985; Luther et al., 1995
T-SR junction*	*Cyprinus carpio*	0.77 ±0.04 μm^{-2}	0.53 ±0.03 μm^{-2}	Shorter T-SR junctions in slow than fast muscle. Influenced the rate of calcium release.	Akster, 1985
Reaction time	*Citharichthus sordidus*	<1s	~100ms	Quicker response in fast than slow muscle. Intermediate muscle had a reaction time +2 s.	Gilly and Aladjem, 1987

* T system-sarcoplasmatic reticulum junction (T-SR junction). Average values are given in ± SE.

1.4 Muscle growth in teleosts

During embryogenesis, the segmental plate divides in a rostral to caudal wave under the influence of Sonic Hedgehog (Shh) and notch (trans-membrane protein), forming the somites which are the precursors of the myotomes (Mullins, 1999). In zebrafish (*Danio rerio*) embryos two sub populations of cells have been identified prior to somite formation (Devoto et al., 1996; Cortés et al., 2003). *Post*-somitogenesis the adaxial cell, located adjacent to the notochord, starts to migrate (takes approx. 5 h at 28.5 ºC) away from the notochord forming a superficial layer of muscle fibres identified as slow muscle fibres (Devoto et al., 1996; Cortés et al., 2003). Chaderin trensmambrane proteins have been identified as a regulator and responsible for the adaxial cell migration (Cortés et al., 2003). Lewis et al. (1999) showed that zebrafish lacking Shh signalling pathway (mutants) resulted in fish with adaxial cells lacking MyoD expression (a member of the myogenic regulatory factor required for muscle lineage determination) (except caudal adaxial cells in the tailbud), reducing the development of slow muscle fibres. Thus the loss of Shh can be partially compensated for by other proteins from the Hedgehog family (Lewis et al., 1999). Shh signalling has also been associated with small-sub populations of fast fibres expressing Engrailed protein (Wolff et al., 2003) and found to be required for fast fibre elongation (Henry and Amacher, 2004). The second sub population of cells arises from the lateral somatic mesoderm and is regulated by distinct signals, the same signals also being involved in the adaxial cell myogenesis (Groves et al., 2005). This second population stays in a medial position and differentiates into fast embryonic muscle fibres (Groves et al., 2005). By utilizing vital fluorophore BODIPY-Ceramide staining and confocal laser scanning in combination with linage tracking techniques it was discovered that by mid somitogenesis, somatic cells of zebrafish had initiated a set of morphometric behaviours that resulted in 90º rotation to that of the initial position (Hollway et al., 2007). The linage technique also showed that individual labelled cells can rotate at different rates and Sdf

cytokine signalling is required for somite rotation (Hollway et al., 2007). Pax3 and Pax7 are both transcription factors expressed in mitotacally active muscle progenitors cells (Relaix et al., 2004). The anterior component of the somite forms the external cell layer expressing these two transcription factors (Hollway et al., 2007; Stellabotte et al., 2007). Further, sub-populations of these cells migrate to become fast muscle fibres during the larval stage while some remain in their position providing a source of myogenic progenitor cells for *post* embryonic muscle growth (Hollway et al., 2007; Stellabotte et al., 2007).

Post-embryonic increase of muscle mass takes place via two distinct mechanisms called hypertrophy and hyperplasia (Johnston, 1999; Rowlerson and Veggetti, 2001). Hypertrophy is the increase in muscle fibre diameter while hyperplasia is the increase in muscle fibre number (recruitment of new muscle fibres). Teleosts recruit muscle fibres throughout the juvenile phase and into adulthood until a certain body size (Weatherley et al., 1988; Kiessling et al, 1991; Zimmerman and Lowery 1999; Johnston et al., 2000c, 2002, 2003a, 2003b), which is different from mammals where fibre numbers are fixed at birth (Rowe and Goldspink, 1969; Stickland, 1981). However, Artic char (*Salvelinus alpinus*) slow muscle fibre recruitment continues after fast fibre number is established (Johnston et al., 2004a). Fish with a small final body size tend to stop recruiting muscle fibres at an earlier stage compared to fish with a large ultimate body size (Weatherley et al., 1988). Since the majority of the slow and fast muscle fibres seldom achieve diameters of more then 50 and 240 μm respectively (Weatherley at al., 1988; Johnston et al., 2000a), fish with a large body size recruit fast muscle fibres for a prolonged period to achieve a large size. For example, a 0.2 kg halibut has ~320000 muscle fibres, while a halibut on 96 kg has ~1.7 million fibres (> 4.3 fold increase, Hagen et al., 2008a). In contrast, the notothenoids (sub-Antarctic family) recruit a modest number of fast

fibres, but compensate by growing unusually large muscle fibres (500-650 μm) allowing them to reach a relatively large final size (Johnston et al., 2003b).

Hyperplastic growth is divided into two distinct phase's occurring at different stages of the fish life cycles, referred to as stratified (Fig. 1.8 A) and mosaic hyperplasia (Fig. 1.8 B) (Rowlerson and Veggetti, 2001). Stratified hyperplastic growth is a process where new muscle fibres are continuously recruited through the early phases of ontogeny, in a germinal zone located in the periphery of the myotome, just beneath the superficial layer of slow muscle fibres (Fig. 1.8 A). These germinal zones are the primary source for new fibres recruitment throughout the late embryonic and larval stage (Rowlerson and Veggetti, 2001) and has been described in the primary Norwegian aquaculture species, cod (*Gadus morhua*) (Galloway et al., 1999a), halibut (*Hippoglossus hippoglossus*) (Galloway et al., 1999b) and salmon (*Salmo salar*) (Johnston and McLay, 1997) including several other species as well (Brooks and Johnston, 1993; Gibson and Johnson, 1995; Johnston et al., 1998; Johnston et al., 2003a).

The timing of stratified hyperplasia varies between species and occurs at different developmental stages. For example, during first feeding of cod larvae (*Gadus morhua*) the number of newly recruited fibres increased significantly in dorsal, ventral and lateral germinal zones of the fast muscle, triggered by endogenous feeding (Galloway et al., 1999a) and was found to be the dominant contributor of muscle growth. In salmon the germinal zones where found at the time of hatching, contributing to myotomal growth in the yolk-sac stage (Johnston and McLay, 1997). In plaice (*Pleuronectes platessa*) germinal growth zones were first observed at the end of the yolk-sac stage (start feeding), and persisted throughout the larval stage until metamorphosis, when being less distinct (Brooks and Johnston, 1993). In halibut, a three time increase in total cross-sectional area of fast muscle was observed between hatching and 10 mm, entirely caused by hypertrophy (Galloway et al., 1999b). A modest

number of small fast fibres were present at hatching, but the number only increased after 10 mm in germinal zones close to the superficial layer in the dorsal, ventral and lateral part of the myotome (Galloway et al., 1999b). Thus, at 13 mm (230 degree days) the contribution of hyperplasia to muscle growth was only estimated to be 4-6% of total fast muscle cross-section area, indicating that hypertrophy was the dominant contributor of myotomal growth during the larval stage (Galloway et al., 1999b).

When the germinal zones are depleted in the late larval stage, mosaic hyperplasia takes over as the main mechanism of muscle recruitment (Rowlerson and Veggetti, 2001). In herring (*Clupea harengus*) the onset of mosaic hyperplasia overlaps with the stratified hyperplasia growth phase (Johnston et al., 1998), an observation made for other species as well (Brooks and Johnston, 1993; Johnston and McLay 1997). During the mosaic hyperplastic growth phase newly recruited muscle fibres are scattered throughout the entire myotome, giving a mosaic appearance with intermingled muscle fibres of different diameters (Fig. 1.8 B). The mosaic hyperplastic contribution is highest during the early juvenile stages and the percentage of small muscle fibres gradually decline with size. Weatherley et al. (1988) revealed that the mosaic hyperplastic growth phase came to a halt at 44% of final body length, and further growth is only achieved by hypertrophy (Stickland 1983; Weatherley et al., 1988; Veggetti et al., 1990; Johnston et al., 2000a). In contrast, growth dynamics of the white seabass (*Atractoscion nobilis*) showed that muscle fibre recruitment did not terminate until 74% of ultimate body size (Zimmerman and Lowery, 1999). As an exception to this, some species within the notothenoid fish seams to lack the mosaic hyperplastic growth phase, and once the germinal zones are depleted (in the stratified hyperplastic growth phase) further growth is entirely achieved through hypertrophy (Johnston et al., 2003b). Until the discovery of the myogenic progenitor cells (Champion, 1984; Carlson 1986; Grounds, 1991), which were identified as the precursors of muscle fibre, the mechanism behind muscle growth was poorly

understood. The myogenic progenitor cells are characterised as small circular shaped cells containing a heterochromatic nucleus with few organelles and are located between the sacrolemma and the basal lamina (Koumans and Akster, 1995). When activated (which a wide range of growth factors and transcript factors are identified being involved in) the fate of the myogenic progenitor cell is decided and the myogenic progenitor cells differentiate into a myoblasts. The myoblast further undergoes a proliferation and is then either absorbed into an existing muscle fibre, or fuses with other myoblasts to produce a myotube which matures into a muscle fibre (Koumans and Akster, 1995; Watabe, 1999; Johnston, 2001) (Fig. 1.9). Newly recruited fibres are observed as small fibres, often on the surface of large muscle fibres (Yablonka-Reuveni and Rivera, 1994; Schultz, 1996). The increase in muscle fibre diameter (hypertrophy) is achieved through the absorption of additional myoblast nuclei to maintain a relative constant ratio of myonuclei to cytoplasm (Koumans and Akster, 1995; Johnston et al., 2003b, 2004a) (Fig. 1.9).

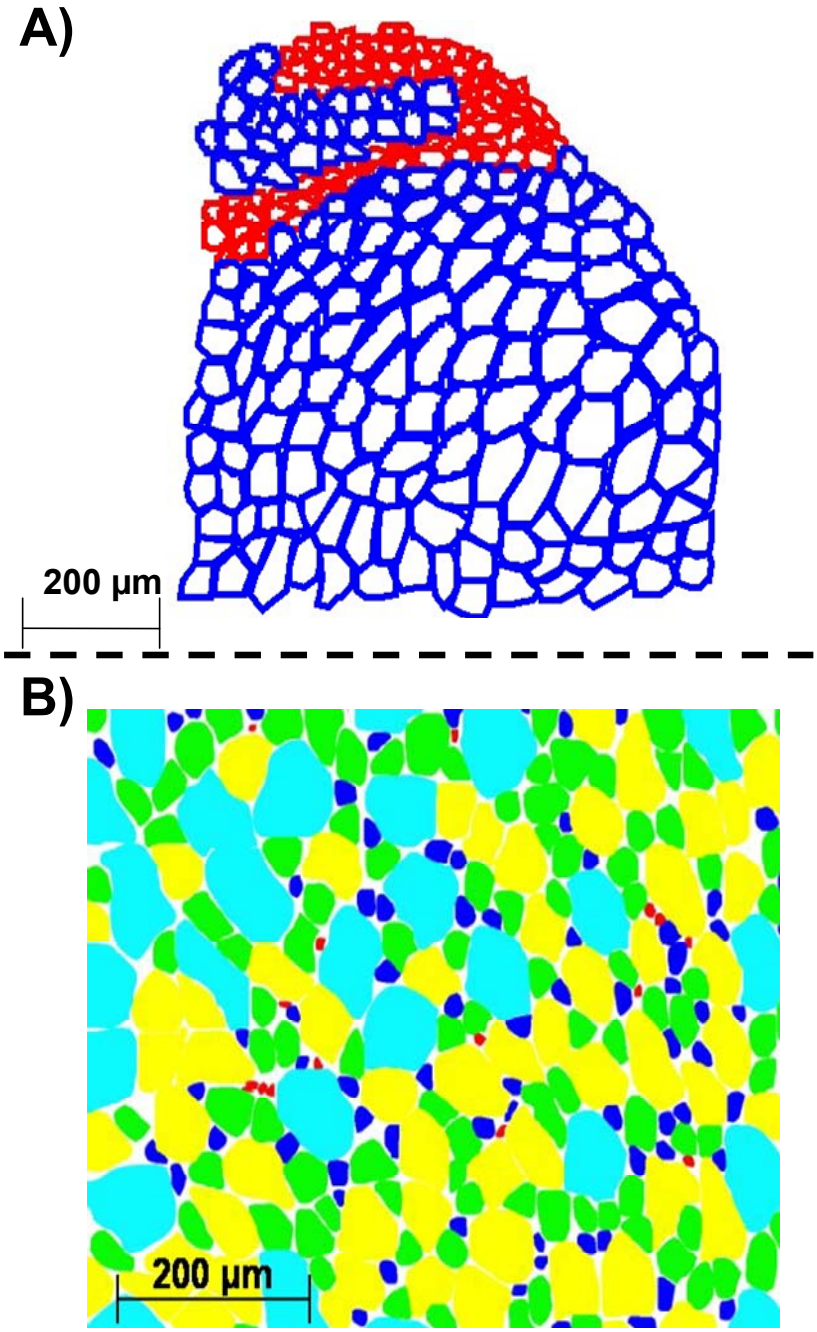

Figure 1.8. (A) A cross-section of the dorsal trunk in a 6 cm juvenile black rockcod (*Notothenia coriiceps*) illustrating stratified hyperplasia. The growth zone containing smaller fibres than the surrounding fast tissue is outlined in red and the fast muscle fibres are outlined in blue. Adapted and modified from Johnston et al., (2003b). (B) A cross-section of the deeper dorsal layer of a 61 cm Atlantic halibut (*Hippoglossus hippoglossus*) illustrating mosaic hyperplasia. The different fast fibres size classes are colour coded (red = 0-29 µm, blue = 30-59 µm, green = 60-89 µm, yellow = 90-119 µm and aqua = 120 µm <), and the newly recruited fibres are outlined in red (Hagen et al., 2008a).

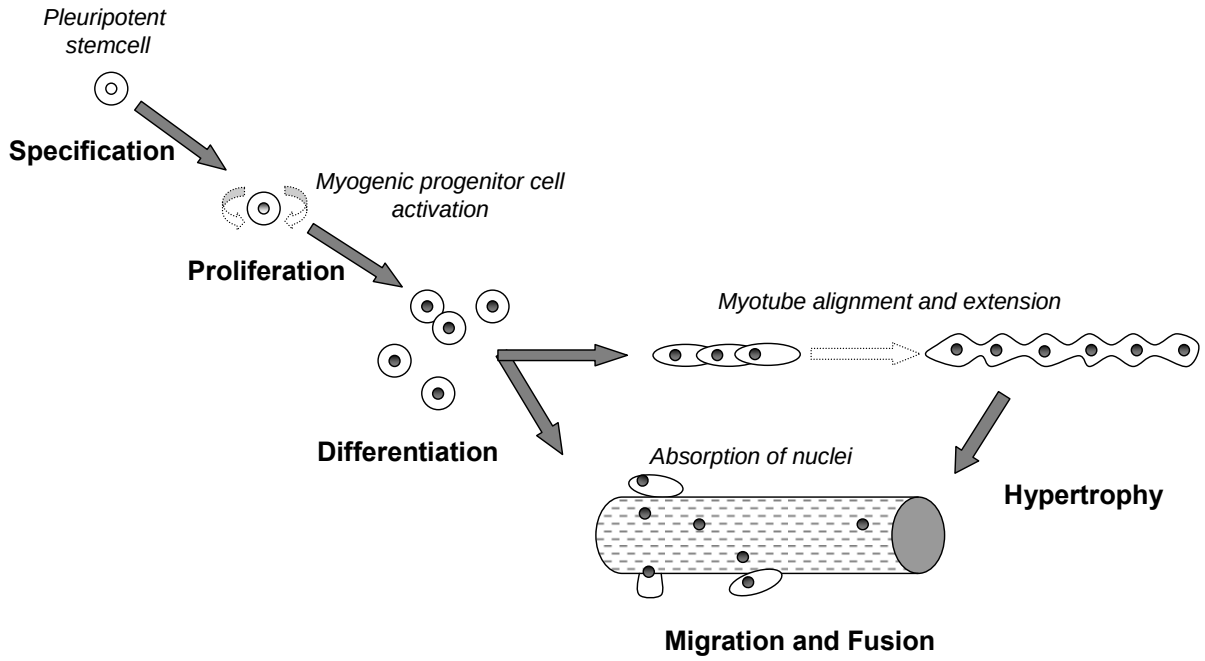

Figure 1.9. A simplified illustration of the fate of the stem cells upon activation. There is a general agreement that the destiny of the stem cells are under both genetic and endocrine control, e.g. the IGF system, which will be discussed in more detail in the following section. The outcome of the myogenic cell cycle is either (a) cell death (apoptosis, not shown), (b) fusion and myotube production or (c) differentiation of fusion with existing muscle fibres. Adapted and modified from Johnston (2006).

1.5 Response of muscle to the environment

With a few exceptions most teleosts are ectotherms, and species living in sub-tropic and/or Arctic environments are subjected to significant seasonal variation regarding abiotic factors such as temperature and light intensity. Numerous studies have illustrated that abiotic as well as biotic factors (e.g. feed) significantly influencing myogenesis in fish.

1.5.1 Temperature

When Atlantic halibut larvae were incubated at 1, 5 and 8 ºC, stage 4 (start-feeding, see Pittman et al., 1990) was reached at 210 and 175 day degrees for 5 and 8 ºC respectively (Galloway et al., 1999b). In the 1 ºC group most of the larvae developed abnormalities and did not survive throughout the experiment. Herring larvae (*Clupea harengus*) kept under different temperature regimes hatch after 26, 13 and 8 days at 5°C, at 10°C and 15°C respectively (Vieira and Johnston, 1992). Both of the above examples illustrate how temperature speeds up cell cycle time and metabolism in ectotherms and a consequence of this is a shortening of the developmental stages. Different fish species have a defined temperature tolerance, which is normally less in the embryonic stage compared with the juvenile and adult stages. When larvae are exposed to temperatures close to their lethal limits, abnormalities, developmental failure and decline in survival are more frequently observed (Bolla and Holmefjord, 1988; Galloway et al., 1999b). The lower and upper lethal temperature limit for Atlantic halibut larvae are 2 and 9 ºC respectively (Bolla and Holmefjord, 1988; Pittman et al., 1989), but temperatures close to the lower limit (4-5 ºC) produce a higher proportion of normally developed larvae (Pittman et al., 1989; Galloway et al., 1999b). For halibut in the on-growing phase the optimal temperature for growth (based on

gained body mass) was inversely related to size and found to be 14, 10.6 and 5.5 ºC for 10-60 g, 100-500 g and 3-5 kg fish respectively (Björnsson and Tryggvadóttir, 1996). Rise in temperature increases metabolism, shortens the embryonic/larval stage, in addition to the alteration of the volume density of mitochondria in both embryonic muscle types, as documented for herring larvae (*Clupea harengus*) (Vieira and Johnston, 1992) and turbot (*Pleuronectes platessa*) (Brooks and Johnston, 1993). The increased mitochondrial density in herring is probably an acclimatisation to sustain a higher activity level at increased temperatures, which also potentially influences the recovery rate after exercise (Vieira and Johnston, 1992). Fluctuations in temperature during the embryonic/larval stage are known to affect muscle growth later on in the larval/juvenile stage (Stickland et al., 1988; Nathanailides et al., 1995; Johnston and McLay, 1997; Johnston et al., 1998, 2000b, 2000c 2003b; Macqueen et al., 2008).

In 1 d old herring larvae, the total cross-sectional area was found to be similar between larvae reared at 5, 10 and 15 ºC, but with significantly different muscle fibre number (muscle fibre number increased with decreasing temperature) (Vieira and Johnston, 1992). In a similar study, herring eggs were incubated at 5 and 8 ºC and transferred to equal temperature at the point of start feeding (Johnston et al., 1998). The temperature experienced during the embryonic stage was evident on myogenesis throughout the larval stage, where eggs incubated at 5 ºC had a higher fibre number. However, different results were found for cod (*Gadus morhua*) (Hall and Johnston, 2003) and plaice (*Pleuronectes platessa*) (Brooks and Johnston, 1993). Cod eggs reared at 10 ºC had 14% more white fibres at the point of hatching compared to larvae reared at 4 ºC and 7 ºC, whereas numbers of superficial red fibres remained unchanged (Hall and Johnston, 2003). These results suggest that muscle fibre number could be influenced by the water temperature which the embryo is exposed to during a critical stage of early development (Vieira and Johnston, 1992).

Atlantic salmon larvae (*Salmo salar*) reared at 10 ºC had 30% fewer fast fibres at the time of hatching compared to the ambient group (1.6 ºC) (Stickland et al., 1988). The 10 ºC group hatched earlier, had 40% larger fast fibre diameter and the myofibrils occupied more of the fast fibre cross-sectional area, which might be explained by increased embryonic movement due to higher temperature stimulating myofibrillar production (Stickland et al., 1988). Similar results were reported by Johnston and McLay (1997), at hatching of salmon alevins the ambient group had more muscle fibres compared to the heated group. However, at the timing of first feeding the heated group had caught up with the ambient group and the difference in fast fibre number was no longer significant due to better utilization and translation of yolk into muscle. The hypothesis that temperature in the freshwater stage of Atlantic salmon affects hyperplasia and hypertrophy of muscle fibres in the seawater stage (same temperature) was confirmed when eggs/larvae up to smoltification were kept at ambient temperature (1-3 ºC less than heated group at 8.4 ºC) displayed 22.4% more fast fibres at slaughter (3.7-3.9 kg) than the heated group ($9.3x10^5 \pm 2.0x10^4$ vs. $7.6x10^5 \pm 1.5x10^4$) (Johnston et al., 2003b). The density of myogenic progenitor cells, calculated using the *c-met* antibody were also higher in the ambient compared to the heated group, which indicates that the difference in fibre number and hence myogenic progenitor cell content must come from the difference in the freshwater stage. A recent experiment on Atlantic salmon (*Salmo salar*) has brought new light on the effect of temperature during embryogenesis on final muscle fibre number in adult stages (Macqueen et al., 2008). Eggs were incubated at 2, 5, 8 and 10 ºC until eye pigmentation was completed and then exposed to similar temperature. At smoltification the 2 and 5 ºC group were smaller than the higher temperature groups, but the 5 ºC group caught up with the 8 and 10 ºC group during the grow-out phase and was similar in size at harvest (three years later). After three years the difference in temperature regime during embryogenesis showed that the 5 ºC group had 14 and 17% more fibres than the 2 and 10 ºC group respectively. In addition,

the 5 ºC group had more muscle fibres in the size class 0-75 µm and a higher myonuclei density compared to the 10 ºC group.

1.5.2 Photoperiod

Light is considered the second most important environmental factor having an impact on the plasticity of myogenesis of fish. Numerous studies has proven that additional light boosts the growth rate in a variety of fish species including salmon (*Salmo salar*) (Krakenes et al., 1991; Hansen et al., 1992; Johnston et al., 2003c), Nile tilapia (*Oreochromis niloticus*) (Rad et al., 2006), halibut (*Hippoglossus hippoglossus*) (Norberg et al., 2001; Imsland and Jonassen, 2005; Haugen et al., 2006;), red sea bream (*Pagrus major*) (Biswas et al., 2005, 2006) and haddock (*Melanogrammus aeglefinus*) (Davie et al., 2007). Another effect of light is that it is crucial for the timing of sexual maturation through the endocrine system making it possible to postpone the spawning season (Davie et al., 2007; Hansen et al., 1992; Rad et al., 2006). However, the knowledge regarding the underlying mechanisms controlling growth at a cellular level is limited and the genes controlling muscle fibre number are not so far identified. Thus a group of salmon (*Salmo salar*) farmed under identical conditions, displaying the same cellular distribution, was split into two groups in sea-cages subjected to ambient light and continuous light in November (Johnston et al., 2003c). At the first sampling point (~100d later) the continuous light group had a marked increase in muscle fibre number, while the ambient group had not recruited fibres since transfer to the sea-stage. The difference between groups was present throughout the experimental period (23% more fibres in the continuous light group), but with increasing day length during the spring the rate of recruitment was similar between groups. Continuous light treatment had a significant impact on the increase in myogenic progenitor cell (quantified using a *c-met* antibody) and coincided

with myotubes production and/or a shortening in cell cycle time. These responses overshadowed the negative effect of declining temperature on myogenesis during the winter (Johnston et al., 2003c). Thus, no evidence supported that the light treatment effected fibre hypertrophy, possibly indicating that the genetic mechanisms are different from those controlling myotube formation (Johnston et al., 2003c). Similar results were documented in another salmon experiment, showing that light treatment resulted in superior growth and muscle fibre number after transfer to sea-cages compared to the ambient group (Johnston et al., 2004b).

1.5.3 Food

The third factor having significant effect on muscle growth is feed and feeding regime. When the yolk sac stage is ended, exogenous feed is required to support further growth and survival. The literature is limited regarding effect of diet on muscle cellularity, but diet has proven to be of significance for myogenesis in teleosts (Galloway et al., 1999b; Johnston et al., 2002). The effect of high (HP) and low protein content (LP) on growth and muscle fibre structure was investigated in two strains (Lochy and Mowi strain) of Atlantic salmon (Johnston et al., 2002). The average weight between groups did not differ, but the Lochy strain feed HP diet had a 15% greater fibre number compared to the LP diet. On the other hand, the Mowi strain did not show any difference in cellularity nor fibre number between the diets.

The contribution of hyperplasia and hypertrophy was investigated in cod larvae fed rotifers and Artemia nauplii (from day 17) containing a high (1.4) and low (0.2) ratio of DHA and EPA (Galloway et al., 1999a). The high ratio feed gave superior growth rate, and a 8.5 mm larvae fed the low ratio feed had a 25% smaller total cross-sectional area than that of the high ratio group. Difference in growth between groups was due to increased in hyperplastic growth

and the relative contribution was 50 and 41% in the high and low ratio group respectively (Galloway et al., 1999a). In another study, *post* feeding of fasted *Notothenia coriiceps* caused a 60% increase of *c-met* positive cells after 24 and 96 hours. At the same time the number of cells expressing the myogenic transcription factor MyoD increased 20% and 44% respectively (Brodeur et al., 2002). Both *c-met* and MyoD are markers for the activation of myogenic progenitor cell *post* feeding.

1.6 IGF-system in fish, a general perspective

The primary structure of IGF-I and IGF-II was discovered and named by Rinderknecht and Humbel (1978). The IGFs were named "insulin-like" because their structures were very similar to that of insulin (~50% amino acid resemblance) and proinsulin, and they had the ability to stimulate glucose uptake in adipose and muscle cells. Before the structure of IGF-I and IGF-II was revealed, they were referred to as none suppressible insulin-like activity I and II respectively. The growth hormone/insulin-like growth factor axis (GH/IGF-axis) is involved in several biological pathways during ontogeny. In teleosts the insulin-like growth factors (IGF-I and IGF-II) are involved in the regulation of somatic growth (Duan et al., 1995, 1998; Chauvigné et al., 2003; Montserrat et al., 2007a,b), gonad development (Berishvili et al., 2006; Huang et al., 2007; Ma et al., 2007; Taylor et al., 2007), osmoregulation and smoltification in salmonids (Duan et al., 1995; Agustsson et al., 2001; Liebert and Schreck, 2006), and possibly metamorphosis (Hildhal et al., 2007). Both ligands (IGF-I and IGF-II) are synthesized as prepropeptide, but to achieve their mature form a subsequent cleavage of the E-peptide is required. Originally hepatic tissue was believed to be the only site of IGF synthesis, having an endocrine effect on peripheral tissues through the circulatory system via the pituitary/growth hormone (GH) axis. Thus, the effect of GH on IGF-II is controversial. More recently, IGF expression has been documented in several none hepatic tissues which mediate a paracrine/autocrine action on muscle growth in mammals (Jones and Clemmons, 1995) and fish (Vong and Cheng, 2003; Biga et al., 2004). The differential splicing of the IGF-I gene yielding distinct isoforms (splice variants) has been documented in for example salmon (Duguay et al., 1994), but also for other fish species (Tanaka et al., 1998; Schmid et al., 1999) and suggests substantial complexity of the IGF-system. Mammalian studies have demonstrated that different splicing variants shows tissue specificity, e.g. *IGFI-Ea* is

equivalent to the liver version of *IGF-I* while *menchano-growth factor* (*MGF*, also referred to as *IGF-IEb*) are expressed in muscle as a response to mechanical stimuli (Goldspink, 2005), causing increased proliferation of muscle progenitor cells and myoblast migration (Ates et al., 2007; Mills et al., 2007). However, most of *IGF-I* and *IGF-II* mRNA expression studies in teleosts have not distinguished between different splice variants. Thus, a recent study showed that three splicing variants (1a, 1b and 1c) in seabream (*Sparus aurata*) were differently expressed in the larvae and adult stage, indicating a possible role of 1a and 1b in early larval formation while 1c are more important for later stages (Tiago et al., 2008).

At least six IGF binding proteins (IGFBPs) have been identified from mammals, but until recently only four IGFBPs (IGFBP1, 2, 3 and 5) have been characterised in fish (Reinecke et al., 2005). However, IGFBP4 and 6 are now characterised from salmon, in addition to two paralogues of IGFBP-2 and three paralogues of IGFBP5 (Bower et al., unpublished). The role of the IGFBPs are heavily debated and poorly evaluated in fish, but mammalian studies indicate that the IGFBPs prolong the half-life of the ligands, influence IGF delivery and enhancing and/or inhibit IGF effects (see review of Clemmons 2001; Jones and Clemmons 1995). IGFI plasma levels are known to correlate in fish showing seasonal growth pattern (Mingarro et al., 2002) and are both stimulated by day length (*Salmo salar*) (McCormick at al., 2000) and increased temperature (*Oncorhynchus tshawytscha*) (Beckman et al., 1998). In fish, circulating IGF-I level is also known to correlate to high feed intake and good growth (Beckman et al., 2004a; Imsland et al., 2007).

The complexity of the interactions between components, multiple tissue expression, different splicing variants and diverse biological functions make the IGF-system one of the most studied and complex endocrine systems (LeRoit et al., 2001; Wood et al., 2005). Figure 1.10 shows the main constituents of the IGF system and their interaction.

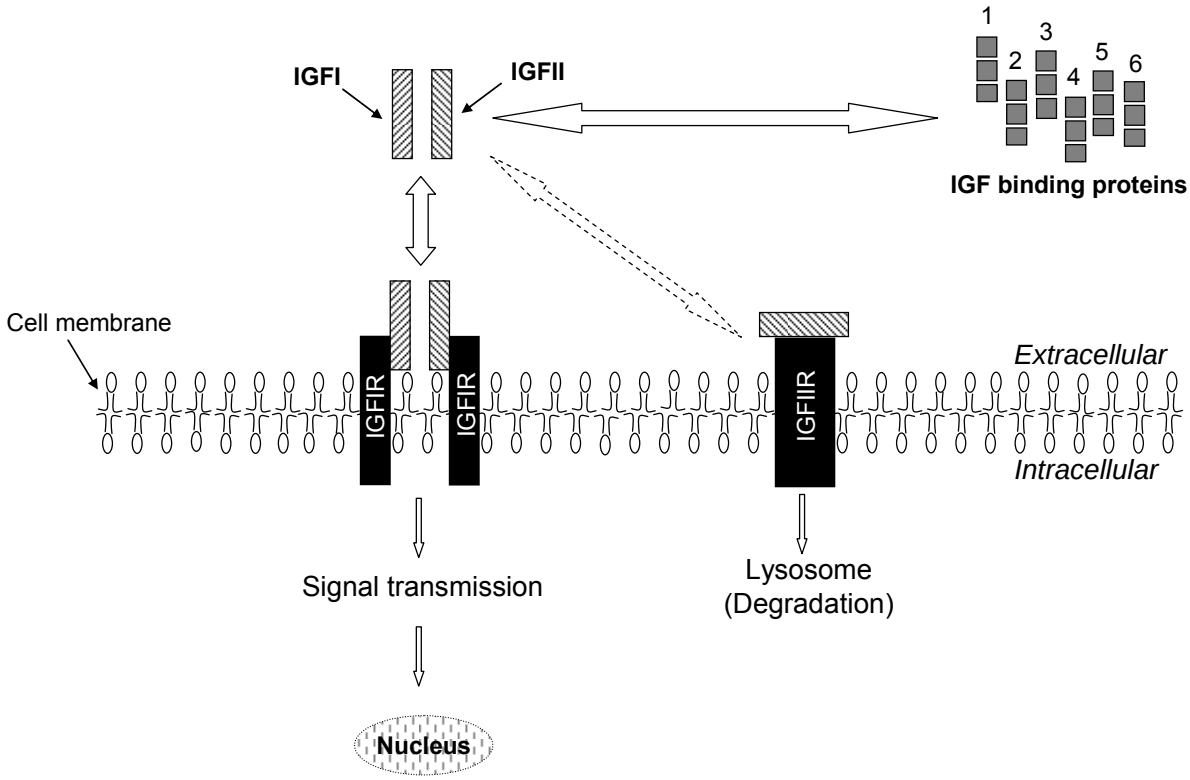

Figure 1.10. Sketch of the IGF system in mammals, illustrating the main constituents and their interaction. Both IGF-I and IGF-II transfer their message through the IGF-I receptor. The IGF-II receptor has a high affinity for IGF.II, but is not considered of importance for the signalling pathway, but rather a control mechanism of IGF-II levels. A total of six IGFBPs (6 binding) have been characterised in mammals and they form complexes with the IGF ligands, functioning as a feed back mechanism in addition to prolonging their bioavailability and half-life. Adapted and modified from Wood et al. (2005).

1.7 Atlantic halibut farming

Farming of salmon (*Salmo salar*) was initiated in Norway in the seventies by a few pioneers and since then salmon farming has been an incredible success story few ever dared dream of, making Norway to the leading salmon producer in the world. The success is due to a number of factors such as the establishment of successful breeding programs, increased research, improved technological designs (netpens, feed distribution units), improved feed recipes and the development of successful vaccines. From 2006 to 2007 salmon (*Salmo salar*) farming had an amazing 21% increase, from 598.000 to 723.000 tons (Boxaspen et al., 2008). In 2006 salmon farming in Norway stood for more than 52% of the total export value from the seafood sector (18.000 mill. NOK, data from the Norwegian Directorate of Fisheries). To underline the economical importance and dimension of both Norwegian fisheries and aquaculture industry, the total export value of both is projected to exceed that of gas in the twenty-first century. It is believed that the fishery industry has reached an upper limit for what is possible to sustainable harvest from wild stocks without the risk of "over-fishing".. This means that further development and increase in volume has to come from better utilization of by-products (fisheries and aquaculture) and increased aquaculture production.

During the last two decades the interest of cultivating other species has increased as a result of fluctuating salmon prices, over production and a genuine belief in other candidate species for aquaculture. Cold water species that have been tried are cod (*Gadus morhua*), wolf fish (*Anarhichas minor*) and Atlantic halibut (*Hippoglossus hippoglossus*). Commercial farming of halibut has been favoured for several reasons, e.g. it is a high priced product, has large final size and a relatively good growth rate compared to other flatfish species (Scott and Scott 1988). Since farming of halibut was first tried in Norway in the eighties the total production volume has increased from two larvae in 1985 (Pittman et al., 1996) to ~1.3 mill larvae and

2000 tons in 2006 (Dahl et al., 2007). The number of farmers has fluctuated during the last two decades, and in 2008 only three companies are still producing halibut in Norway (Boxaspen et al., 2008). However, the companies that are left have increased their production volumes considerably (being more efficient). The main export markets for Norwegian farmed Atlantic halibut are UK (~27 mill NOK), USA (~17 mill NOK), Sweden (~5.2 mill NOK), Netherlands (~3.8 mill NOK) Germany (~2 mill NOK) and Denmark (~0.8 mill NOK) (data from the Norwegian Directorate of Fisheries).

There are several reasons why the expected breakthrough in halibut farming has not yet been achieved. The largest bottlenecks having the most significant impact on the development of the industry are a reliable, sustainable and cost efficient production of high quality larvae. High mortality in early life stages up to metamorphosis has been one of the largest problems limiting rapid expansion of the industry (Pittman, 1996; Olsen et al., 1999; Shields et al., 1999). The high mortality is caused by numerous factors such as poor quality eggs, lack of knowledge regarding broodstock and larval nutrition and larval deformities (e.g. yolk-sac oedema and jaw deformities) (Olsen et al., 1999; Shields et al., 1999). One type of deformity called “gaping”, related to salinity (Lein et al., 1997a) and suboptimal temperatures (Lein et al., 1997b), is a very serious disorder during larval development and is associated with the locking of the jaw cartilage. The “gaping” larvae are not capable of closing the jaw and can therefore not eat, leading to a certain death due to starvation when the yolk is absorbed. Other deformities are also present during larval development and metamorphosis which are not necessarily lethal, but unwanted in commercial farming (Pittman, 1996; Sæle et al., 2004; Lewis and Lall, 2006).

In addition to the problems in the early life stages, halibut in the grow-out phase are facing relatively poor growth due to precocious maturation of males and low temperatures during the winter months (Jákupsstovu and Haug, 1988; Björnsson, 1995; Norberg et al., 2001). The

precocious maturation of males does not only cause a halt in growth, but the flesh quality is also significantly reduced (Hagen et al., 2006; Roth et al., 2007a).

Commercial halibut farmers are also facing technological challenges in respect of netpen design. When not active, halibut like other flatfishes rest on the seabed. In a farming situation this is solved by operating with "steel-frame" facilities (see Fig. 1.11 A), where the netpens are equipped with a flat double-netted bottom in addition to a shelve system alongside the walls of the netpens (see Fig. 1.11 B). Apart from the financial disadvantage of investing in expensive netpen designs, the shelve system also make it difficult to monitor the fish (e.g. watching feeding behaviour and the removal of dead fish).

Compared to the "Norwegian flagship" in aquaculture, the Atlantic salmon, halibut has proven to be challenging to farm with its special needs in respect of biology, live feed (early life stages) and netpen designs. Nevertheless, increased research regarding larval rearing and on-growing during the last decade has helped the industry achieve better results. Light manipulation of Atlantic salmon has been very successful and is used as a tool to speed up growth and delay maturation (Hansen et al., 1992; Krakenes et al., 1991). However, light manipulated Atlantic halibut has not responded in the same way, and the success has been limited (Aune et al., 1997; Jonassen et al., 2000; Norberg et al., 2001; Imsland and Jonassen 2005). Since the research activities concerning Atlantic halibut have mostly focused on early life stages, feed, feeding regimes and growth optimisation in the grow-out phase are all areas of potential improvement. The need for more knowledge is crucial for the future success and survival of the halibut industry in Norway (and the rest of the world), and is to be considered a national research priority rather than just being the Atlantic halibut farmer's burden.

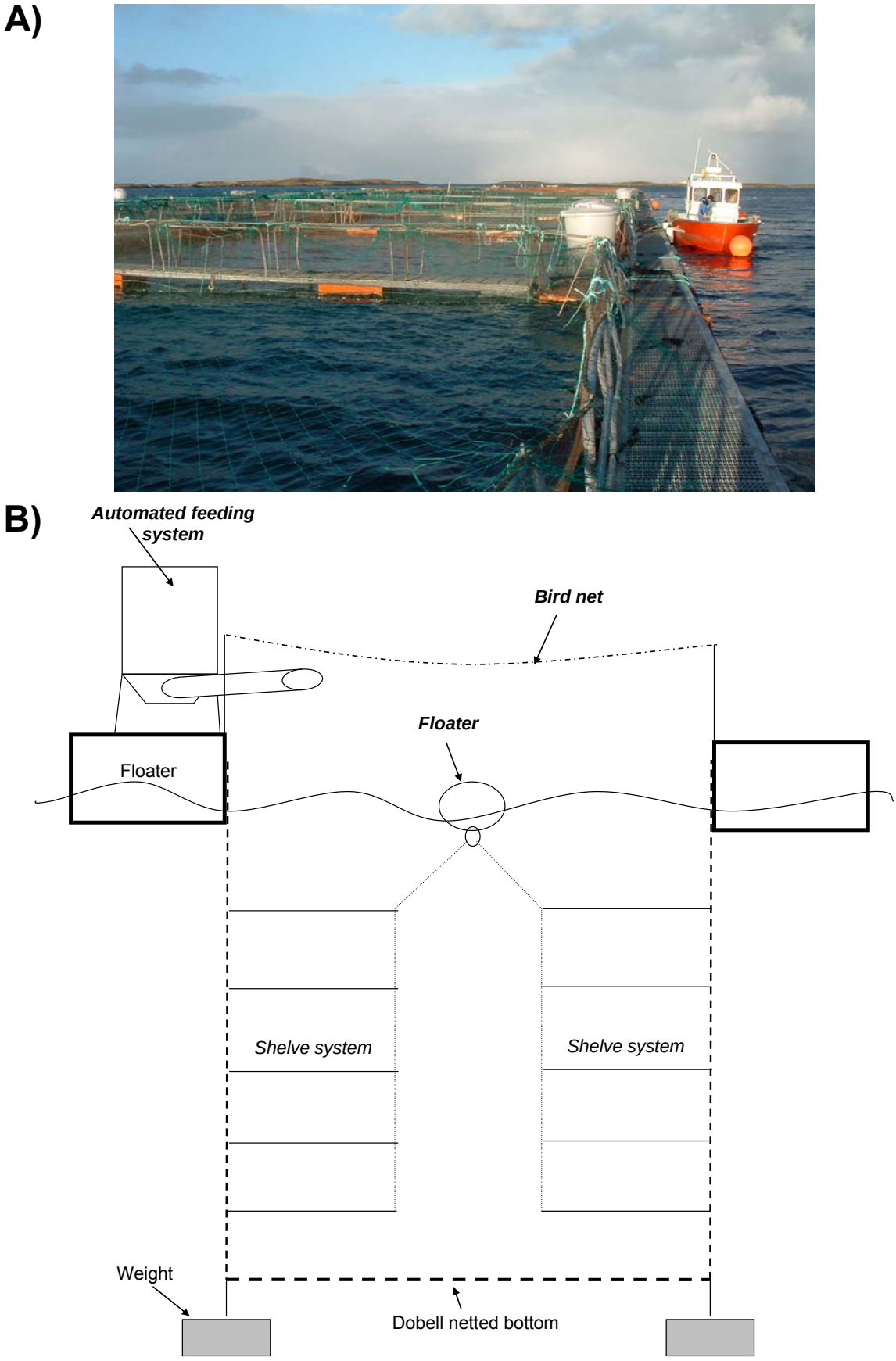

Figure 1.11. Picture (A) and an illustration (B) of a commercial Atlantic halibut farm (Aga Marin, Helgeland, Norway).

1.8 General parameters influencing flesh quality in fish

There are several definitions of flesh quality, but one definition frequently referred to is: "A *products ability to satisfy the consumer expectations and requirements*". However, what is quality and what defines it? One interesting aspect of quality is that the preferences differ between people and nations, and these differences are often linked to traditions. In other words, a fish can have poor or good quality at the same time depending on the tradition and nation of the consumer.

Once fish quality is spoiled, it can not be adjusted or fixed. Harvesting and storage conditions are known to affect the flesh quality (Hultin 1985), so proper handling routines will at least make sure that the quality of the fish caught in the fisheries is maintained. In aquaculture the picture is quite different. A good farmer should have superior control of the farming conditions, at least in respect of feeding, feeding routines, light conditions, biomass and the time of harvest. This should favour aquaculture bred species in respect of flesh quality and make reared fish superior to that of wild caught fish. However, to be able to do so it is necessary to understand what factors are able to affect the flesh quality, and how they can be used as tools to ensure a high quality product (e.g. harvesting fish at the right time). Some of the most important factors affecting flesh quality and the consumer acceptability of a product are chemical composition, colour, water holding capacity, proteolytic degradation and texture (see below).

1.8.1 Chemical composition

The chemical composition varies considerable between species, fishing ground, sex and season (Love, 1988). Based on the fat content, fish can be divided into four classes, lean (<2%), low fat (2-4%), medium fat (4-8%) and high fat (>8%) fish species (Ackmann, 1990). Fat is either evenly distributed in the muscle, deposited in organs (especially in the liver and viscera) or both (Love, 1988; Ackmann, 1990; Lynnum, 1996). Wild Atlantic halibut are reported to contain up to 3% fat (Haug, 1990), placing halibut in the low fat fish class (Ackmann, 1990). However, the farmed counterpart are reported to have as much as ~7.5% (Olsson et al., 2003a), and this would place farmed halibut amongst the medium fatty fishes. Most of the fat in halibut is located in fat deposits as notches at the fin basis (Fig. 1.5 A), under the skin and some is stored in the muscle. The protein content in most fish species is relatively stable, varying from 16 – 20%, while the fat fraction varies from 0.3% in cod to 25% in herring (Love, 1988; Lynum, 1996). However, deep sea fishes have shown an extraordinary adaptation to the hydrostatic pressure in which the water content of the body tissues increases with depth, while the fat and protein content decreases (Childress and Nygaard, 1973). Fat influences flesh quality in two ways, first it significantly influences the flavour of the fish (Skonberg et al., 1993; Waagbø et al., 1993; Einen and Skrede, 1998) and secondly the flesh texture (Dunajski, 1979; Andersen et al., 1994, 1997), although the latter is debated (Bjørnevik et al., 2004; Young et al., 2005). See section 1.9 for more details about texture.

1.8.2 Colour

Due to today's strict food safety regulations, fish fillets/steaks are often vacuum-packed and/or frozen. In the cases where the fish is freshly stored on ice, it is normally out of reach behind a glass window at the fishmonger's. This strictly limits the consumer's opportunities to evaluate the product and decisions are often only based on the visual appearance of the product. In fact, as much as 40% of consumers make a decision based on colour only (Rasekh et al., 1970). Colour is therefore regarded as one of the most important quality parameter in fish and can be evaluated in several different ways. Colour assessments in salmonids are often preformed using a "salmo fan", a subjective method where the colour of the flesh is visually compared to the "salmo fan" which contains cards labelled with numbers increasing with the redness of the card. This method is quick and easy to perform, but is not very objective and the results are influenced by both the light conditions and human errors. If this is the method of choice, it is important that the colour assessment is done under identical light conditions (stable) and by the same person to limit the human errors. A more objective method, often preferred for research purposes, is the use of the Minolta which determines the colour after the well known L* a* b* system, representing the whiteness, redness and yellowness of the flesh respectively. The red colour of salmonid flesh comes from carotenoids, especially astaxanthin added through the diet. In the flesh astaxanthin is attached to the muscle fibres (Choubert et al., 1994), and high fibre density is known to give a brighter red colour of the salmon (*Salmo salar*) flesh (Johnson et al., 2000d). Several other factors are also known to affect the colourization of fish flesh *pre-* and *post-* harvests, such as bioavailability (Torrisen et al., 1981), feed lipid content (Einen and Roem, 1997) and freeze storage (Ingemansson et al., 1993). Atlantic halibut does not accumulate astaxanthin in the flesh, but a clear white flesh would be associated with a product of good quality.

1.8.3 Water holding capacity

The term "water holding capacity" (WHC) of the fish muscle refers to the muscle's ability to retain its water despite the application of a force (Van Laack et al., 1994). There are several reasons why WHC is important, but the two most important are financial and consumers acceptability. Poor WHC can cause a significant liquid loss, especially during processing and storage, leading to less biomass and profit. For example, cod (*Gadus morhua*) caught in August and stored for 7 days on ice are reported to have a liquid loss of ~26% (Olsson et al., 2007). From a quality point of view poor WHC is not desirable because the water content and its distribution in the muscle highly influence the sensory properties (e.g. juiciness). pH below or above the isoelectric point (net charge of the proteins are zero) will cause a swelling of myofibres and an increased WHC (Morrissey et al., 1987). Other factors affecting WHC are heating (Ofstad et al., 1993, 1995), salt additives (Ofstad et al., 1995), *post-mortem* muscle structure alteration (Olsson et al., 2003b) and bacterial contamination (Olsson et al., 2003c, 2007). The myofibrillar proteins are the primary source of water binding in the muscle (especially myosin and actin), and the water is held within the muscle by capillary forces and the unequal distribution of positive and negative electrical charges (hydrogen-bounding and hydrophobic attractions) (Hamm, 1986; Wismer-Pedersen, 1987). According to Hamm (1972), myofibrillar, non-protein components of the sarcoplasma and sarcomplasmic proteins contributes to WHC by 50, 47 and 3% respectively.

Several techniques are utilised to evaluate the WHC of fish flesh, but one of the most frequent methods used is a technique which involves centrifugation, described by Ofstad et al. (1993).

In respect of weight gain, protein deposition is favoured to that of fat deposition. One gram of fat deposition in salmon (*Salmo salar*) leads to a one gram weight gain, while one gram of protein deposition will lead to ~five gram of total weight gain, due to the binding of three to

four grams of water (Kryvi and Totland, 1997). Wismer-Pedersen (1987) arranged the water binding to proteins in muscle into three distinct regions around a protein molecule, 1) primary hydration shell (bound water), 2) secondary hydration shell (loosely bound water) and 3) undisturbed water (free water) (Fig. 1.12). In meat, the primary and secondary hydration shell contains between 20-27% of the total water.

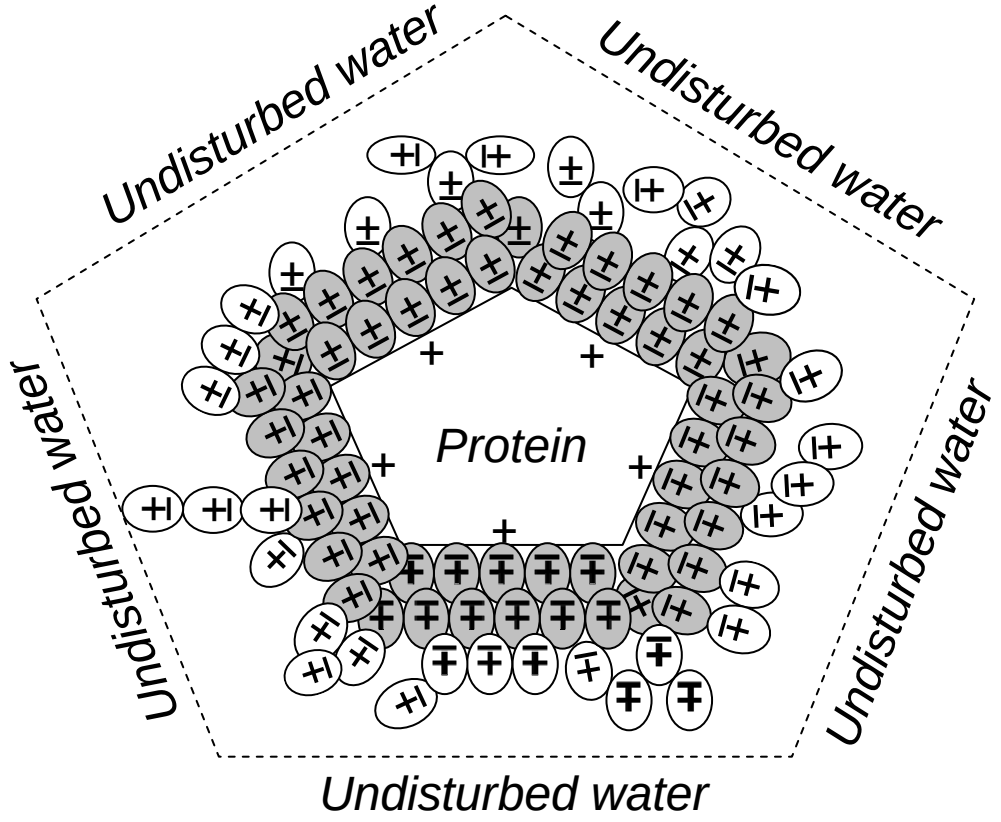

Figure 1.12. Illustration of the three different water compartments surrounding a protein molecule. Grey water molecules represent the primary hydration shell (bound water), white water molecules represent the secondary hydration shell which consists of more random bound water molecules (loosely bound water). The second hydration shell merges into the last region of unperturbed water molecules (outside the dotted line, free water). Adapted, modified and re-drawn from Wismer-Pedersen (1987).

1.8.4 Texture

Texture is one of the most crucial quality parameters for producers, processors and consumers since several fish species do not have their own characteristic flavour (Hyldig and Nielsen, 2001). The most frequent applied methods to evaluate texture are either by instrumental or sensory assessment (test panels). The disadvantage with the instrumental method is that varying results are obtained due to the heterogeneity of the fish muscle and sample location. Other variables affecting texture measurements are different instrumental settings, fluctuating temperature and sample size (Hyldig and Nielsen, 2001). On the other hand, the advantage with the instrumental method is that it is quick, cheap, easy to perform and has high throughput.

By utilizing a taste panel several variables can be assayed together, but the taste panels has to be highly trained which is time consuming, expensive and only a limited number of fish can be assayed (Hyldig and Nielsen, 2001). A number of different biological, environmental and dietary factors are reported to influence texture including season (Espe et al., 2004; Roth et al., 2005), exercise (Bugeon et al., 2003), stress (Sigholt et al., 1997; Stien et al., 2005) chemical composition (Ackman, 1990; Andresen et al., 1997; Nortvedt and Tuene, 1998), size (Fauconneau et al., 1995), slaughtering method and storing temperature and time (Andresen et al., 1997; Sigholt et al., 1997; Skjervold et al., 2001; Roth et al., 2007b). In addition, structural factors such as muscle fibre diameter distribution (Hatae et al., 1990; Hurling et al., 1996; Johnston et al., 2000d; Periago et al., 2005), collagen (Sato et al., 1986; Li et al., 2005; Johnston et al., 2006a) and collagen cross-links (Li et al., 2005; Johnston et al., 2006a) also influences texture. The proteoglycans impact on flesh texture is vague, but could also in theory have some influence (Ofstad et al., 2006). A serious problem related to texture and the appearance of the fillet is a phenomena called “gaping”. Gaping can be seen as splits and tears

in a fillet where the myocommata fails to hold together the myotomal muscle (Love 1988), which is affected by handling, storage temperature and nutritional value (Lavéty et al., 1988; Love 1988; Robb et al., 2000). The knowledge of the mechanisms behind the phenomena are limited, but gaping is believed to be caused by a degradation of the myotendious junctions (the connection between ECM and the myofibres) (Fletcher et al., 1997; Espe et al., 2004; Ofstad et al., 2006) caused by enzymatic degradation during storage (Ando et al., 1991). Johnston et al. (2002) showed that muscle fibre density has a significant influence on the presence of gaping. It was concluded that in salmon (*Salmo salar*) having a fibre density of > 95 fibres/mm, little or no gaping was present.

In recent years the interest of cod (*Gadus morhua*) farming especially in Norway, Iceland, Scotland, Faroe Island and Canada has increased considerably (Björnsson et al., 2007). However, earlier research on cod has shown that farmed cod are more subjected to softer texture and gaping than their wild counterparts (Rustad, 1992; Ofstad et al., 1996a), representing a serious challenge in the cod farming industry. Textural problems such as gaping are not documented in farming of Atlantic halibut. A texture evaluation of Atlantic halibut using a sensory method revealed that a diet with high lipid content gave a juicier texture, but no differences were found in fattiness, hardness or roughness (Nortvedt and Tuene, 1998). Texture evaluation in other species have shown contradictory results in respect of fat vs. firmness. For example, in salmon (*Salmo salar*) it was found that the texture of the flesh significantly decreased with increasing fat content (Andresen et al., 1994, 1997), which is confirmed by Dunajski (1979). In contrast, fat content of diploid and triploid salmon did not influence flesh texture (Bjørnevik et al., 2004), similar to that reported by Nortvedt and Tune (1998) and Young and co workers (2005).

1.8.5 Proteolytic enzymes

It is widely accepted that *post-mortem* degradation and muscle softening is caused by the action of endogenous enzymes. This is a biochemical process known as autolysis when the enzymes are no longer capable to distinguish between the organisms own tissue and other organic material. The activity of enzymes in fish is less studied than that of mammals, but in the last two decades the number of manuscripts studying the effect enzymes *post-mortem* has increased. In contrast to mammals, fish muscle undergoes a rapid softening which can make it unappealing to consumers (Ashie and Simpson, 1997; Carpo et al., 1999). Z-band disruption is suggested to be a likely candidate to muscle tenderisation (Hultin, 1984; Hatae et al., 1985; Seki and Tsuchiya, 1991; Tachibana et al., 1993; Taylor et al., 1995), but it is not fully understood to what extent Z-band disruption impacts *post-mortem* softening. In contrast, no obvious breakdown of the Z-disc was found for rainbow trout (*Oncorhynchus mykiss*) (Ando et al., 1991, 1995), sardine (*Sardinops melanosticta*) or tiger puffer (*Fugu rubripes*) (Sato et al., 1997) and it was concluded that *post-mortem* tenderisation during chill storage was more likely due to disintegration of collagen fibres of the pericellular connective tissue. The impact of ECM breakdown to *post-mortem* softening has been suggested by several authors (Bremner and Hallet, 1985; Hallet and Bremner, 1988; Sato et al., 1991, 1997; Ando et al., 1991, 1993, 1995). Compared to meat, the I-band of the myofibrils in fish seems to be more stable against degradation (Taylor et al., 2002).

A total of five classes of enzymes are known to impact muscle degradation and softening. These are the calpains, the lysosomal cathepsins, the proteasome complex, caspases, serine peptidases and the matrix metalloproteases, including collagenase (Sentandreu et al., 2002). In this thesis cathepsins and collagenase have been studied and are considered in more detail below.

1.8.5.1 Lysosomal cathepsins

The cathepsins belong to a group of enzymes that are associated with the lysosomes and consist of different members of the serine, cysteine and aspartic peptidase families. So far 13 different cathepsins are known to be associated with the lysosomes, but only 6 cathepsins have been found to exist within the muscle (cathepsin A, B, C, D, H and L) (Goll et al., 1983). The cathepsins have an acidic pH optimum and are synthesised as proenzymes (Turk et al., 1999; 2000). To achieve its final mature and active form the N-terminal propeptide has to be degraded by peptidases or by proteolytic removal (Turk et al., 2000).

In relation to fish muscle degradation and softening cathepsins have without doubt been the most studied group of enzymes, especially cathepsin B, D and L (Tokiwa and Matsumiya, 1969; Konagaya, 1985; Yamashita and Konagaya, 1990a,b, 1991, 1992; Aoki and Ueno 1997; Aoki et al., 2002; Ladrat et al., 2003; Delbarre-Ladrat et al., 2004; Hultmann and Rustad 2004; Sovik and Rustad 2006; Chéret et al., 2007). One of the main reasons why cathepsin D and L has received more attention compared to the rest of the cathepsins, is that they are believed to be of major importance in autolytic degradation due to their capability of sustaining activity over a wide pH range (Delbarre-Ladrat et al., 2006). The distribution and activity of cathepsins were examined in slow and fast muscle of 24 different fresh and salt water fish species, and it was concluded that cathepsin B, L, D and H were the major cathepsins of the fish lysosomes (Aoki et al., 2000), in agreement with a more recent study (Chéret et al., 2007). Compared to the other major cathepsins, cathepsin H is believed to have a minor role in *post-mortem* degradation of fish and bovine muscle (Chéret et al., 2007).

The cathepsins are capable of degrading a wide range of proteins such as structural myofibrillar components (e.g. actin, myosin, troponin, tropomyosin, desmin), collagen and possibly proteoglycans of the ECM (Burleigh et al., 1974; Etherington, 1974; Schwartz and

Bird, 1977; Matsukura et al., 1981, 1984; Noda et al., 1981; Okitani et al., 1980; Kim and Haard, 1992; Sato et al., 1997; Ladrat et al., 2003; Baron et al., 2004).

In Chum salmon (*Oncorhynchus keta*) the activity of cathepsin L has been suggested to be the major contributor to increased muscle softening during spawning migration, making the muscle unappealing for consumption (Konagaya, 1985, Yamashita and Konagaya, 1990a,b). Cathepsin activity in fast muscle of matured Chum salmon was found to be several folds higher than that of immature Chum salmon (Yamashita and Konagaya, 1992). Thus, cathepsin activity of slow muscle was similar in mature and immature fish (Yamashita and Konagaya, 1990b), which is in agreement with results from sockeye salmon (*Oncorhynchus nerka*) (Mommsen et al., 1980), suggesting a spearing of slow muscle. Yamashita and Konagaya, (1992) estimated that cathepsin L activity of fast muscle alone contributed to 80% of the total autolysis during spawning, while the remaining 20% was due to cathepsin D and E. Evidence for higher cathepsin activity during the spawning period are also reported from sardine (*Sardina pilchardus*) (Gómez-Guillén and Batista, 1997) and ayu (*Plecoglossus altivelis*) (Yamashita et al., 1990). In contrast to this information in salmon, sardine slow muscle had in average 2-3 fold higher cathepsin D activity of fast muscle except during the spawning period (April and August) when the activity levels peaked, being 4-5 times higher in slow than fast muscle (Gómez-Guillén and Batista, 1997).

Even though the function of the cathepsins has been addressed during the recent years, their impact on *post-mortem* muscle degradation is still not understood (Delbarre-Ladrat et al., 2006)

1.8.5.2 Matrix metalloproteases

Matrix Metalloproteinases (MMPs) are a large family consisting of at last 26 calcium-dependent zinc-containing metalloendopeptidases members, divided into 5 distinct classes (collagenases, gelatinases, stromelysins, matrilysins and membrane type MMPs). The MMPs are under the control of both endogenous and tissue MMP inhibitors (Verma and Hansch 2007). The MMPs are responsible for much of the degradation and turnover of the constituents of the ECM, including collagens, matrix glycoproteins, elastins and proteoglycan, and are secreted from different cells including fibroblasts, osteoblasts, macrophages, endothelial cells, lymphocytes and neutrophils (Verma and Hansch, 2007). The MMPs are secreted as zymogenes which require the action of other enzymes to achieve its active form. Compared to the cathepsins, the MMPs are a less studied group of enzymes, but are a possible candidate for *post-mortem* muscle softening in fish (Kubota et al., 2001, 2003). By comparing the control group (not treated) with a group that had MMPs inhibitors injected into the bloodstream , the texture evaluated at the time of sacrifice and 6 hours *post-mortem* indicated that tenderization was significantly suppressed in the MMPs inhibitor treated group (Kubota et al., 2001). In general, the triple helix of collagen is attacked by specific collagenases which in turn open the structure for other proteases, leading to a further disintegration of the macro molecules (Kristjansson et al., 1995). Collagenases, a member of the MMPs, are good candidates which might be involved in the disintegration of ECM, leading to textural changes and possibly gaping (Ando et al., 1995; Bremner and Hallett, 1985). For example, in blue sardine (*Macruronus novaezelandiae*) during chilled storage the endomysium were degraded and the muscle fibres were detached from the myocommata (Bremner and Hallet, 1985, Hallet and Bremner, 1988). In cod, collagenase activity was found to increase with storage time on ice, having the highest activity level at the termination of the experiment (9 days on ice)

(Heranández-Herrero et al., 2003). It was concluded that endogenous collagenases and other proteases were responsible for the disintegration of collagenous fibrils in the fish muscle.

1.10 Project aims

Due to commercial problems in the Atlantic halibut larval rearing, survival and production most of the previous research has been directed towards the initial stages of production. Little attention has been directed towards the on-growing phase of juvenile and adult fish and to examine the flesh quality of this species. As a consequence of this there is little knowledge about the growth dynamics and flesh quality traits of the Atlantic halibut. The few growth and flesh quality studies that have been carried out throughout the last years are all small scale laboratory experiments and no commercial scale trials has been preformed.

The main objective of this project was to provide the background information on normal muscle growth in farmed Atlantic halibut needed to develop strategies for optimizing product quality, particularly in relation to the season of harvesting. The sub-goals in the project are listed below:

1. Provide an accurate method to determine muscle fibre numbers in a large dorsal-ventral fish, the Atlantic halibut.

2. Investigate muscle fibre recruitment (number, size distribution and density) and growth through a one year commercial production cycle.

3. Study the development of the collagen compartment and concentration of the connective tissue matrix, including the amount of hydroxylysyl pyridinoline cross-links during a one year commercial production cycle.

4. Investigate the proteolytic enzyme activities of cathepsin B, D, L, H and collagenase over a one year commercial production cycle.

5. To determine flesh quality parameters including texture, water holding capacity, pH, and chemical composition over a one year production cycle and to explore its relationship to normal muscle growth.

6. Study the gene expression of some muscle regulatory genes during fasting and re-feeding in Atlantic halibut.

Chapter 2

Sexual dimorphism of muscle growth in Atlantic halibut (*Hippoglossus hippoglossus*) results in a lower final fibre number in males.

2.1 Abstract

A sampling method is described to accurately determine the number of fast myotomal muscle fibres (FN) in a large flatfish species, the Atlantic halibut (*Hippoglossus hippoglossus*). An unusual feature of the fast myotomal muscle is the presence of internalised strips of slow muscle fibres. In fish of 1.5 to 3.5 kg (n=24), the total cross-sectional area (TCA) of fast muscle was 18% greater in the dorsal than ventral myotomal compartments ($P<0.05$), whereas there was no significant difference between left-hand and right-hand sides of the body. Due to the bilateral asymmetry, muscle blocks (5x5x5 mm) were prepared to systematically sample each myotomal quadrant (dorsal, ventral, left-side, right-side) and the diameters of 150 fast fibres measured per block. Smooth nonparametric probability functions were fitted to a minimum of 800 measurements of fibre diameter per quadrant (n=5). There were no significant differences in the distribution of muscle fibre diameters between myotomal compartments and therefore fibre number (FN) could be estimated from a single quadrant. The number of blocks required to estimate FN with a repeatability of ± 2.5% increased from 6 at 300 g body mass (Mb) to 17 at 96.5 kg, caused by variation within and between blocks. Gompertz curves were fitted to measurements of fibre number and fork length (cm). The estimated final fibre number was 8.96×10^5 ($7.99\text{-}9.94 \times 10^5$, 95% confidence intervals) for males and 1.73×10^6 ($1.56\text{-}1.90 \times 10^6$, 95% confidence intervals) for female fish. The estimated fork length for cessation of fibre recruitment in the fast muscle of female fish (177.5 cm) was almost twice that in males (81.0 cm), reflecting their greater ultimate body size.

2.2 Introduction

Three main skeletal muscle types are usually found in the trunk musculature of adult teleosts, classified according to colour/contraction speed as: red/slow, pink/intermediate and white/fast (Johnston et al., 1977; Bone, 1978). Each fibre type expresses distinct isoforms of myosin heavy chain that are preceded by developmental-stage specific isoforms during the early stages of growth (Rowlerson et al., 1985; Scapolo et al., 1988; Crockford and Johnston, 1993; Ennion et al., 1995). The axial musculature in yolk-sac halibut larvae consists of a superficial layer of embryonic slow fibres and an inner core of fast muscle fibres (Galloway et al., 1999b) in common with other teleost species (Van Raamsdonk et al., 1978; El-Fiky et al., 1987; Stickland et al., 1988; Vieira and Johnston, 1992; Galloway et al., 1998). Growth of the fast muscle involves the hypertrophy of the embryonic fibres until around 150 degree days (10 mm LS) after which additional myotubes are formed (Galloway et al., 1999b). Initially, fast muscle fibres are added from discrete germinal zones at the extremities of the myotomal cones (Galloway et al., 1999b), a growth phase known as stratified hyperplasia (Rowlerson and Veggetti, 2001). Later, new fibres are formed on the surface of fibres formed earlier in ontogeny, producing a mosaic of fibre diameters (mosaic hyperplasia) (Weatherley et al., 1979; Carpené and Veggetti, 1981; Stickland, 1983; Johnston et al., 2003a). However, little quantitative data exists on the growth of myotomal muscle in juvenile and adult halibut (Hagen et al., 2006; Haugen et al., 2006).

Atlantic halibut (*Hippoglossus hippoglossus*) is the largest of all flatfish species in the Atlantic Ocean and females of 300 kg have been recorded, whereas males seldom exceed 50 kg (Moen and Svensen, 1999). In aquaculture halibut are rarely farmed to more than 4-6 kg. Metamorphosis in flatfishes is different and more extensive than in other teleosts species. Flatfish go through a morphological transformation from being larvae to becoming a sexually

immature juvenile which has most of the adult phenotypic characteristics. In Atlantic halibut metamorphosis takes place at 10-30 mm total length (Osse and Van den Boogaart, 1997) and results in morphological changes in most organs (Sæle et al., 2004). The two most pronounced phenotypic changes during metamorphosis are the migration of the eye to the right hand side (eye migration to the left occasionally happens) and the transformation from a pelagic bilaterally symmetrical larva to an asymmetric benthic juvenile. These morphological changes are under both endocrine and genetic control (Yamano et al., 1991; Power et al., 2001; Bao et al., 2005; Tagawa and Aritaki, 2005).

The determination of fibre number for a species is important, since flesh quality traits such as texture, are related to the number and diameters of muscle fibres (Hurling et al., 1996; Johnston et al., 2000d). These traits are of interest to potential halibut breeding programs since the final number of fast muscle fibres (FN_{final}) for other aquaculture species, such as Atlantic salmon, is known to vary between families and populations (Johnston et al., 2000a), has a moderate heritability (Vieira et al., 2007) and correlates with growth rate (Johnston et al., 2003b). For a large flatfish species, such as the Atlantic halibut, the large number of muscle fibres present, and potential differences in the distribution of fibre diameters between dorsal and ventral and left and right sides of the trunk complicate quantitative studies of muscle growth. The aim of this project was to provide information on muscle fibre growth patterns in the fast myotomal muscle of Atlantic halibut (*Hippoglossus hippoglossus*) using morphometric and immunohistochemical methods. Since no previous study has attempted to estimate FN_{final} in such a large flatfish species, the sampling methods needed to accurately estimate fibre number were investigated.

2.3 Material and methods

2.3.1 Fish

A total of 47 Atlantic halibut (*Hippoglossus hippoglossus*) were used to study muscle fibre recruitment patterns including 40 farmed fish (2 g–13 kg, 1993-2006 generations), three broodstock fish (80, 96.5 and 97.5 kg) all provided from Mørkvedbukta Research Station (Bodø University College, Norway) and four wild fish between 6-50 kg (caught in Saltenfjorden outside Bodø in Norway, by local fishermen). The broodstock were originally wild caught fish that had been held in seawater tanks at ambient temperature and photoperiod since 1990. The fish in the 2 g - 13 kg group had been fed commercially produced feed (Skretting, Norway) since weening, while the broodstock fish had been provided wet feed produced from herring at the research station (extra vitamins and minerals added). In addition, 24 commercially farmed halibuts (1.5-3.5 kg) from Aga Marin AS (Dønna, Norway) were used to investigate any potential differences in fast muscle cross-sectional area between, dorsal, ventral, left-hand and right-hand myotomal compartments. All the fish included in the study had normal eye migration (to the right-hand side). Fish were killed with a sharp blow to the head, except for the broodstock halibut which were killed with an overdose of anaesthetic (MS-222, Argent Chemical Laboratories, Washington, USA). Twenty seven of the halibut for the morphometric study (including the broodstock fish) and the fish from Aga Marin AS were sampled and analysed at Bodø University College. The remaining fish were transported in polystyrene boxes on ice to the Gatty Marine Laboratory (University of St Andrews, Scotland) and sampled *post-rigor* three days after sacrifice.

2.3.2 Sample preparation

A myotomal steak of 0.5 cm thickness was prepared at 0.55 L_F (fork length) using a large sharp knife (Fig. 2.1 A). A photograph was taken of all steaks containing a scale bar, making scaling of the picture possible when calculating the total cross-sectional area of fast muscle (TCA) using SigmaScan Pro (v. 5.0, Systat Software, Inc., Point Richmond, USA). From 1 to 60 blocks (5x5x5 mm), dependent upon the Mb of the fish, were prepared from the fast muscle on one or both sides of the body in order to systematically sample all areas of the myotome containing fast muscle fibres. Blocks were mounted on small pieces of cork sheets (1x1 cm) and oriented so the muscle fibres would be cut transverse to their longitudinal axis. All blocks were covered in Cryomaterix (Anatomical pathology/Bergmann AS, Oslo, Norway), and frozen for 45 s in 2-methyl butane (isopentane) cooled to near its freezing point (-159 ºC) in liquid nitrogen. *Post*-frozen blocks were wrapped in *pre*-labelled tinfoil and stored in a liquid nitrogen container. Blocks were acclimated to -18 ºC for ~1 hour and 7 µm sections were cut using a cryostat (Microm HM 550, MICROM International/Bergmann AS, Oslo, Norway). Serial sections were mounted on poly-L-lysine-coated slides and air dried, to prevent sections coming off the slides during washing and/or staining procedures.

2.3.3 Histochemistry and Immunohistochemistry

Serial transverse sections were stained for succinic dehydrogenase (SDHase) a marker of mitochondria and aerobic capacity (Nachlas et al., 1957), used to separate slow from fast muscle (Johnston 1977). 50 mM phosphate buffer/80 mM sodium succinate stock solution were prepared and the final pH was adjusted to 7.6. The slides was placed horizontally in a

humidity tray (plastic box with lid containing wet paper under a rack), inscribed with a circle using an immedge pen (Vector laboratories, Burlingame, UK), covered with the working solution (~500 µl) containing 2 mg/ml nitroblue tetrazolium and incubated in room temperature for 1 hour in the dark. Sections were rinsed in water and mounted using glycerol gelatin (Sigma, Oslo, Norway).

Sections were also stained using the *S58* antibody against chicken slow muscle myosin (obtained from the Developmental Studies Hybridoma Bank, University of Iowa, USA) (Crow and Stockdale, 1986) that has been shown to identify slow muscle fibres in a number of fish species (Devoto et al., 1996; Johnston et al., 2003a; 2004). Briefly, sections were fixed in acetone for 10 min and air dried (10 min). Subsequently, sections were re-hydrated in 5% normal goat serum (v/v), 1% Triton X-100 (v/v), 1.5% BSA in PBS (w/v) for 30 min to block non-specific binding sites, followed by a 3 x wash in PBS (3 min) before overnight incubation (4 ^{o}C) in 1:10 dilution of the *S58* antibody (v/v) in 1% Triton X100 (v/v), 1.5% BSA in PBS (w/v). Sections were washed 3x3 min in PBS and incubated in 1:20 anti-mouse IgA-biotin conjugated (v/v) secondary antibody (Sigma, St Louis, USA) for 1 h at room temperature. Following 3x3 min washes in PBS sections were incubated in 1:20 ExtrAvidin-Peroxidase (v/v) for 30 min, washed 3x3 min in PBS and developed with 3-amino-9-ethylcarbazole (Sigma) which produces an insoluble red end product. The reaction was stopped by washing in distilled water and the slides mounted using glycerol gelatine (Sigma).

The third method used to separate slow and fast muscle was myosin ATPase staining (mATPase, Guth and Samaha 1970, modified by Johnston et al., 1974). This method needs optimization regarding the pH of the *pre*-incubation buffers (alkaline or acidic) for different species. Sections were incubated in both acidic and alkaline *pre*-incubation buffer with different pH and for Atlantic halibut an alkaline *pre*-incubation of pH 10.2 gave the best differentiation of fibre types. Briefly, the slides were placed in a staining jar and incubated for

30 s in the alkaline *pre*-incubation buffer consisting of 10 mM Alkaline buffer (Sigma, Oslo, Norway) and 20 mM calcium chloride (Sigma, Oslo, Norway) (pH adjusted to 10.2). The slides were washed 2x1 minutes in distilled water and then incubated in an ATP incubation buffer (freshly made before use) consisting of 0.2 M Tris-Cl, 18 mM calcium chloride and 2 mM ATP (Roche, Oslo, Norway) (pH adjusted to 9.5) for 25 minutes with stirring. Slides were then washed for 3x3 minutes in 1% (w/v) calcium chloride solution. *Post*-washing, slides were incubated in a 1% (w/v) chloride cobalt solution for 10 minutes, followed by 5x1 minute washes in distilled water. Slides were then developed in a 2% (v/v) ammonium polysulfide solution for 30 s in a fume hood, followed by 5x1 minute washes in distilled water. The reacting tissue had a darker color than the surrounding tissue. Slides were mounted using glycerol gelatin.

2.3.4 Morphometric studies

Only regions of the myotome composed entirely of fast muscle fibres were sampled. For fish ≥ 1 kg it was possible to distinguish the internal strips of slow muscle from the digital photograph of the entire myotomal cross-section (Fig. 2.1 B). All sections were counterstained with Harris' hematoxylin (Sigma, Oslo, Norway) for ~8 minutes. The hematoxylin was then decanted and the sections washed in running tapwater for 10 minutes prior to mounting using glycerol gelatine.

The outlines of ~150 muscle fibres per block were digitized using an Image Analysis System (Axioskop 2 mot plus, Carl Zeiss, Oberkochen, Germany) with Axiovision 4.2 software, (Carl Zeiss, Oberkochen, Germany) and the average muscle fibre cross-sectional area was calculated. For the small fish (2-10 g) where TCA was sampled in one to two blocks ~1200 muscle fibres were measured. The fibre number (FN) was estimated as:

*FN = TFC * TCA/FA*

TFC was the cumulative number of fibres counted per block; TCA was the total cross-sectional area of fast muscle in mm^2 and FA was the cumulative cross-sectional area of the measured fields in mm^2. The diameters of 1200 to 8200 muscle fibres were measured in the dorsal, ventral, left-hand and right-hand compartments in 20 fish. For the remaining 27 fish (dependent upon size, see Fig. 2.3) at least 1200 fibres were measured from muscle blocks prepared from the dorsal left-hand side compartment. The colour coding of fast muscle fibres according to their diameter range (see Fig. 2.5 A&B) was performed using SigmaScan Pro (v. 5.0).

2.3.5 Statistics

TCA in dorsal vs. ventral, left-hand vs. right-hand compartments in males and females was compared using a one-way ANOVA (SPSS Inc, v. 14.0.1., Chicago, IL, USA). Nonparametric statistical techniques were used to fit smoothed probability density functions (pdfs) to the measured muscle fibre diameters using a kernel function as described in Bowman and Azzalini (1997). The application of these methods to the analysis of muscle fibre diameters has been described in detail (Johnston et al., 1999). Briefly, bootstrap techniques were used to distinguish underlying structure in the distributions from random variation (Bowman and Azzalini 1997; Davison and Hinkley 1997; Johnston et al., 1999). The Kolmogorov-Smirnov two-sample test statistic was used to test the null hypothesis that the probability density functions of groups were equal over all diameters. To supplement this test, density curves for each treatment were compared graphically by constructing a variability

band around the density estimate for the combined populations using the mean smoothing parameter. Values for the smoothing parameter, h, (Bowman and Azzalini, 1997) ranged from 0.132 to 0.133. The variability band provided an indication of which part(s) of the distribution of diameters that were significantly different. The relationship between maximum fibre diameter (FD_{max}) and fish fork length was investigated using a linear regression analysis (SPSS Inc, v. 14.0.1., Chicago, IL, USA).

Fibre number is known to increase until a certain fork length and then reach a plateau at the final fibre number. Two horizontally asymptotic growth models were initially considered based on von Bertalanffy and Gompertz curves respectively. The curves were fitted in the non-linear curve fitting package the nlme (Pinheiro and Bates, 2000) library in R (R Development Core Team, 2007). Models were considered for each sex separately and combined. Model selection was by Akaike Information Criterion (AIC). As there was evidence of heterogeneity in the residuals, the models were fitted with a variance power function proportional to the value of the fitted value (Pinheiro and Bates, 2000). Various models were considered with different subsets of parameters varying by sex. The final best fit model was considered to be the Gompertz curve for each sex considered separately, with the following equation:

$$FN_i = \alpha_j \times \exp(-\exp(\beta - \gamma \times FL_{ij}))$$

Where i is an index of the ith fish. α, β and γ are parameters to be estimated for each sex. α represents the asymptote, γ describes the rate at which the curve ascends and β is a constant. The fork length at which fibre number reached within 1% of the estimated mean fibre number determined from the asymptote of the Gompertz curve was also calculated.

2.4 Results

2.4.1 Muscle fibre differentiation

Red muscle in Atlantic halibut is not restricted to the superficial layer under the skin, but was also found in narrow internalised strips (Fig. 2.1 B). The fibres in these internal strips of red muscle stained for slow muscle myosin (Fig. 2.1 C) and had a more intensive staining for SDHase than the surrounding fast muscle fibres which were only weakly stained (not shown). Slow muscle fibres stained darkly compared to the intermediate and unstained fast muscle fibres after alkaline *pre*-incubation (Fig. 2.1 D). The slow muscle fibres in ≤ 1kg fish were best distinguished using the *S58* antibody (Fig. 2.1 C).

2.4.2 Muscle fibre size and number

The TCA of fast muscle in the dorsal myotomal compartment was 18% greater than in the ventral compartment for fish of 1.5-3.5 kg (one-way ANOVA, n=24, $P<0.05$). No significant difference was found in TCA between left-hand and right-hand compartments. There was no significant difference ($P>0.05$) between the distribution of muscle fibre diameters between either the dorsal versus ventral or left-hand versus right-hand myotomal compartments (n=5, 280-375 g, Fig. 2.2 A&B). Similar results were obtained for the three broodstock fish (data not shown).

The number of blocks required to estimate fibre number with a repeatability of ± 2.5% increased from one in fish of 2.6 g (not shown), to six in fish of 300 g and 17 in a fish of 96.5 kg (Fig. 2.2 C&D, Fig. 2.3). On the other hand, for the smallest size classes of fish where the

TCA was sampled in one to two blocks, 800 muscle fibres were found necessary to achieve a reliable estimate.

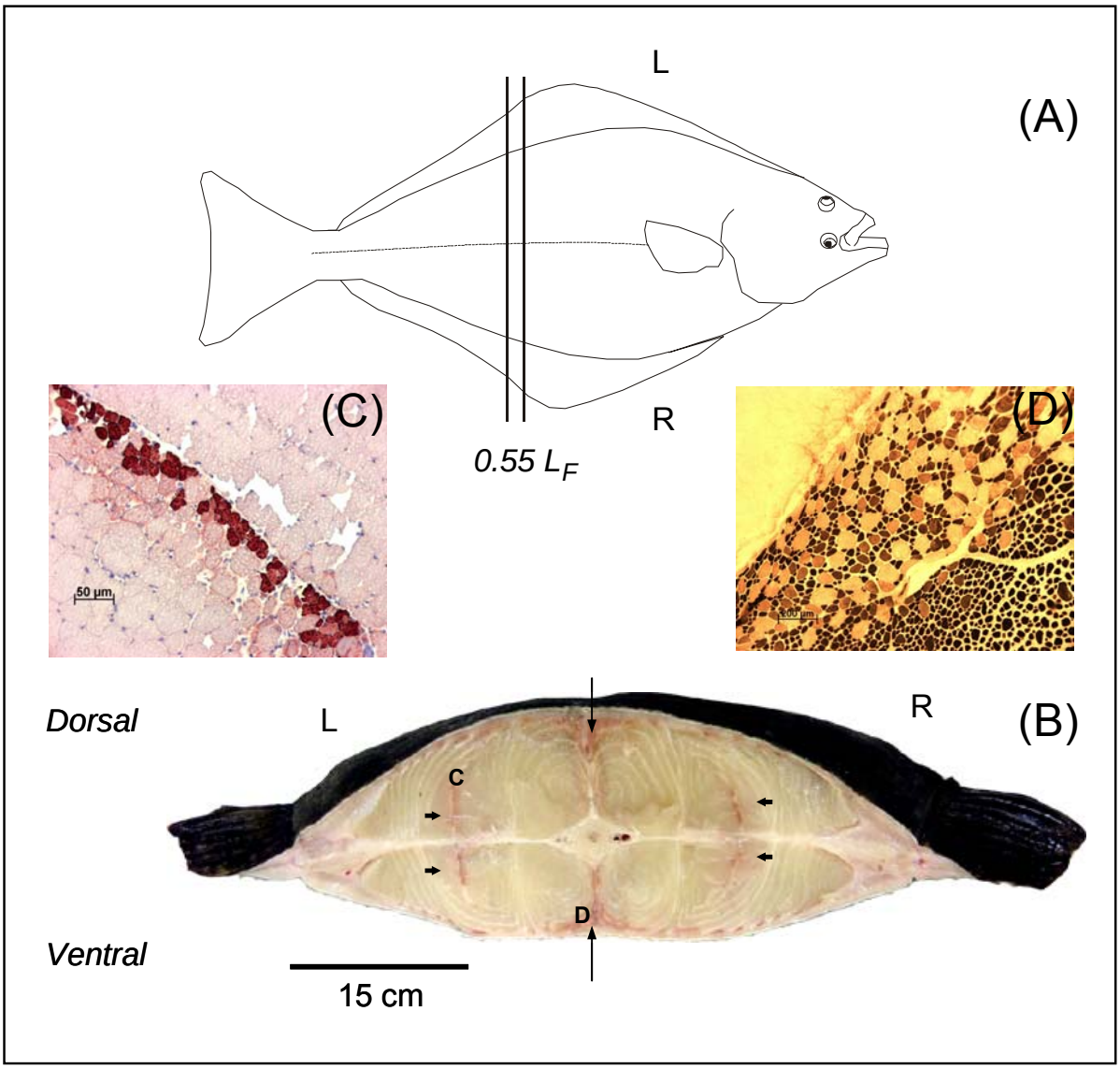

Figure 2.1. Muscle growth was estimated at 0.55 L_F (A). The distribution of slow muscle (B) is indicated by arrows (horizontal septum and internal strips). Insert C&D show *S58* antibody and mATPase staining of slow muscle from a 2.6 g and ~4 kg halibut respectively.

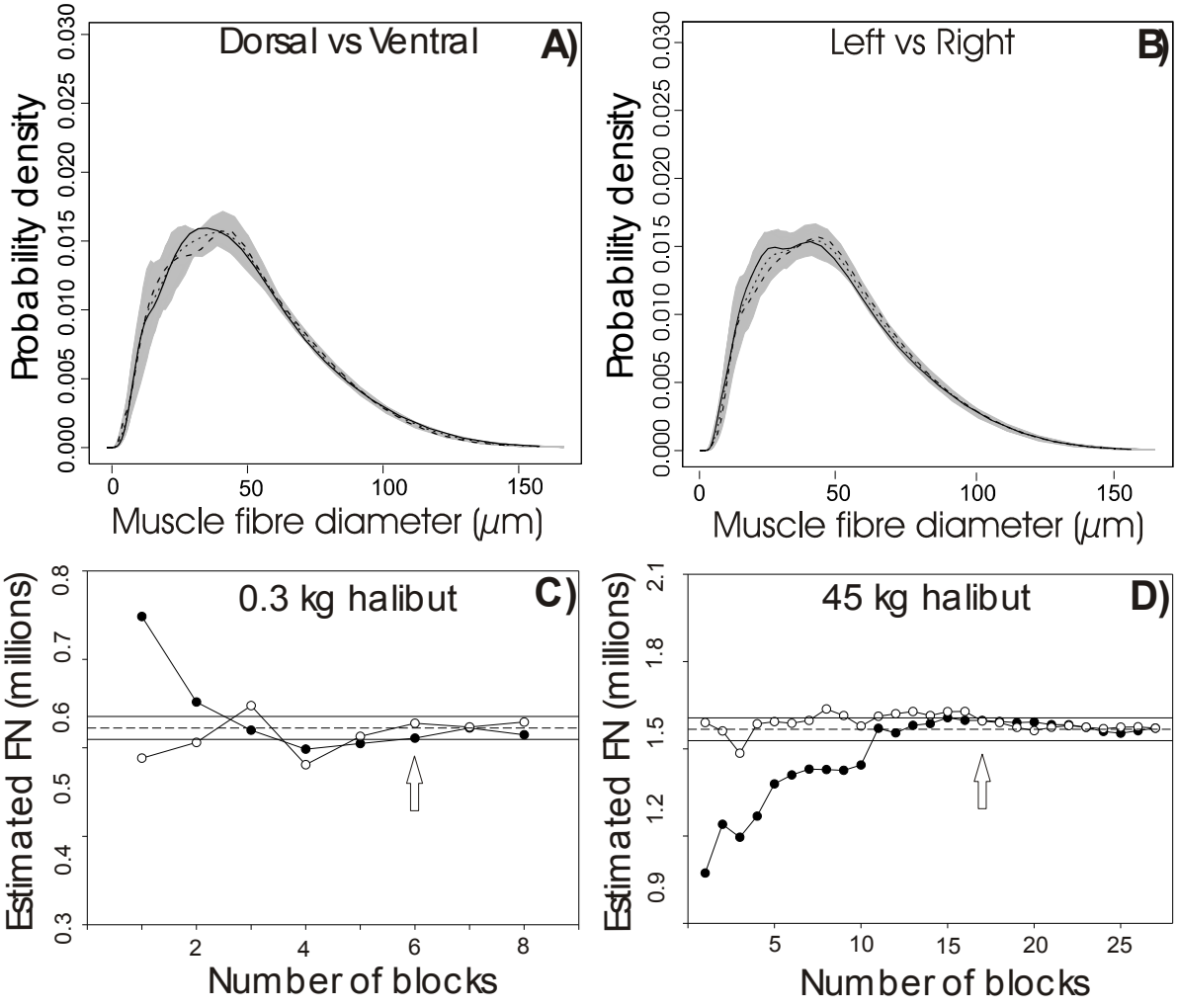

Figure 2.2. The distribution of fast muscle fibre diameters in dorsal (dotted line) and ventral (solid line) (A), left-hand (dotted line) and right-hand (solid line) (B) myotomal compartments (P>0.05). The dashed line in A&B represents the average value. The number of blocks (~150 fibres/block) needed to estimate the fibre number in a 0.3 kg juvenile (C, > 6 blocks) and a 96 kg adult halibut (D, > 17 blocks) with a repeatability of ± 2.5% are indicated by arrows. The dashed line (C&D) represents the muscle fibre number estimated using all measured blocks. Open and filled circles in C&D represent dorsal and ventral compartment respectively.

Smooth distributions were fitted to 1200 measurements of fibre diameter per fish using a kernel function and the corresponding probability pdfs were plotted. Figure 2.4 A shows a selection of the data from seven out of 47 halibut. With increasing Mb there was a right-hand shift in the distribution of muscle fibre diameter, illustrating the hypertropic growth of the muscle fibres. There was a linear relationship between the FD_{max} (calculated from the 97^{th} percentile of diameters) and fish fork length (Fig. 2.4 B) ($n=47$, $r^2=0.89$, $P<0.001$). In the wild caught fish of 45 kg FD_{max} was 360 µm, while FD_{max} for the broodstock varied from 280–330 µm (Fig. 2.4 B).

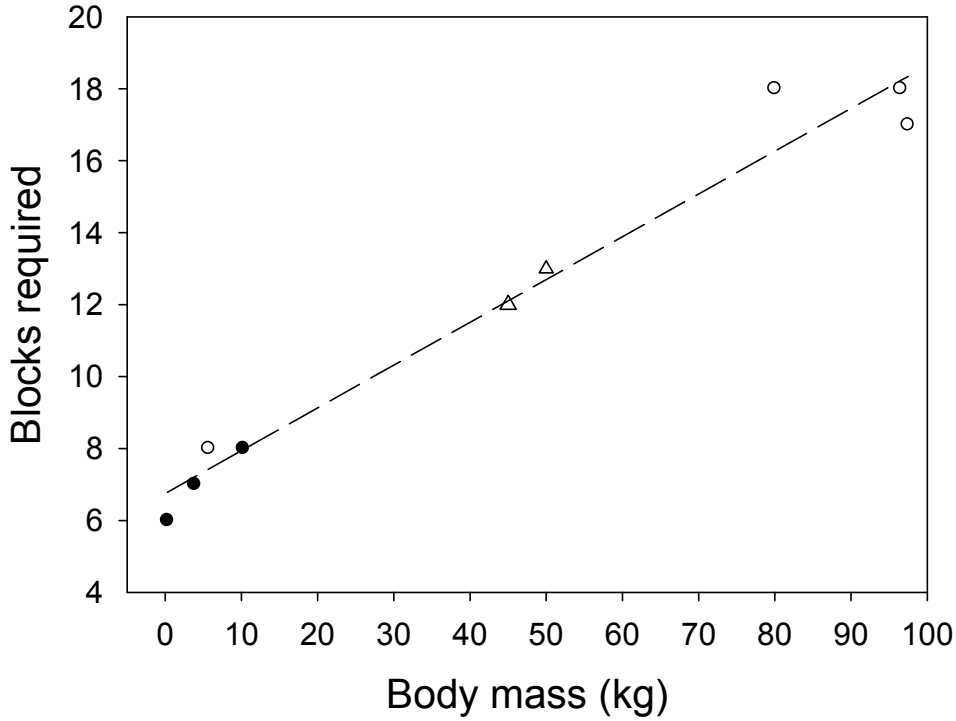

Figure 2.3. The relationship between the number of blocks needed to estimate FN within 2.5% accuracy and body mass. Males (●), females (○) and wild fish (Δ, all females). Based on size and FN_{final} the 45 kg wild fish is likely to be a female. The line presents a first order linear regression (n=9, r^2=0.96, P<0.001).

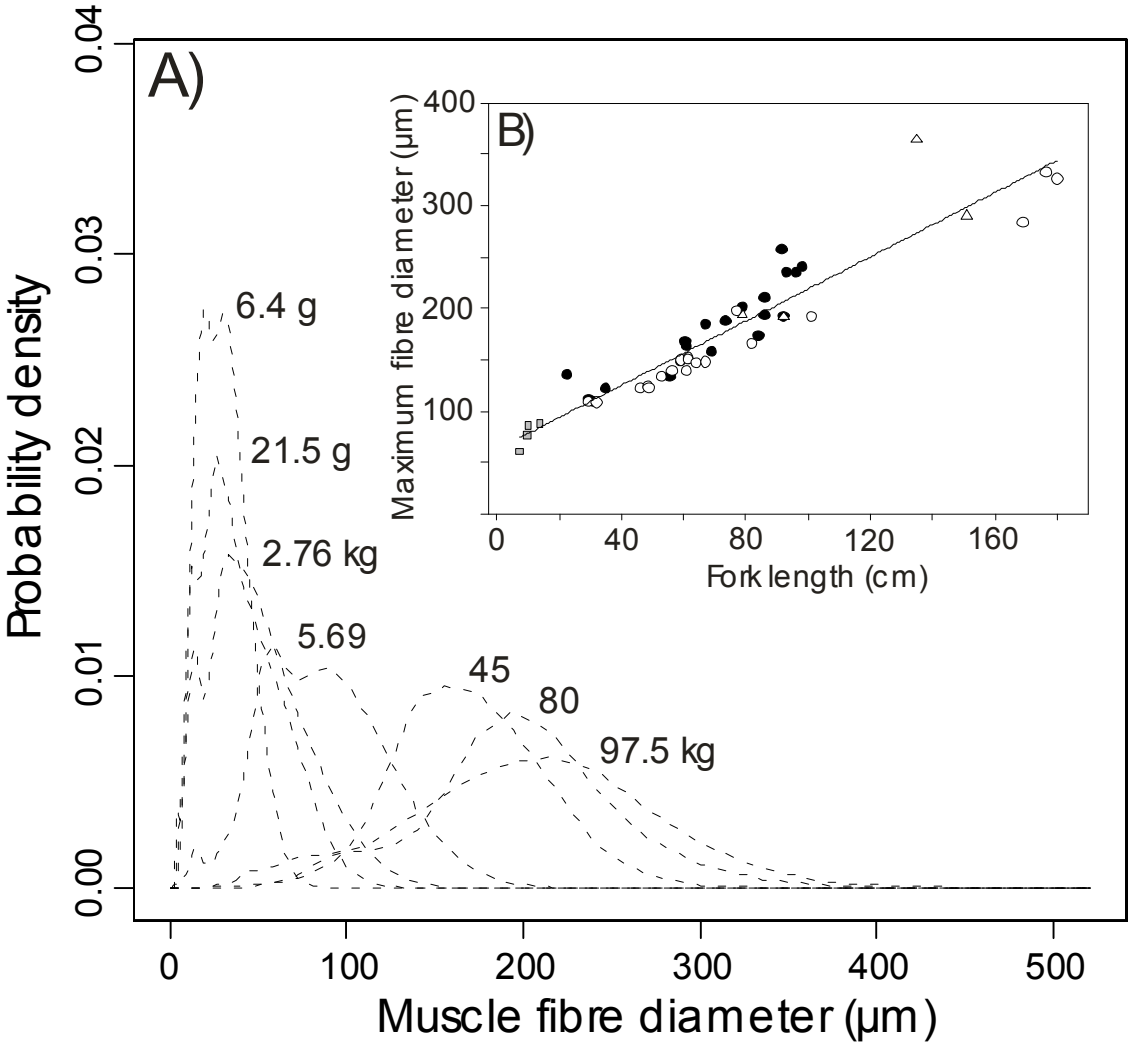

Figure 2.4. (A) The probability density functions of fast muscle fibre diameter for 7 of 47 halibut investigated. (B) A linear regression was fitted to the maximum fibre diameter calculated from the 97th percentile of the distribution and L_F (n=47, r^2=0.89, P<0.001). Males (●), females (○), sex unknown (■) and wild fish (Δ, all females). Based on size and FN_{final} the 45 kg (135 cm) fish is likely to be a female.

Fast muscle fibres sampled from the same location (dorsal left side close to the horizontal septum) of one male and one female of identical size (61 cm) were given colour codes based on the difference in fibre size range to illustrate the difference between sexes (Fig. 2.5 A&B). This pictorial representation of fibre size distribution shows that male fish have very few small fibres compared to female fish (Fig. 2.5 A&B). Comparison of the distribution of fast fibre diameters in five males and five females of similar size (~60 cm) showed that males had fewer muscle fibres in the smaller size classes (10-50 μm), but a higher percentage of large diameter fibres (90-200 μm) than females (Fig. 2.5 C) (Kolmogorow-Smirnov test, $P<0.05$).

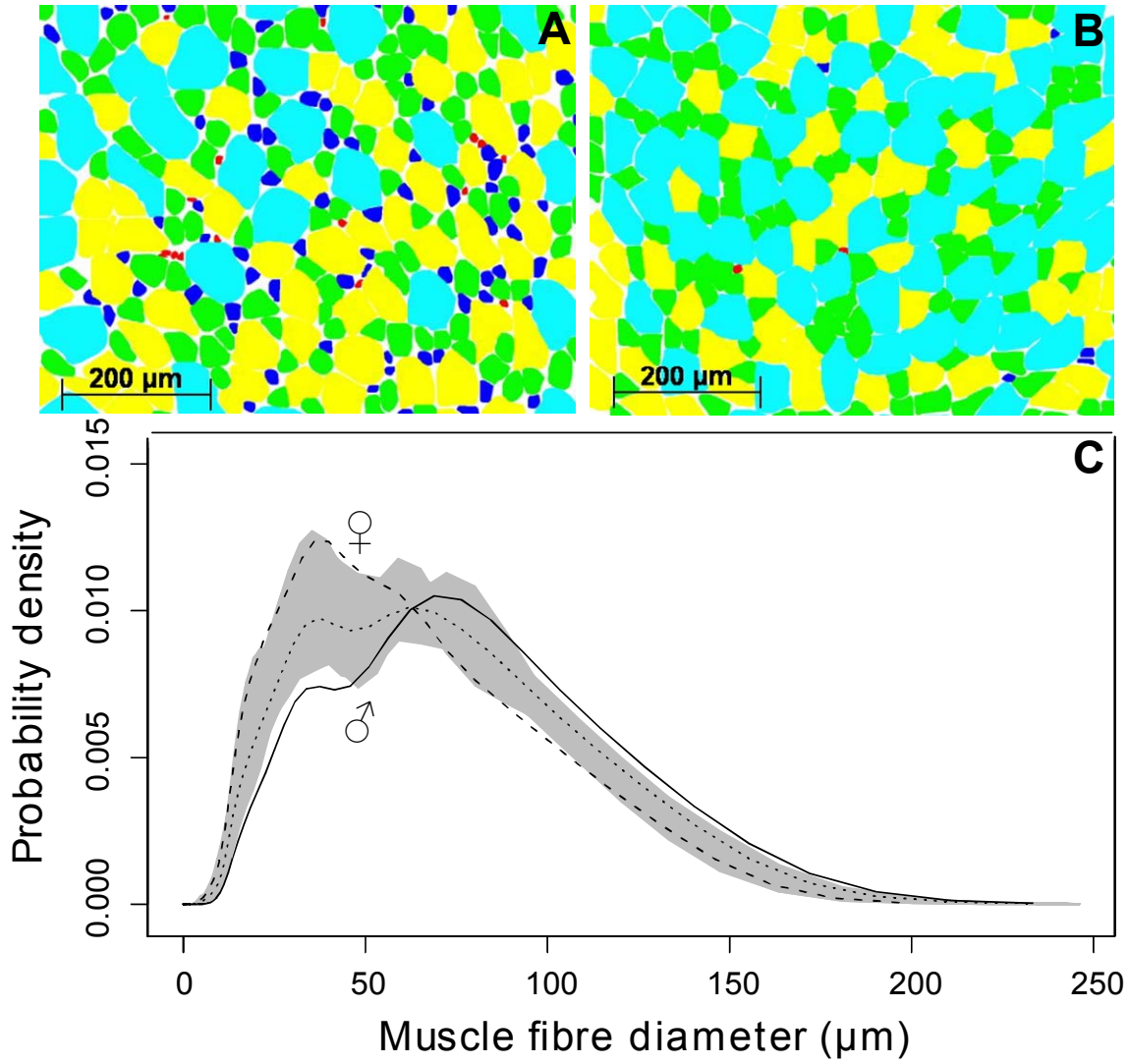

Figure 2.5. Fast muscle fibres sampled from the same location from one female (A) and one male (B) of identical length (61 cm) were colour coded and divided into size classes. Red = 0-29 µm, blue = 30-59 µm, green = 60-89 µm, yellow = 90-119 µm and aqua = 120 µm <. Nonparametric probability density functions were fitted to measurements of fast fibre diameter of 5 male (solid line) and 5 female (dashed line) fish of ~60 cm (C). The dotted line represents the average probability density function of the combined population.

In order to obtain estimates of the final fibre number and the fork length at which fibre recruitment ceased, the data on fibre number was fitted to separate Gompertz growth models for each sex. The final fibre number was set to 1% of the asymptotic value of the curve to obtain a model output that was broadly consistent with the information collected on muscle fibre diameters (Fig. 2.6). The estimated final fibre number was 8.96×10^5 ($7.99 - 9.94 \times 10^5$, 95% confidence intervals) for males and 1.73×10^6 ($1.56 - 1.90 \times 10^6$, 95% confidence intervals) for female fish. The estimated fork length for cessation of fibre recruitment in the fast muscle of female fish (177.5 cm) was more than twice of that in males (81.0 cm)

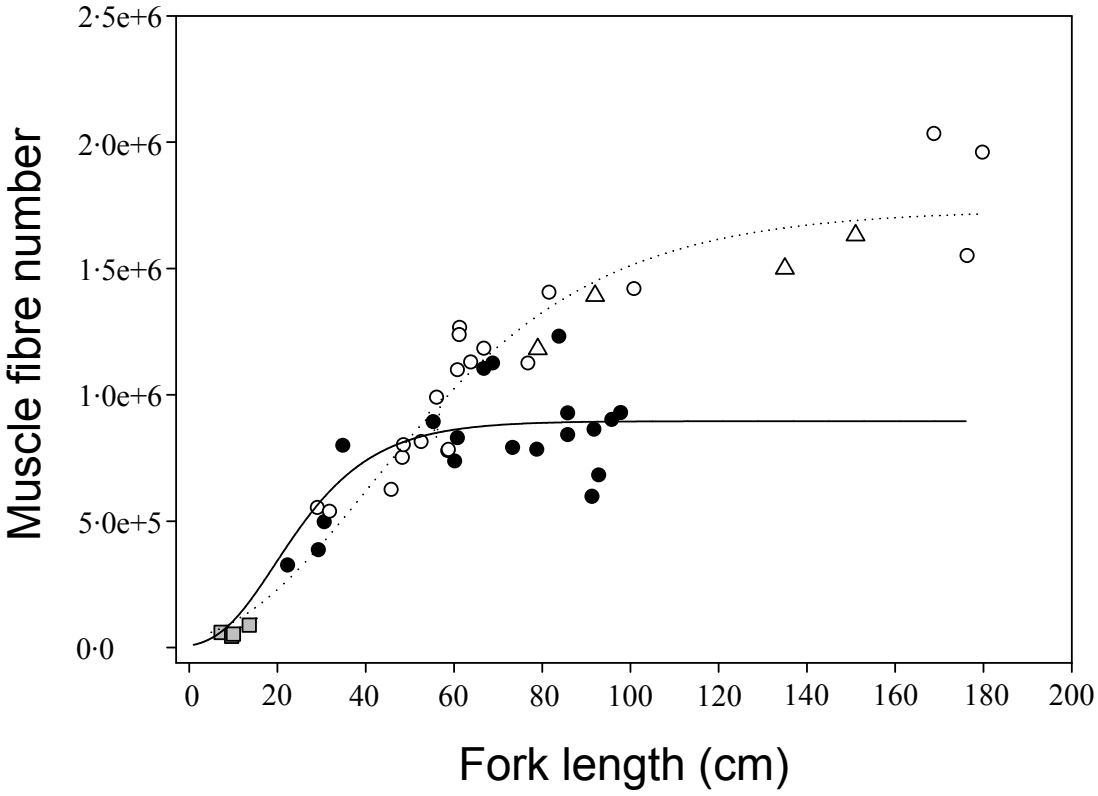

Figure 2.6. Gompertz curves were fitted separately to measurements of muscle fibre number and fork length for male (solid line) and female (dotted line). Males (●), females (○), sex unknown (■) and wild fish (Δ, all females).

2.5 Discussion

In the present study it was shown that the distribution of fast muscle fibre diameters in Atlantic halibut is similar between myotomal compartments (Fig. 2.2 A&B), such that FN can be estimated from measurements of any quartile. Furthermore, the number of blocks (~150 fibres/block) required to sample FN with a repeatability of ± 2.5% has been determined for fish up to 96.5 kg. In such large fish, it is necessary to quantify the cross-sectional areas of more than 2500 individual fibres distributed between 17 muscle blocks (5x5x5 mm) to obtain a reliable estimate of FN. It has recently been suggested that discrete growth zones are present in the myotomes of adult halibut (Haugen, 2006), however, no evidence for this was found in the present study.

Previous studies with 1-2 kg halibut had shown that females had a higher FN than males (Hagen et al., 2006). In the present study we have established that FN_{final} was ~1.9-fold higher in female (1.73×10^6) than male (8.96×10^5) fish reflecting the greater ultimate size of females. Furthermore this increase in fibre number of fast muscle was achieved by delaying the switching-off of myotube production at longer body lengths in female (~177.5 cm L_F) than in male (~81 cm L_F) fish (Fig. 2.6). These estimates of the fork length at which fibre recruitment stops should be regarded with some caution due to the relatively small number of fish studied. Male fish matured at a slightly shorter fork length (Jákubsstovu and Haug, 1988) than the length at which recruitment stops (Hagen et al., 2006). It is interesting to note that females do not become sexually mature until 110-115 cm (Jákubsstovu and Haug, 1988), such that muscle fibre recruitment ceases at a larger body length than sexual maturity which is similar to the situation in males. The literature regarding maximum length of female and male Atlantic halibut is not consistent, and data on maximum length in males is not very well established. However, assuming that the maximum fork length is ~190 cm (Bowering, 1986)

and ~300 cm (Moen and Svensen, 1999) for males and female respectively, the recruitment of fast muscle fibres stopped at ~43% and ~59% of the ultimate body length. In ten fresh water species, fast muscle hyperplasia was found to cease at 44% of the ultimate fork length (Weatherley et al., 1988).

Evidence for a similar sexual dimorphism in fast muscle fibre recruitment patterns has been reported for the Argentine hake (*Merluccius hubbsi*) in which females also reach a larger body size than males (Calvo, 1989). In mammals, males are often larger than females and this sexual dimorphism is associated with greater muscle mass (Shahin, 1995). It has been suggested that this sexual dimorphism is attributable to differences in androgens (Bardin and Catterall, 1981), insulin-like growth factor I (Liu et al., 2000), growth hormone (Udy et al., 1997) and the processed form of myostatin (McMahon et al., 2003). The physiological mechanisms regulating differences in muscle fibre recruitment between male and female fish showing a sexual dimorphism in body size are unknown.

At all stages of ontogeny muscle growth involves an increase in the length and diameter of muscle fibres less than FD_{max}. The 45 kg wild halibut was identified as an outlier in Fig. 2.4 B, having the largest FD_{max}, considerable larger that the largest of the broodstocks. This fish was in an exceptional good condition having the largest condition factor of all halibut investigated and a TCA almost as large as the 80 kg broodstock halibut. The maximum diameter is probably limited by diffusional constraints and the need to avoid an anoxic core in the centre of the fibre (Egginton et al., 2002). Resting and maximum mass specific metabolism scales with $Mb^{-0.25}$(Schmidt-Nielsen, 1984), and thus the maximum permissible diameter is expected to increase with body size (Fig. 2.4 B) as diffusional constraints are relaxed (see Johnston et al., 2003a for further discussion). FD_{max} was a linear function of length in Atlantic halibut as has been reported for sub-Antarctic and Antarctic notothenioids living at cold temperatures (Johnston et al., 2003a). In contrast, in some species e.g. Atlantic

salmon (*Salmo salar*), the maximum diameter reaches a limiting value and then becomes independent of L_F (Johnston et al., 2003b). The relationship between FD_{max} and L_F was the same for male and female halibut suggesting that similar diffusional constraints operate in the muscle fibres of both sexes. In contrast, ~60 cm L_F males have a higher percentage of muscle fibres with diameters in the largest size classes less than FD_{max} than females (Fig. 2.5 A, B&C). It has previously been proposed an optimum fibre number hypothesis to explain intra and inter-specific differences in muscle fibre size (Johnston et al., 2003b; 2005; 2006b). Theoretically, as the surface to volume ratio of muscle sarcolemma decreases with an increase in fibre diameter, so do the passive leak of ions across the muscle membrane and hence the cost of ionic homeostasis involving ATP-dependent pumps of various kinds. Thus routine maintenance costs are minimised if the fibres are as large as possible without incurring diffusional constraints which sets the fibre number at some optimal level. In the case of Atlantic halibut, growth to a large body size by females presumably brings benefits in terms of fecundity. However, in order for female fish to achieve a larger ultimate body size than males which mature earlier, a higher final fibre number is required, and this may bring a penalty in terms of a higher routine maintenance cost due to the associated smaller average fibre diameter, in particular for immature individuals.

Chapter 3

Sexual dimorphism of fast muscle fibre recruitment in farmed Atlantic halibut (*Hippoglossuss hippoglossus*).

3.1. Abstract

Commercially farmed Atlantic halibut (*Hippoglossus hippoglossus*) were reared at the Aga Marin's facility located on Dønna (Norway) under ambient environmental conditions in duplicate 15x15x8 m netpens (May 2004 to May 2005). Twenty fish were sampled five times over a twelve month production period during which time the average body weight increased from 1.26 to 2.08 kg (n=100 fish). Body mass (Mb), fork length (L_F), and the number and size distributions of fast muscle fibres were determined in male and female fish. All males matured during the autumn whereas no maturation was observed in females. From the point of maturation females had superior growth performance to males and Mb and the total cross-sectional area of muscle were 1.4-fold ($P<0.05$) and 1.3-fold higher ($P<0.01$) respectively by May 2005. The total number of fast muscle fibres per trunk cross-section at 0.55 L_F was 24.5% higher in females (7.58×10^5) than males (5.80×10^5) in May 2004. In females, muscle fibre recruitment slowed with short days and low water temperatures (<6.5 ^oC), but had increased to 1.01×10^6 by May 2005 ($P<0.001$). In contrast, there was no growth and no increase in muscle fibre number in males following the onset of maturation. The cross-sectional area of 1200 fast muscle fibres was measured per fish and smooth distributions fitted using a kernel function. For L_F matched male and female fish sampled in August the distributions of fibre diameter were significantly different ($P<0.05$, nonparametric Kolmogorov-Smirnoff test): the peak probability density function of diameter was shifted towards larger sizes in males relative to females whereas females had a higher proportion of the smallest size class of fibres (<10 μm). The results illustrate a sexual dimorphism of muscle fibre recruitment patterns related to differences in Mb and highlight the adverse affects of sexual maturation in males on muscle growth.

3.2 Introduction

Atlantic halibut exhibits relatively slow growth in culture, particularly males due to precocious sexual maturation and this represents one of the most significant problems for farming the species in the grow out phase (Jákupsstovu and Haug, 1988; Björnsson, 1995; Norberg et al., 2001). Small scale laboratory experiments with halibut have attempted to stimulate growth and inhibit maturation, by manipulating photoperiod (Aune et al., 1997; Norberg et al., 2001; Imsland et al., 2005), temperature (Björnsson and Tryggvadóttir, 1996; Jonassen et al., 1999) or a combination of both factors (Jonassen et al., 2000). However, to date the success of such environmental manipulations in reducing male maturation and slow growth has been limited.

Muscle growth in teleosts involves the recruitment and subsequent expansion of muscle fibres. In slow muscle, fibre number increases continuously with fish length, whereas in fast muscle recruitment stops at around 40% of the maximum length and subsequent growth is by hypertrophy alone (Weatherly et al., 1988; Rowlerson et al., 1995; Johnston et al., 2000a, 2004). For example, in Atlantic salmon (*Salmo salar*) approximately 80% of fast fibres are produced in seawater stages and the maximum fibre number is reached in fish of about 2 kg, with summer growth to a commercial harvest weight (4-5 kg) involving hypertrophy of the fibres formed earlier in development (Johnston et al., 2000a, 2003a,b). The maximum fibre number (FN_{max}) shows significant variation within and between population (Johnston et al., 2000a) and can be altered by environmental conditions at critical stages in the life cycle (Johnston et al., 2003a,b), resulting in significant variation in muscle fibre density for fish of the same Mb. The maximum muscle fibre number has recently been reported for male (8.96×10^5) and female (1.73×10^6) Atlantic halibut (Hagen et al. 2008a).

Galloway et al. (1999b) investigated the ultrastructure and cellularity of myotomal muscles in 1 d-old halibut larvae. The trunk muscle of halibut is not bilaterally symmetrical, and in an earlier study potential differences in muscle cellularity between dorsal and ventral myotomes on the left and right side of the fish were investigated (Hagen et al., 2008a). It was established that for relatively small fish (<5 kg) an accurate estimate of total fibre number could be obtained from sampling a single quadrant. Olsson et al. (2003) found a higher proportion of small diameter fibres in the fast muscle of farmed compared to wild halibut, consistent with a higher intensity of fibre recruitment. Recently we demonstrated that in Atlantic halibut, displaying sexual dimorphism in final body size, males terminate the mosaic growth phase at smaller size (~81 cm) than females (177.5 cm) (Hagen et al., 2008a). Thus, it is not known if sexual dimorphism in muscle fibre recruitment is present during the grow-out phase in commercial aquaculture (<6 kg). It is known from Argentine hake (*Merluccius hubbsi)* that females have a higher proportion of small diameter fibres and are capable of sustaining fibre recruitment to a larger body length than males (Calvo, 1989).

The first objective of the present study was to investigate muscle fibre recruitment in Atlantic halibut farmed commercially under ambient photoperiod condition in the grow-out phase. The second objective was to compare muscle fibre recruitment and the distribution of muscle fibre diameters with respect to the effects of precocious sexual maturation on muscle growth in males.

3.3 Material and methods

3.3.1 Fish husbandry

Atlantic halibut (*Hippoglossus hippoglossus*) were obtained from the commercial production of Aga Marin AS, located at Dønna in Helgeland, Norway. The fish were hatched by Brandal Havbruk AS in 2001 and on-grown for one year (2002) before being sold to Aga Marine AS (then called Dønna Oppdrettsmiljø AS). The fish were three years old at the beginning of the trial. The feed used in the trial was Biomarine kveite (pellet, size 15 mm), a newly developed test feed for Atlantic halibut farming, with a composition of 50% crude protein, 22% crude fat, 6% nitrogen free extract, 0.3% crude fibre and 8.6% ash (w/v, data provided by BioMar AS, Trondheim, Norway). Fish were fed to satiation in the morning and afternoon, using an automated feeding system with additional hand feeding if necessary. Rearing was carried out at ambient temperatures and day length in 15x15x8 m netpens, with seven shelves (25 m^2 each) per netpen. During the experiment, 24.05.04–05.05.05, fish were harvested five times over a twelve months production period. Ten fish were randomly sampled from each of two netpens. Fish were harvested in the afternoon and killed with a sharp blow to the head, the gill bows were cut and the carcass stored on ice in polystyrene boxes until next morning prior to transportation to Bodø by the local speedboat. The fish were then collected at the harbour and driven up to Bodø University College. Biological data such as Mb, fork length (L_F), condition factor and gonad somatic index were recorded.

The condition factor was calculated using the following formula:

$$Condition\ factor = \frac{Body\ mass\ (g)}{Length\ (cm)^3}\ x\ 100$$

Gonad somatic index (GSI) was calculated for all maturing/matured fish according to the formula:

$$GSI = \frac{Gonad\ (g)}{Body\ mass\ (g)}\ x\ 100$$

A total of one hundred Atlantic halibut were sampled during the trial comprising 56 females and 44 males. The sex of the fish sampled is shown in Table 3.1.

3.3.2 Morphometric studies

A myotomal steak (0.5 cm) was prepared at 0.55 of the L_F and a digital photograph taken. From the digital image the total cross-sectional area of the fast muscle was measured (SigmaScan pro. 5.0, Systat, Inc). From each steak a total of six blocks was prepared with a scalpel and mounted on cork using cryomatrix (Shandon Cryomatrix, Thermo Electron Corporation, UK). All blocks (5x5x5 mm) were prepared from the dorsal left quartile of the fillet (Fig. 3.1). Measurements were restricted to the fast muscle only which compromises more then 90% of total muscle mass. To prevent contamination with slow twitch muscle fibres areas immediately adjacent to the internalised layers of slow muscle were not sampled (shown in Fig. 3.1). Blocks were frozen in 2-methylbutane cooled to near its freezing point (-159 ºC) in liquid nitrogen and stored in liquid nitrogen until analysis. Blocks were cut at -18 ºC in a cryostat (Microm HM 550, MICROM International GmbH) and 7 µm thick sections were mounted on poly-L-lysine treated slides, air dried and stained with Harries' hematoxylin (Sigma, Oslo, Norway). After staining sections were mounted using glycerol gelatine (Sigma, Oslo, Norway). Sections were analysed using a light microscope (Axioskop 2 mot plus, Carl Zeiss) and an image analysing software (Axiovision v. 4.2 software, Carl Zeiss). The outlines

of 200 random selected muscle fibres were measured per block, a total of 1200 muscle fibres per fish. Muscle fibre diameter and number was calculated as previously described (see section 2.1.1 and Johnston et al., 1999).

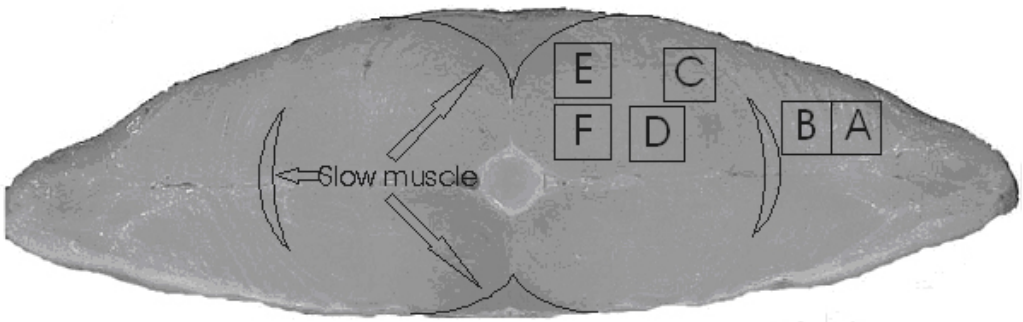

Figure 3.1. Sampling site for studies of muscle cellularity, six blocks in total (A-F). Outlines illustrate the location of the slow muscle.

3.3.3 General chemistry

The whole fillet (dorsal and ventral side) anterior to 0.55 L_F was minced, after 4 d storage on ice (*post-rigor*). From the mince 5 g was measured into a tinfoil cup and dried over night. The water content was estimated in duplicate as weight loss overnight at 104 ºC (16 h) using the following formula:

$$Water\ (\%) = \frac{Dry\ weight}{Wet\ weight}\ x\ 100$$

For the crude protein content 1 g of mince was accurately measured into a kjeldahl weighing boat (nitrogenous free paper) (Whatman, San Diego, USA) and added to a glass tube

containing 30 ml concentrated sulphuric acid (51%) and 2 Kjeltabs (catalyst) (Foss, analytical, Höganäs, Sweden). The glass tubes were then heated inside a fume hood to 420 ºC using a block heater with cold water-suction for removing of the sulphuric gas. The crude protein content was estimated using a Kjeltec 2300 Analyser instrument (Foss Tecator AB, Höganäs, Sweden) and a factor of 6.25.

Fat extraction was performed using ethyl acetate according to Norwegian Standards (1994). Briefly, 10 g of mince was mixed together with 20 g of anhydrous sodium sulphate using a mortar. The mixture was then transferred to a 100 ml pyrex glass with a screw cap and 50 ml of ethyl acetate was added. Samples were left on a stirring board for 2 h in room temperature for fat extraction. After extraction the mixture was filtered and the ethyl acetate containing the fat fraction was collected. 20 ml of the ethyl acetate was removed into a *pre*-weight glass container using a volumetric glass pipette, and placed over a steam bath inside a fume hood. *Post* evaporation samples were put in an oven (104 ºC) for ~10 minutes to remove any remaining water. After cooling down to room temperature (~15 minutes) the glass containers with the fat fraction were weighted. All extractions were preformed in duplicate and the fat content was calculated using the formula:

$$\textit{Fat content (\%)} = \frac{\textit{10300 x remaining (g)}}{\textit{(40 – 2.17 x remaining (g)) x sample weight}}$$

where 10300 is a constant and 40-2.17 is a calibration factor.

3.3.4 Statistics

Unless otherwise stated SPSS version 12.0.1 (SPSS Inc., Chicago, IL, USA) was used for all data analysis. Before analysis the assumptions for the statistical methods used were tested. To analyse for significant differences in L_F and Mb between netpens a one-way ANOVA was used. Smooth nonparametric distributions were fitted to 1200 measurements of fast fibre diameter using a kernel function (Johnston et al., 1999) with an average value of the smoothing coefficient h of 0.131 (Bowman and Azzalini 1997). Groups to be compared were matched for L_F (n=10 for both sexes). A nonparametric Kolmogorov-Smirnov two sample test was applied to test the hypothesis that the average probability density function for male and female fish differed from the combined population from both sexes. A one-way ANOVA was used to analyse for significant difference between sexes in total muscle fibre number. The 97^{th} percentile of muscle fibre diameter, calculated from smooth distribution of the muscle fibres was used as an estimate of the maximum fibre diameter (FD_{max}). A Mann-Whitney U-test was used to analyse for significant difference in length and Mb, condition factor and water content, total cross-section area (TCA), FD_{max} and average muscle fibre diameter. In order to satisfy the assumptions for General Linear Methods (GLM) when analysing for significant difference (Grafen and Hails, 2002) between sexes in the presence of muscle fibres <10 μm, a square root transformation of the data was applied. Statistical significance was established at a P value of 0.05 or less.

3.4. Results

3.4.1 Growth characteristics

Now differences in Mb or L_F (tank effect) between netpens were seen (P>0.2). The ambient photoperiod and temperature during the trial is shown in Fig. 3.2 A. The shaded area between 6-7 ºC represents the temperature threshold for feeding. For a substantial period (December – end of April) the temperature was less then 6 ºC, and the fish showed a loss of appetite. The highest and lowest average temperature recorded during one week in the trial were 14.8 ºC in August 2004 and 3.5 ºC in March 2005 respectively. Commercially farmed Atlantic halibut males reared under ambient conditions had similar growth rates to that of females until the point of maturation, which occurred in November 2004 (Fig. 3.2 B). All males matured during the autumn, whereas no maturation was observed in females. The GSI was 4.4% in males in November and decreased to 3.4% by February when all males had started to spawn. Due to spawning it was not possible to get an accurate measurement of the gonad weight.

A significant and progressive divergence in growth performance between males and females was evident by February 2005 and this difference increased until the end of the trial in May 2005 (Fig. 3.2 B). From November to May males decreased in Mb (P=0.072), but L_F (P=0.8) was about the same. Females also showed a growth stagnation period during winter, but to a lesser extent then males. They increased in length during the whole trial (P<0.005), but did not gain weight from Nov 2004–May 2005 (P>0.05, Fig. 3.2 B).

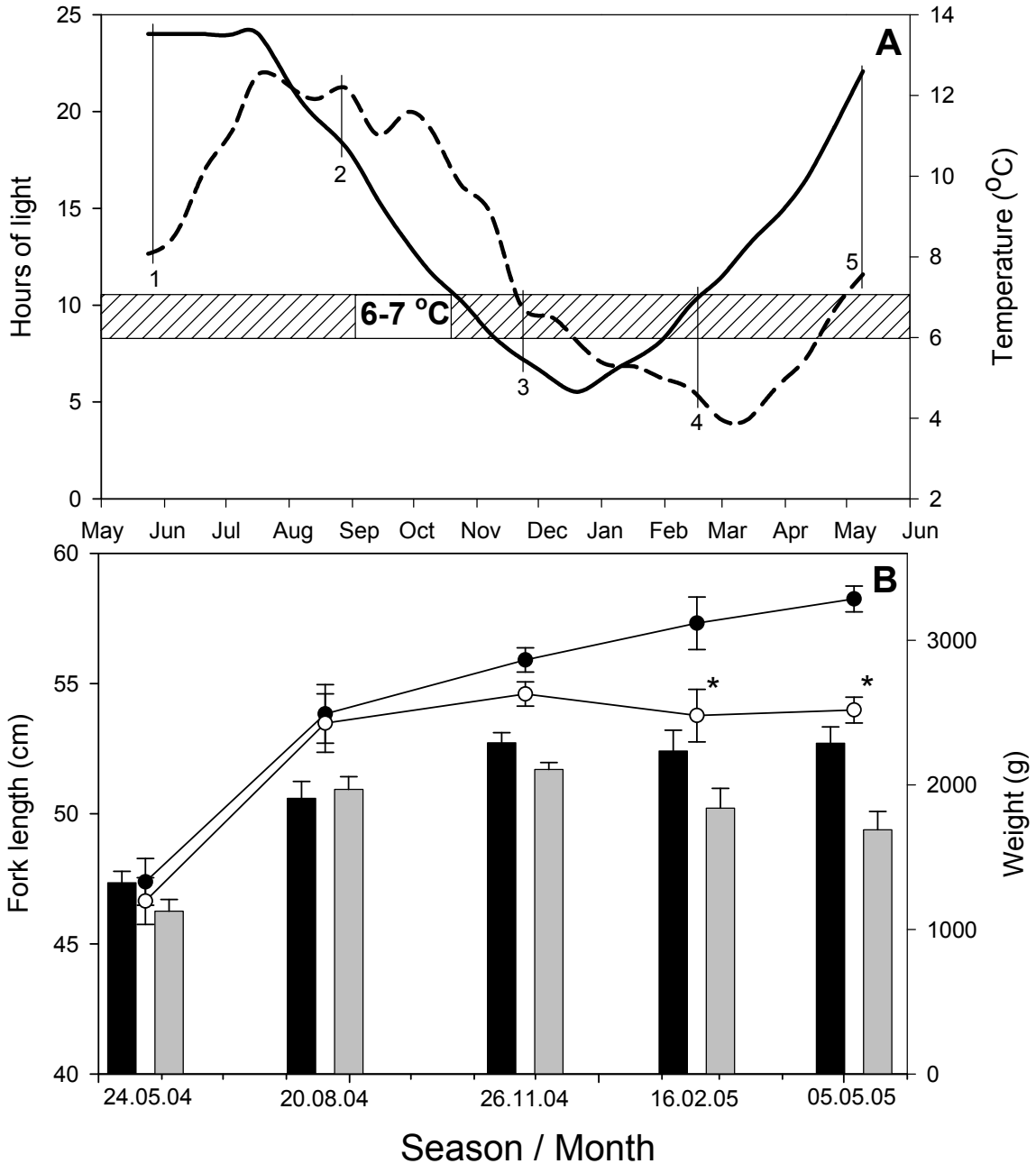

Figure 3.2. Changes in environmental conditions (temperature and light), fork length and body mass through the trial. A: Seasonal changes in day light (solid line) and temperature (dotted line). 6-7 ºC (hatchd area) represent the threshold for feeding. B: Filled (●) and open (o) circles represent fork length in females and males respectively. Black and grey bars represent body mass (g) of females and males respectively. Significant sexual differences are indicated with asterisks. Error bars are given as mean ± SE.

3.4.2 Condition-factor, GSI and general chemistry

At the beginning of the trial condition factor was significantly lower in males than females ($P<0.05$), but this difference disappeared in subsequent samples (Table 3.1). Condition factor in males and females showed the same trend throughout the experiment, a significant increase in both sexes ($P<0.05$) from May to November 2004 and then a progressive drop until the end of trial ($P<0.005$). The condition factor did not change in males between August and November. During the winter, males and females showed a drop in condition factor towards values observed at the start of the trial ($P<0.05$). The water content of the muscle showed a similar trend in males and females during the trial, with a significant decrease between May and November in males ($P<0.005$) and females ($P<0.05$) (Table 3.1). The lowest water content recorded was for males in August (77.3% ± 0.6, mean ± SD) and for females in November (77.2% ± 0.7). From November until the end of the trial, water content in both males and females increased significantly ($P<0.005$), with the highest values for both females (80.2 ± 1.4) and males (81.8 ± 1.3) recorded in May 2005. At the end of the trial muscle water content was significant higher for males than females ($P<0.05$). While flesh water values increased during winter, protein and fat content decreased significantly in males, but were relatively unaffected in females (Table 3.1), displaying the lowest levels in May 2005. Changes in condition factor were correlated with changes in water content of the fast muscle ($r^2=0.51$, Fig. 3.3) and crude protein ($r^2=0.49$) (results not shown).

Table 3.1. Gonadal somatic index (GSI), muscle water (%), condition factor (C-factor), protein (%) and fat (%) content of the male and female (#) fish in the trial (mean ± SD).

	Sex	24.05.04	20.08.04	26.11.04	18.02.05	05.05.05
Sex (#)	♀	13	9	11	10	13
	♂	7	11	9	10	7
GSI (%)	♀	-	-	-	-	-
	♂	-	-	4,4 ±1.0a	3.4 ±0.59b	-
C-factor	♀	1.23 ±0.07a*	1.21 ±0.08a	1.31 ±0.08b	1.17 ±0.03ac	1.15 ±0.13c
	♂	1.10 ±0.13a*	1.28 ±0.06b	1.28 ±0.08b	1.17 ±0.11ac	1.06 ±0.10ac
Water (%)	♀	78.2 ±1.5a	78 ±1.6a	77 ±0.7b	78.6 ±1.8b	80.2 ±1.6c**
	♂	80.3 ±2.3a	77.3±0.6b	77.4 ±1.4b	79.5 ±0.9a	81.8 ±1.3c**
Protein (%)	♀	19.29 ±1.3a	20.41 ±0.5a	20.92 ±0.4b	19.88 ±1.4a	19.83± 0.6a**
	♂	18.47 ±1.5a	20.58 ±0.4b	20.26 ±1.0ab	19.48 ±0.7ab	16.95 ±1.2c**
Fat (%)	♀	1.51 ±0.77a*	1.17 ±0.67a	1.13 ±0.55a	0.47 ±0.23b	0.25±0.14b
	♂	0.93 ±0.68a*	1.36 ±0.25b	1.19 ±0.31ac	0.31 ±0.07d	0.2 ±0.1e

Different letters in the same row indicates significant difference ($P<0.05$) between sampling points. Asterisks (*) indicates significant difference between sex in the same sampling (* = $P<0.05$, ** = $P<0.005$).

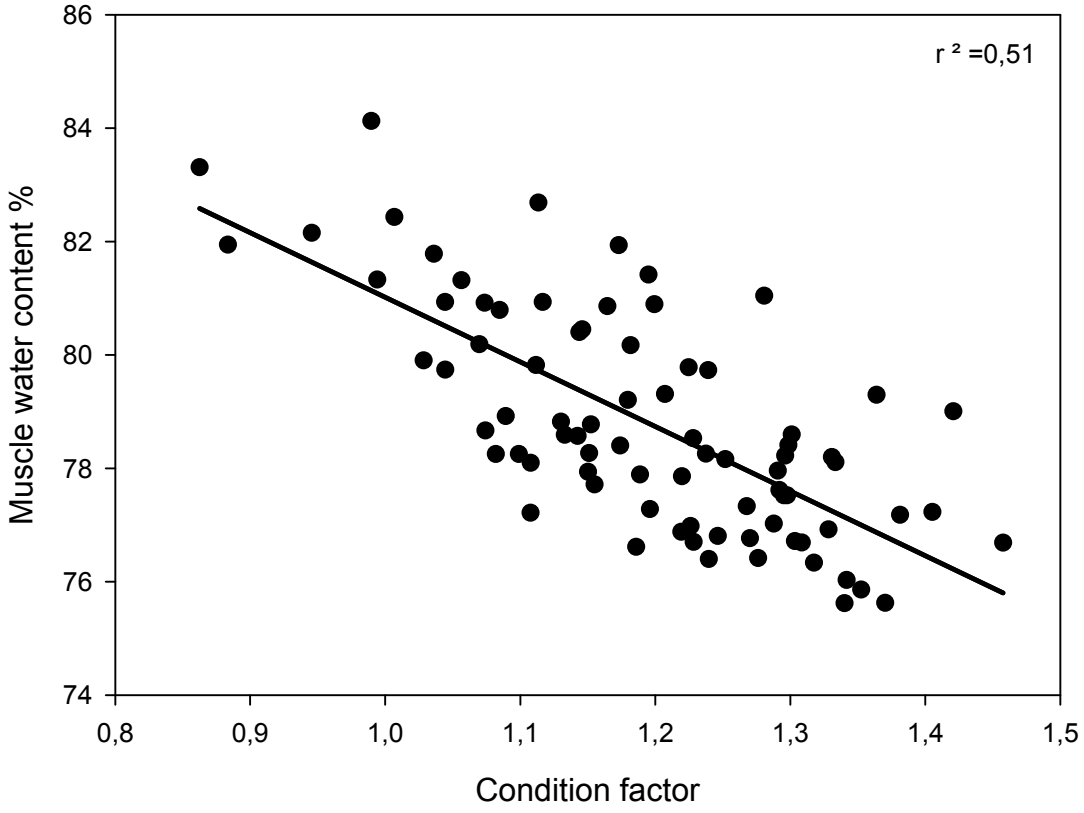

Figure 3.3. Correlation between water content of the muscle and condition factor of Atlantic halibut (r^2=0.51, n=100).

3.4.3 Muscle growth pattern

3.4.3.1 Total cross-section area of fast muscle

From May to August 2004, TCA increased in both sexes (Fig. 3.4). Between August and November only females showed a further significant increase in TCA. The cross-sectional area of fast muscle in female halibut did not increase significantly between November 2004 and May 2005. TCA in males on the other hand decreased 17% between August 2004 and May 2005 ($P<0.05$). At the last sample period the TCA in males was 24.2% less than in females ($P<0.01$), equivalent to 946 mm^2 for fish with an average weight of 1690 g.

3.4.3.2 Change in muscle fibre number

The number of fast muscle fibres was determined and plotted against L_F (Fig. 3.5). Both sexes showed a significant increase in fork length and fast muscle fibre number between May and August, but from this point only females showed further muscle fibre recruitment. Females increased the number of fast muscle fibres per cross section by 22.3% from $7.6 \times 10^5 \pm 1.0 \times 10^5$ (May 2004) to $9.8 \times 10^5 \pm 1.3 \times 10^5$ (August 2004) fibres (mean ± SD), an increase of 251 fibres per mm^2 cross-section area. Over the same period males also grew and recruited fibres, and fibre numbers increased by 27.7%, from $5.8 \times 10^5 \pm 1.2 \times 10^5$ to $7.7 \times 10^5 \pm 1.5 \times 10^5$ (mean ± SD), equivalent to 120 fibres per mm^2 cross-section. This was the only period during which growth was observed for males, and no additional muscle fibres were recruited during the winter and spring months following sexual maturation. The shaded bar (Fig. 3.5) illustrates the maturation and spawning period in males (November–February). In May 2005, little or no testis was observed in *post* spawning males, although they showed the same distinctive

features as sexually matured fish (lean, with a low condition factor and high muscle water content), implying that the spawning period had just ended. Between August and November females only displayed a modest increase in fibre number of 3.3% compared to the first period ($P>0.05$), 53 fibres per mm^2 cross-section. Females recruited fibres throughout the winter, but at a lower rate compared to the summer. Through the next six months female grew by an average of two cm in L_F and showed an 8.4% increased in fibre number to $1.1 \times 10^6 \pm 2.9 \times 10^5$ (mean ± SD). Difference in muscle fibre number between sexes were highly significant ($P<0.001$) at all five sample points. The presence of muscle fibre <10 μm was used as an indication of recent fast muscle fibre recruitment (Fig. 3.6). Females had a significantly higher percentage of fast muscle fibres <10 μm then males throughout the experiment ($P<0.001$, GLM). The presence of fast muscle fibres <10 μm was highest in May 2004, 2.7 ± 1.6% and 3.8 ± 2.1% (mean±SD) for males and females respectively ($P>0.05$). The presence of small fibres decreased through the trial in both sexes, reaching only 0.3 ± 0.1% and 0.8 ± 0.5% (mean ± SD) in May 2005 for males and females respectively.

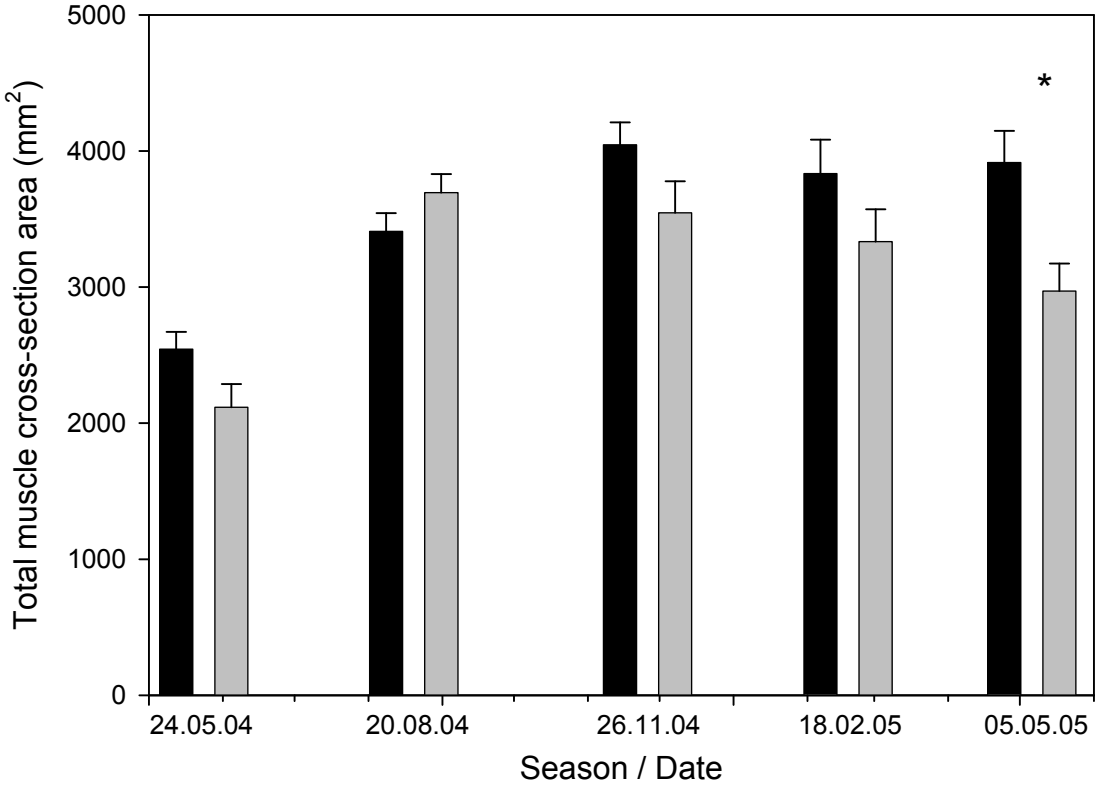

Figure 3.4. Total cross-section area of fast muscle in Atlantic halibut through a one year production cycle. Black and grey bars represent females and males respectively. Significant sexual differences between sexes are indicated with asterisks. Error bars are mean ± SE.

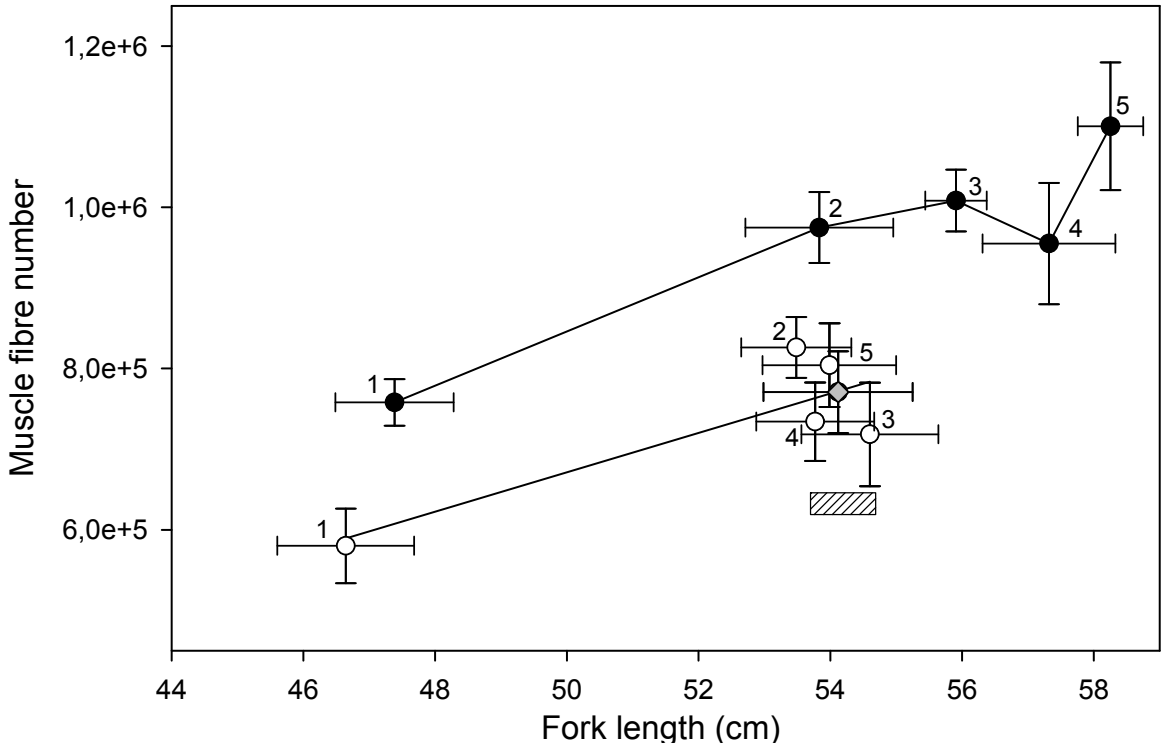

Figure 3.5. The relationship between the number of fast muscle fibres and fork length of commercially farmed Atlantic halibut. Filled (●) and open (o) circles represent females and males respectively, rectangular plot represents the mean of the males in samples 2-5. Bar below male graph represents the point of maturation. Error bars are mean ± SE.

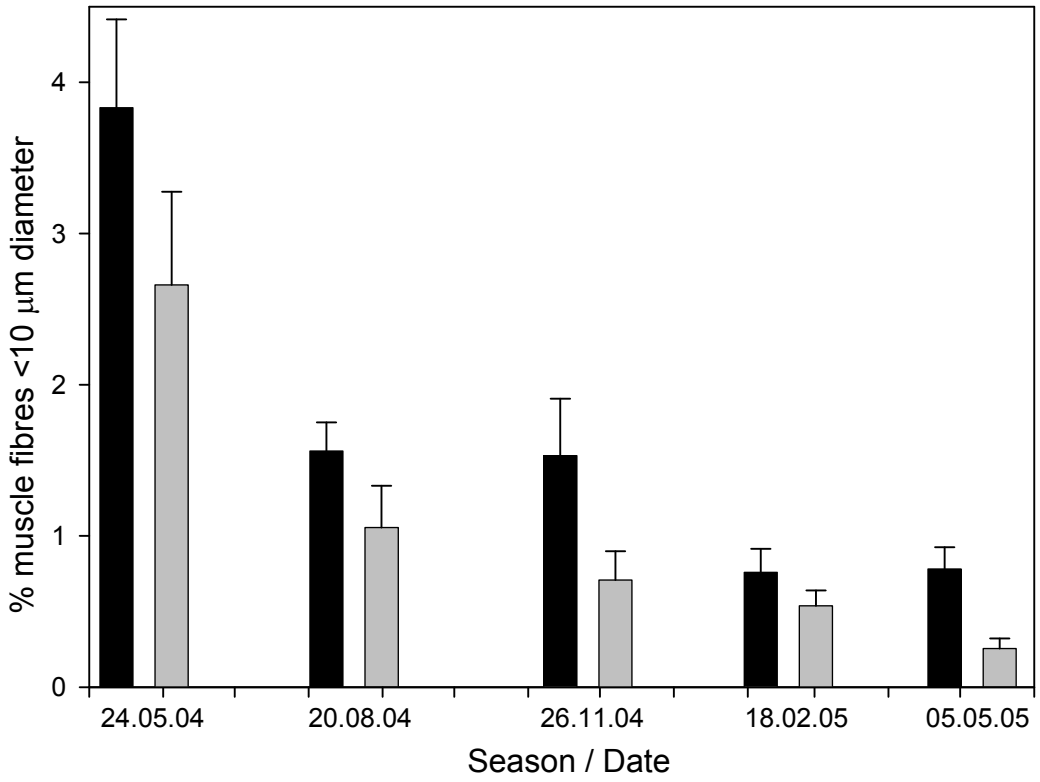

Figure 3.6. Seasonal variation in newly recruited fast muscle fibres (<10 µm diameter) in commercially farmed Atlantic halibut reared under ambient conditions. Black and grey bars represent females and males respectively. Error bars are mean ± SE.

3.4.3.3 Muscle fibre diameter and distribution

Changes in the average diameter of fast muscle fibre for males and females are shown in Fig. 3.7. Average fibre diameter was significantly higher for males than females from August 2004 to February 2005, and fibre diameter increased with growth ($P<0.01$). Males displayed a maximum average diameter at the end of November (65.3 ± 4.3 μm) and females in the middle of February (72.6 ± 8.2 μm). From then, FD_{av} declined in both sexes until May 2005. The FD_{max}, calculated from the 97^{th} percentile of the smooth fast muscle fibre distribution, showed the same trend as for average diameter (Fig. 3.7). Significant differences between males and females with respect to FD_{av} were observed in August and November 2004 and between FD_{max} in August 2004 and February 2005, indicated with asterisks in Fig. 3.7.

The individual and average fast muscle fibre distributions in males and females from the second sample period are shown in Fig. 3.8 A&B. Dotted and solid lines represent individual and average muscle fibre distributions respectively. Females showed a unimodal distribution shifted towards the left (smaller diameter fibres), while males had a bimodal distribution located to the right of females (larger diameter fibres). When global fibre distributions in males and females of the same length were compared, females showed a higher percentage of small fast muscle fibres in the size class of 10-40 μm in diameter (Fig. 3.8 C). The presence of fibres in the smallest size class (10-20 μm) indicates more recent fibre recruitment relative to males. In contrast, males displayed the opposite trend, a significantly lower percentage of fast fibres in the smallest size class, but a larger proportion of fast muscle fibres in the size range 70-150 μm. Regions of the areas where the average probability density function (dotted lines females and solid lines males) fell outside the 100 bootstrap values of the combined populations (males plus females) indicate areas where the fibre distribution differed. The

smooth curves for males and females were significantly different using a nonparametric Kolmogorov-Smirnov test ($P<0.05$).

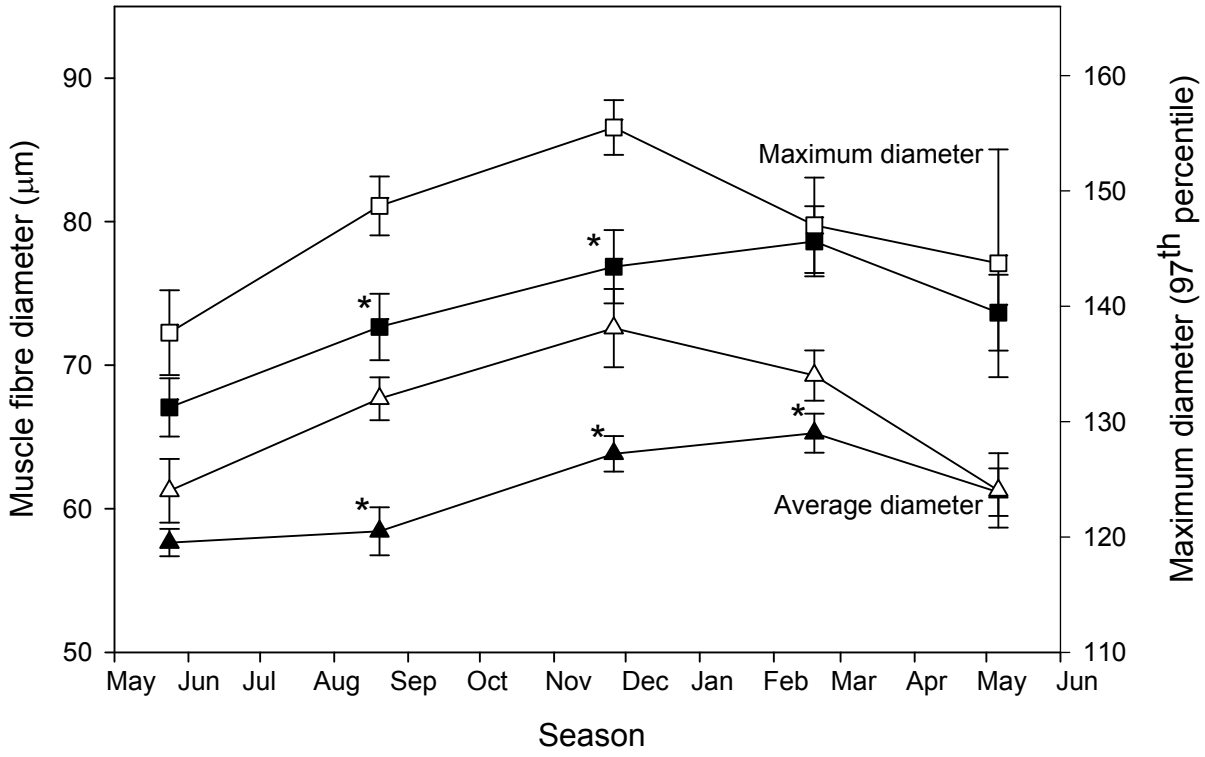

Figure 3.7. Changes in average and maximum fast muscle fibre diameter in relation to season. Black and white plots represent females and males respectively, triangles and quadrangles represent average fibre diameter and maximum fibre diameter respectively. Significant difference between sexes in FD_{av} and FD_{max} is indicated with asterisks (*). Error bars are given as mean ± SE.

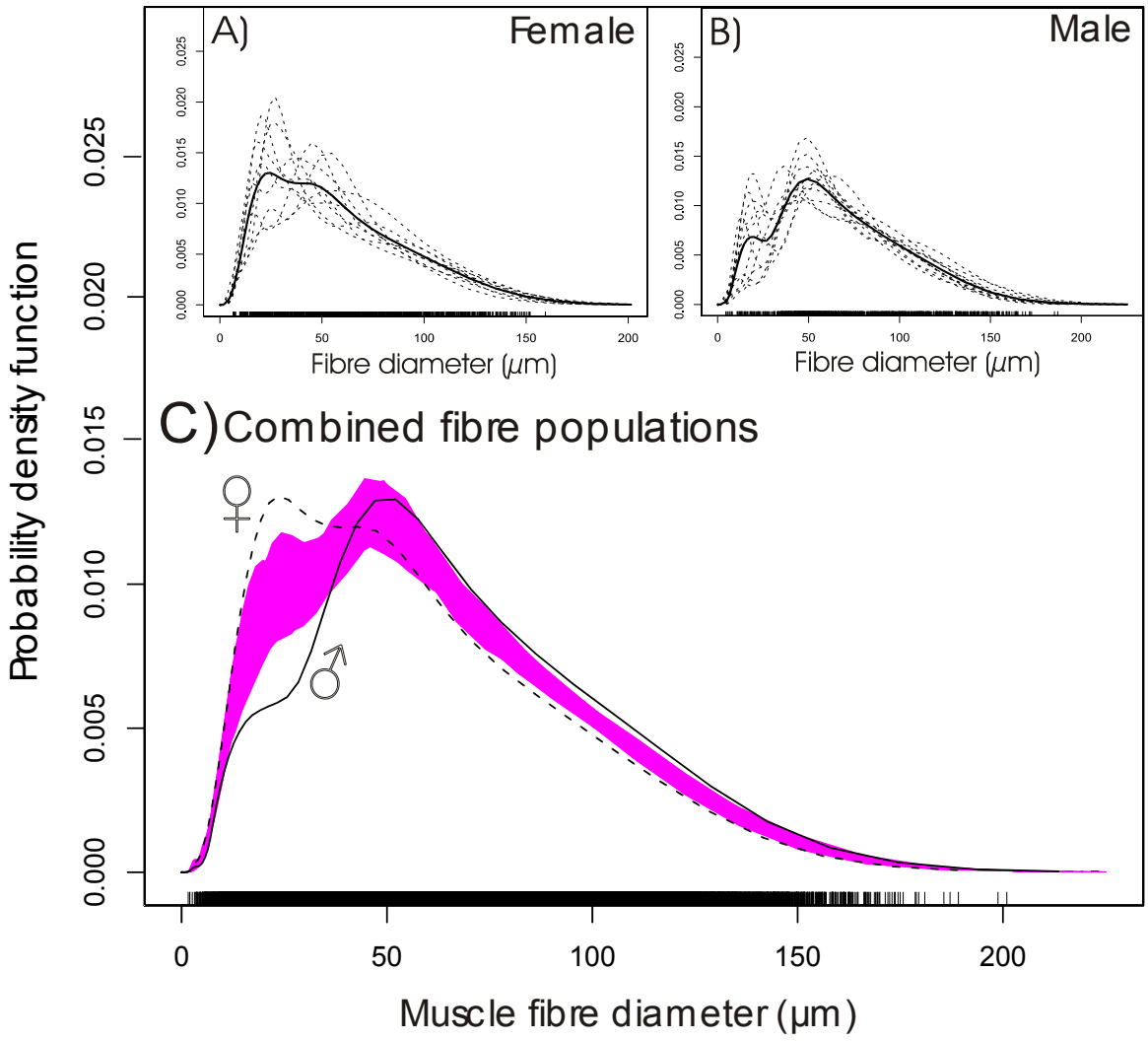

Figure 3.8. Distribution of muscle fibre diameter in female (A, n=10) and male (B, n=10) Atlantic halibut of same fork length. Dashed lines and solid line represent individual and average muscle fibre distribution respectively. C: Average muscle fibre distribution of males and females compared for underlying structural differences. When solid and dotted line falls outside the shaded area a statistical significant difference in muscle fibre distribution between sexes of equal size exist. Solid and dotted line represent males and females respectively.

3.5 Discussion

Early maturation in males is known to represent one of the largest, if not the largest problem in halibut farming in the grow-out phase (Jákupsstovu and Haug, 1988; Björnsson, 1995; Norberg et al., 2001). Environmental factors such as temperature and daylight are thought to be required to initiate the reproductive program in teleosts (Jobling, 1995; Björnsson et al., 1998). Temperature not only affects maturation, but also has a huge impact on growth rate in ectotherms (Hallaråker et al., 1995; Burel et al., 1996; Jonassen et al., 1999; Ruyet et al., 2004, 2006). Based on previous assumptions (Björnsson and Tryggvadóttir, 1996), halibut in the present trial was exposed to suboptimal condition due to low (<6 ºC, mid December–mid April) or high temperature (>11 ºC, mid July–October, see Fig. 3.2 A). Ideally, halibut should be resting on the bottom except when the feed is distributed. Several factors are believed to cause unwanted swimming activity (Karlsen, 2004), but little is known about halibut behaviour. Individuals frequently seen at the surface have a poorer growth rate compared to individuals resting on the bottom (Kristiansen et al., 2004). Swimming activity was not systematically monitored during the present trial, but a relatively high activity level was observed in periods during the winter due to poor weather conditions and maturation. A higher activity level in males combined with maturation and sub-optimal environmental conditions (Haug, 1990; Björnsson, 1994) are likely causes of the winter growth stagnation observed in this species, especially in males. One possible explanation for the early maturation in male halibut is that the threshold for maturation is reached at an earlier age due to the excess of high-energy feed (Norberg et al., 2001; Imsland and Jonassen, 2005). Nordberg et al. (2001) reported a growth reduction three months *pre*-spawning and during the spawning period, which is in good agreement with the present observation (Fig. 3.2 B). Males are known to display more or less the same growth rate as females until maturation

(Jákupsstovu and Haug, 1988; Norberg et al., 2001), which is also supported by the present findings (Fig. 3.2 B). When male halibut started investing energy into the gonads in the period from November 2004 to April 2005, it was reflected in the muscle tissue as a total halt in muscle fibre recruitment (Fig. 3.5). This has not been reported before, and provides at least part of the explanation for the growth stagnation observed.

Biomass loss is a serious problem for a farmer who is trying to make halibut farming a profitable business (Fig. 3.2 B). This not only results in direct biomass loss, but also as an increase in production time with a considerable fraction of the best growth period spent recovering from muscle depletion the previous winter. Gonad investment in males during the winter and loss of appetite resulted in an increase in water content and a decrease in condition factor (Table 3.1), showing a significant negative correlation (r^2=-0.51, Fig. 3.3). A cod with muscle water content above 81% is considered as starved (Love, 1988). If the same is true for halibut, the average muscle water levels in males in May 2005 of 81.8 ± 1.3% indicated muscle depletion. Sexual maturation is known to result in a major increase in muscle water content in some marine fish (Love, 1988).

A right-hand shift in muscle fibre distribution was observed in both sexes until February 2005, and is caused by an increase in fibre diameter (hypertrophy) (results not shown). During the winter period a decrease in average muscle fibre diameter was seen, especially in males, resulting in a shift towards the left indicating depletion. This is confirmed when looking at the decrease in maximum fibre diameter calculated from the 97th percentile (Fig. 3.7), indicating shrinkage of the fibres due to low temperatures, short day length and low feeding activity. The common carp (*Cyprinus carpio*) is capable of going through long periods without food, showing little or no signs of muscle fibre depletion (Love, 1980). In contrast, white sturgeon

(*Acipenser transmontanus*) displayed signs of depletion after only two weeks of fasting (Kiessling et al., 1993). During starvation periods either caused by maturation, spawning or lack of food, fish will use the fast muscle as a source of energy when a critical level is reached (Johnston and Goldspink, 1973, Johnston, 1981). Black and Love (1986) reported that cod preferentially utilise liver lipids during starvation followed by liver glycogen, fast muscle glycogen, slow muscle glycogen and protein respectively. Several studies have shown that white muscle proteins are mobilized before red muscle during starvation and this can be seen as muscle fibre shrinkage due to the breakdown of myofibrils (Johnston 1981; Beardall and Johnston, 1983, Love, 1988; Kiessling et al., 1993). 14 week starvation of plaice (*Pleuronectes platessa*) produced a 6% increase in the water content of fast muscle, whereas slow muscle was relatively unaffected (Johnston and Goldspink, 1973). During starvation in white sturgeon proteins from dorsal fast muscle are mobilized to energy, but myofibres close to the lateral line were apparently spared (Kiessling et al., 1993).

Female halibut increased in length over the whole trial although growth in Mb was minimal over the winter months. Males on the other hand only grew during the first fall prior to sexual maturation. Sexual dimorphism in body size is a common phenomenon in teleosts, with males often larger than females (Quinn and Foote 1994; Toguyeni et al., 1997; Bonnet et al., 1999). For flatfish species including halibut (*Hippoglossus hippoglossus*) (Jákupsstovu and Haug, 1988), dab (*Limanda limanda*) (Lozán, 1992), and turbot (Scophthalmus maximus) (Imsland et al., 1997) the opposite is true, females are larger then males. So far muscle fibre distribution has only been examined in one example of sexual dimorphism in body size, namely the Argentine hake (*Merluccius hubbsi*) (Calvo, 1989). In the present study, fibre number prior to maturation in male Atlantic halibut was significantly greater (28%, August 2004) in females than males (Fig. 3.5, $P<0.001$). This difference in fibre number was

accentuated after maturation in males because fibre recruitment ceased in males, but continued in females once day length started to increase in the spring (Fig. 3.5). These differences in fibre recruitment between sexes were reflected in the distribution of fibre diameters, with a higher proportion of the smallest size class in females compared to males (Fig. 3.8). FD_{av} and FD_{max} were also greater in males than females of the same body size (Fig. 3.8, $P<0.01$). The halt in fibre recruitment in males with maturation is likely to be temporary since it is known that FN_{max} in males is ~100.000 fibres higher compared to the FN_{max} in this study (Hagen et al., 2008a). A seasonal decline in the rate of fibre recruitment during the winter is a common feature of fish from Northern latitudes resulting from low temperatures and short days. For example, in the seawater stages of Atlantic salmon continuous light treatment is able to partially overcome the winter depression of muscle fibre recruitment (Johnston et al., 2003c).

In conclusion, a sexual dimorphism in muscle fibre growth pattern is present in Atlantic halibut, with males having a significant lower fibre number and a significant higher average diameter. When size and TCA was accounted for, females had on average 12% more fast muscle fibres than males through the trial, possibly allowing females to have a greater growth potential than males. We believe that this difference is under genetically control as previously proposed for another species (Holtby and Healey, 1990) and is not an effect of sexual maturity, even if sexual dimorphism in body size is not expressed before this stage.

Chapter 4

Biochemical and structural factors contributing to seasonal variation in the texture of farmed Atlantic halibut (*Hippoglossus hippoglossus*) flesh.

4.1 Abstract

Factors contributing to the texture of fish flesh including pH, water content, density of fast muscle fibres and the concentration of collagen and hydroxylysyl pyridinoline (PYD) cross-links, were investigated *post-rigor* in commercially farmed Atlantic halibut (*Hippoglossus hippoglossus*). The fish were sampled every quarter for a 12 month period from May 2004 to May 2005. Hydroxyproline (HYP) as a measure of collagen and PYD were determined using an HPLC method. An ANCOVA model with fork length and season as covariates were used to explore the seasonal effects on texture, pH, muscle fibre density, alkaline-insoluble collagen (a-i HYP), alkaline-soluble collagen (a-s HYP) and PYD cross-links. A multiple linear regression (MLR) showed that the most important factors contributing to texture were PYD > water (%) > a-i HYP > fibre density, while pH and a-s HYP did not show any correlation to texture. The outcome of the MLR was consistent with the results obtained from a Partial Least Squares regression (PLS). The contribution of fast muscle fibre density to texture was found to vary between sexes and with season, contributing more in males and in the spring. The most important parameter affecting texture was PYD, explaining 64% ($P<0.001$) of the total variation in a linear regression analysis respectively. It is concluded that cross-linking processes are of great importance for the rigidity and strength of the collagen in Atlantic halibut flesh. Farmed halibut should be harvested in the fall or early winter when texture and nutrition are good in order to obtain optimal quality.

4.2. Introduction

Texture is one of the most important sensory characteristics determining the eating quality of fish flesh. During the last decade texture and the factors which are believed to influence texture have been intensively studied including the effects of season (Touhata et al., 1998; Espe et al., 2004; Roth et al., 2005), stress (Stien et al., 2005; Kristoffersen et al., 2006), exercise (Bugeon et al., 2003), temperature and pH (Love et al., 1972, 1974; Roth et al., 2005), chemical composition (Love et al., 1974; Dunajski, 1979; Andersen et al. 1997; Fauconneau et al., 1995), muscle cellularity (Hatae et al., 1990; Hurling et al., 1996; Johnston et al., 2000d; Periago et al., 2005), and collagen content (Hatae et al., 1986; Sato et al., 1986, 1991; Johnston et al., 2006a; Li et al., 2005). The texture of fish flesh also varies with body size (Haard, 1992; Fauconneau et al., 1995) and between species (Sato et al., 1986). This underlies the importance of research on each of the major commercial species, particularly farmed fish where there is a measure of control over the rearing and slaughter conditions. Atlantic halibut (*Hippoglossus hippoglossus*) is one of the marine species which is being considered for large-scale farming in Norway (Boxaspen et al., 2005), but little work has been done on understanding the textural characteristics of its flesh (Olsson et al., 2003a; Haugen et al., 2006).

The extracellular matrix (EMC) of fish has a complex organisation and compromises several molecular species including, collagen, noncollagenous glycoproteins and proteoglycans, often referred to as ground substance (Kierszenbaum, 2002). The bulk of the collagen in fish is located in the myocommata separating the muscle segments, the myotomes. Apart from the myocommata, collagenous sheets surrounds individual muscle fibres (endomysium) and bundles of muscle fibres (perimysium) (Bremner and Hallet, 1985). The collagen super-family comprises 85% of the relative area (calculated using TEM) of the extracellular matrix

(EMC) (Ofstad et al., 2006). Fish skeletal muscle has only ~3% of the collagen content of mammalian muscle (Sato et al., 1989b). Collagen type I and V have been identified respectively as the major and minor constituents of fast muscle which comprises the major portion of fish fillets (Sato et al., 1988, 1989b). In fish type III, VI and IV have also been characterized (Brüggemann and Lawson, 2005), and the amount of collagen in the EMC are found to vary between fish species (Sato et al., 1986; Yoshinaka et al. 1988, 1990; Johnston et al., 2006a). The myocommata has been reported to increase in thickness with age (Love et al., 1976), but relatively little is known corresponding changes in its molecular composition (see review of Bailey et al., 1998, Bailey 2001). The most important and striking alteration in the ECM with age is probably the formation of matured intermolecular cross-links between collagen fibres (Bailey 2001), which contributes to maintainance of the physical structure and rigidity of the collagen matrix (see chapter 1).

The overall aim of the present study was to investigate the relative contributions of *post-rigor* pH, muscle cellularity, and the concentrations of collagen and pyridinoline cross-links to the texture of flesh in farmed Atlantic halibut in relation to the season of slaughter. Since Atlantic halibut show sexual dimorphism in growth and muscle fibre recruitment patterns (Hagen et al., 2006), the influence of sex on texture and the chemical composition of the flesh were also investigated.

4.3. Material and methods

4.3.1 Fish husbandry

For details about fish husbandry see section 3.3.1.

A total of 100 fish were sampled from Aga Marin's commercial production. Fish were shipped to Bodø (Norway) on the public speed boat, arriving Bodø University College approximately 20 h *post-mortem*. Biological data and samples for morphometric studies were taken (see Hagen et al., 2006) and the fish were stored on ice for a total of 4 d in a cold chamber (2 ºC) in plastic bags until further processing *post-rigor*.

Fast muscle fibre density was calculated as followed:

$$\text{Fibre density} = \frac{FN_{measured}}{Area_{cum}}$$

were $FN_{measured}$ is the number of fast muscle fibres measured and $Area_{cum}$ is the cumulative muscle cross-sectional area in mm^2.

4.3.2 Instrumental texture measurement

Texture (shear work) measurements were made anterior of the 0.55 L_F cut (see Figure 4.1). The texture of the flesh was determined in duplicate using a TA-XT2 texture analyser with Texture Expert Exceed 2.52 software from Stable Micro Systems (Surrey, England) using a shear test (see Johnston et al., 2004b) Muscle blocks were cut from the deeper layer of dorsal fast myotomal muscle using a standardized frame (2.5x2.5x1 cm) (Figure 4.1). The fish were filleted in groups of five and kept on ice prior to texture measurements to ensure fillets were

not exposed to temperature fluctuations. The probe used in the shear test was a 60º knife edge blade (not sharpened). A 25 kg load cell was used and test speed was set to 1 mm s^{-1}. The texture profile was analysed using the Texture Expert Exceed 2.52 software, to determine maximum shear force described as the highest peak value measured in Newtons (N) whilst the area under the curve during shearing until fracture corresponds to the work done in millijoules (mJ) (Veland and Torrissen, 1999).

A pilot study containing 5 halibut of similar size (obtained from Mørkvedbukta Research station, Bodø University College) was carried out before the trial. The goal of the pilot study was to evaluate the texture variation over the fillet in front of the 0.55 L_F cut and identify which region was most suitable for texture measurement (e.g. having least variation between replicates).

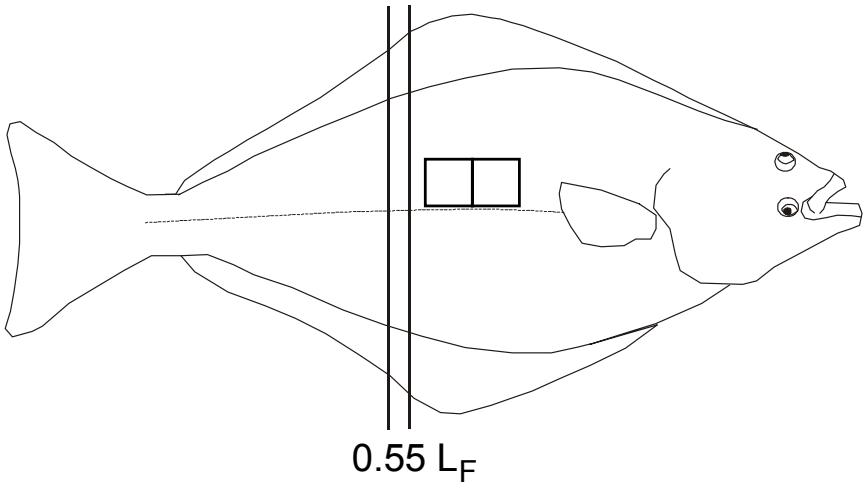

Figure 4.1. Sample sites for instrumental texture measurement (quadrangles) and tissue samples for the collagen and cross-link assays. Tissue samples for the collagen and cross-link were taken from the minced fillet anterior to the 0.55 L_F cut, both dorsal and ventral sides of the body.

4.3.3 Sample preparation

The location of samples taken for biochemical analyses is shown in Figure 4.1. Red muscle close to the horizontal septum was discarded and the fast (white) muscle was minced using a food processor for one minute at full speed (10000 rpm). The pH of the mince was measured using a PHM92 pH meter (Radiometer Analytical, Copenhagen, Denmark). 1 g of the mince was accurately measured into four 50 ml centrifuge tubes which were stored in a -80 ºC freezer until determination of hydroxyproline (HYP) and hydroxylysyl pyridinoline (PYD). Muscle water content were analysed in duplicates of 5 g mince, as described in section 3.3.3.

4.3.4 Collagen and crosslink assay

The assay used for collagen and crosslink analyses was as described by Li et al. (2005). 9 ml of cold deionised water (4 ºC) was added to each of the 50 ml tubes containing exactly 1 g of fast muscle. The sample was homogenised at full speed (22000 rpm) for one minute using a Polytron (mod. PT 1200CL, knife (mod. PT-DA 1207/2EC)). After homogenization 10 ml of cold 0.2 M NaOH was added to each tube, making it up to a total volume of 20 ml 0.1 M NaOH solution. The tubes were then placed on a rotator (Stuart rotator SB3, Bibby Sterilin LTD, Straffordshire, UK) in a cold room (2 ºC) for 4 h. Immediately after NaOH extraction tubes were centrifuged (Eppendorf mod. 5804R) at 10000 g for 30 minutes at 4 ºC. From the supernatant, containing the a-s collagen fraction, 0.5 ml was removed to a 4 ml glass vial with screw cap containing 0.5 ml of 12 M HCl. The remaining of the supernatant was gently poured off. The a-i collagen fraction (pellet) was re-suspended in 2 ml of 6 M HCl and transferred to an 8 ml hydrolyse vial with screw cap. The a-s and a-i collagen fraction was hydrolysed in an oven at 110 ºC for 20 h. 10 µl and 2 µl was then removed from the a-s and a-

i collagen fraction respectively, and re-suspended in an 1.5 ml eppendorf tube containing 100 µl of deionised water. The rest of the a-i collagen fraction, was used for hydroxylysyl pyridinoline (PYD) cross-link analysis, and was dried down together with the re-suspended a-s and a-i collagen samples using a vacuum rotary evaporator (Jouan RC1022, Nantes, France). The dried PYD cross-link fraction was diluted in 3 ml consisting of deionised H_2O, acetic acid and butanol (v/v/v, 1:1:4). For PYD extraction a cellulose column (Varian Ltd., Oxford, UK) was used (Black et al., 1988; Eyre et al., 1984). The cellulose columns were attached to a vacuum block (vacuum (15 Hg) generated by a water suction) (Varian/Holger, Oslo, Norway), and the re-suspended PYD fraction was loaded on to the column, followed by a series of washings (3x3 ml of 1:1:4 buffer). The PYD fraction was eluted from the cellulose column by adding deionised water which was collected in a glass tube, dried down and re-suspended in 300 ml injection sample buffer (5% CH_3CN, 1% HFBA) containing 250 nM pyridoxine (internal standard). The re-suspended PYD samples were filtered through 4 mm syringe filters (0.2 µm PVDF membrane) into micro-inserts in HPLC vials before being loaded on to the HPLC. Before each HPLC run (PYD and HYP) a validation sample was added in front of the sample series to check for any instrumental inaccuracies.

The HPLC system (ProStar, Varian/Holger, Oslo, Norway) consisted of an 800 interface box, 410 autosampler with intergraded sample tray cooling, 240 Inert SDM Solvent delivery module and a 363 fluorescence detector. System operation, data calculation and processing were managed using Star system control v. 6.30, run under windows XP. The column (4.6x150 mm; Micropack ODS-80 TM; 5 µm spherical silica particles with 80 Å pore size; Tosoh Bioscience (Varian/Holger, Oslo, Norway)) temperature was held constant (40 ºC) using the autosampler column oven. Eluent flow rates used for the PYD assay are given in Table 4.1, and are the same described by Li et al. (2005). Excitation (λ_{ex}) and emission (λ_{em}) wavelengths were set to 295 and 400 nm respectively. Results were calculated based on a 6

point standard curve (r^2=0.999) made of purified hydroxylysyl pyridinoline (kindly gifted by Simon Robbins, Rowler Institute, Aberdeen) using the Star system control v. 6.30 software.

The samples for the HYP analysis (dried a-s and a-i collagen fraction) were re-suspended in 200 µl 0.1 M borate buffer (pH 11.4) containing 10 µM homargenine (internal standard) and 200 µl 6 M fluorenylmethoxycarbonyl (FMOC) derivatization solution (Bank et al., 1996). The derivatization was terminated after 40 minutes and the excess reagent was removed by adding 0.5 ml pentane and the mixture was vortexed vigorously. The supernantant pentane layer (containing the excess reagents) was removed using a pasteurized glass pipette and the pentane extraction step was repeated twice. 50 µl of the derivatized a-s and a-i collagen fraction was re-suspended in 1 ml of injection buffer consisting of 25% (v/v) CH_3CN in 0.25 M boric acid (pH 5.5) in a HPLC vial and loaded on to the HPLC. Eluent flow rates used for the HYP assay is given in Table 4.2, and is the same described by Li et al. (2005). The Star system control software was used to identify and quantify the homargenine and hydroxyproline peak based on a 6 point standard curve (r^2=0.999) made of collagen hydrolysate (Sigma, Oslo, Norway). Excitation (λ_{ex}) and emission (λ_{em}) wavelengths were set to 254 nm and 630 nm respectively. The chromatograms for a-i and a-s HYP and PYD are shown in Figure 4.2.

Table 4.1. Flow rate, eluent mixture (%) and setup during the hydroxylysyl pyridinoline assay including the estimated amounts of eluents needed pr sample.

Time (min)	Flow rate (ml/min)	Eluent A (%)	Eluent C (%)
0	1	100	0
15	1	100	0
30	1	85	15
31	1.4	20	80
40	1.4	20	80
41	1.4	100	0
49	1.4	100	0
Vol/sample		46 ml	12.5 ml

Table 4.2. Flow rate, eluent mixture (%) and setup during the hydroxyproline (collagen) assay including the estimated amount of eluents needed pr sample.

Time (min)	Flow rate (ml/min)	Eluent A (%)	Eluent B (%)	Eluent C (%)
0	1.4	75	0	25
11.5	1.4	60	0	40
13	1.4	60	0	40
13.1	1.4	0	64	36
18	1.4	0	62	38
25	1.4	0	30	70
30	1.4	0	25	75
32	1.4	0	25	75
32.1	1.4	75	0	25
39	1.4	75	0	25
Vol/sample		21 ml	12 ml	24 ml

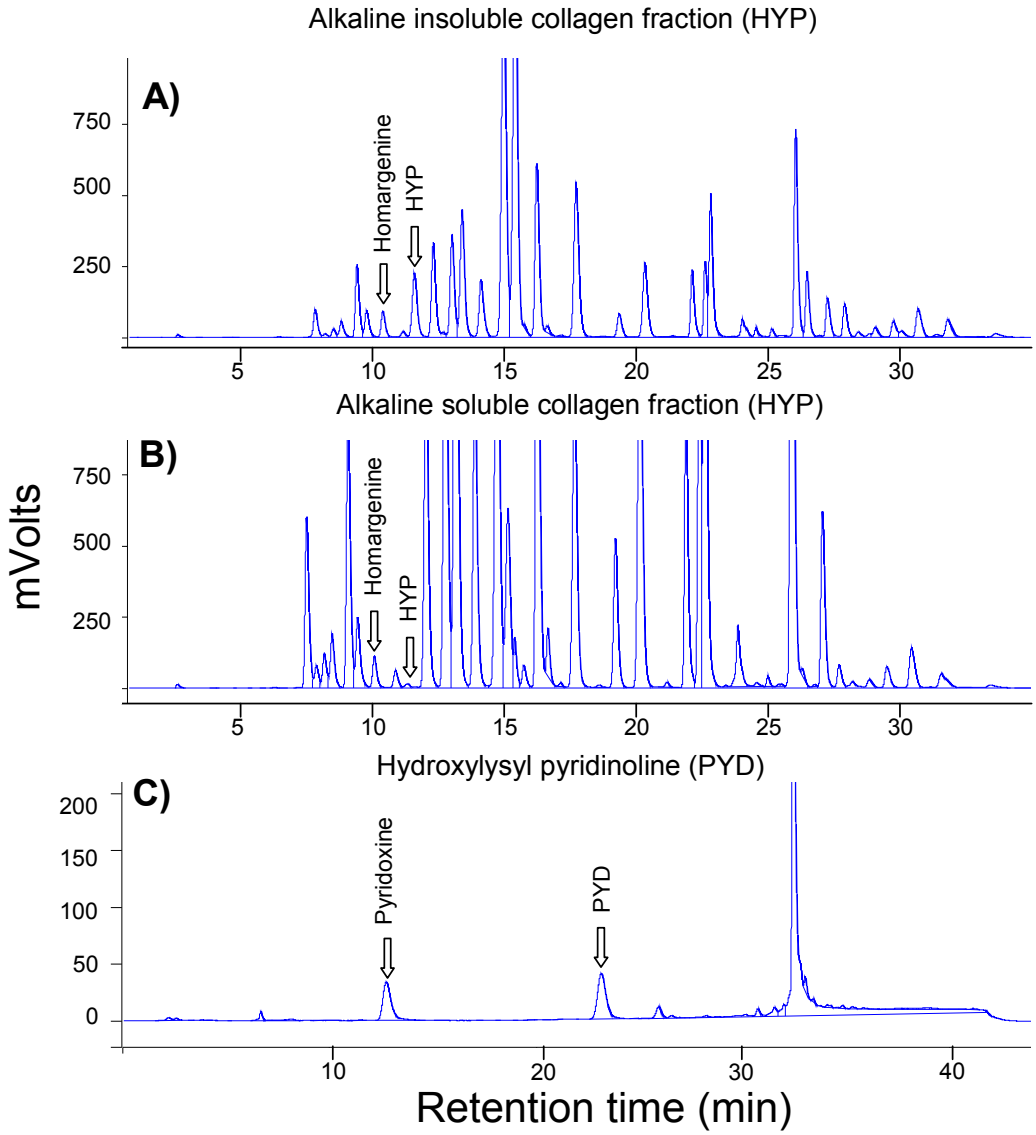

Figure 4.2. Chromatogram showing the elution profiles and retention times of hydroxyproline in (A) the a-i, (B) a-s tissue fractions (homoargenine used as an internal standard) and (C) the PYD cross-link fraction (pyridoxine used as an internal standard). The peaks of interest in the different chromatograms are indicated with an arrow.

4.3.5 Statistics

The statistical tests were preformed using Minitab (v 13.20, Minitab Inc, USA), SigmaPlot (v. 8.0, SPSS Inc, USA) and Unscrambler v. 9.2 (CAMO Software AS, Oslo, Norway. The size distribution of the fish were analysed using a General linear method (GLM). An ANCOVA model (independent variable = season + sex + season * sex) was used to analyse the seasonal effects on the variables. Fork length and season were used as covariates. PYD and texture data (pooled data) were subjected to a square root transformation, while a-i HYP were subjected to a log transformation (left skew of distribution) to comply with the assumptions for the test. Scatterplot matrices of all independent variables were used to study the relationships between them. All independent variables and their relative importance to texture (dependent variable) were further explored using a multiple linear regression (MLR). A connection between PYD and water content and a-i HYP was observed from the scatterplot matrices. To overcome the over lapping effect of one variable on another, two MLR were preformed, one with PYD included, excluding a-i HYP and water and one MLR without PYD. A linear regression analysis was used to show the most important relationships graphically. Unscrambler was used to compose a Partial Least Squares regression (PLS). The PLS is a very convenient and powerful tool where it is not necessary to take covariates into consideration and the results confirmed those of the MLR. The PLS gave a general overview of how the variables interacted and identifying which of the variables that where related to the texture of the fish.

4.4 Results

4.4.1 Validation of the texture over the fillet (pilot study)

The pilot study showed that the texture of the Atlantic halibut flesh varied considerable in front of the 0.55 L_F cut which underlines the importance of being accurate and consistent when the block for texture measurement were taken (Fig. 4.1). The texture of the flesh increased in a posterior-anterior direction, and had least variation in region A, closest to the 0.55 L_F cut (Fig. 4.3). Region A was therefore standardised as texture sampling location for the experiment.

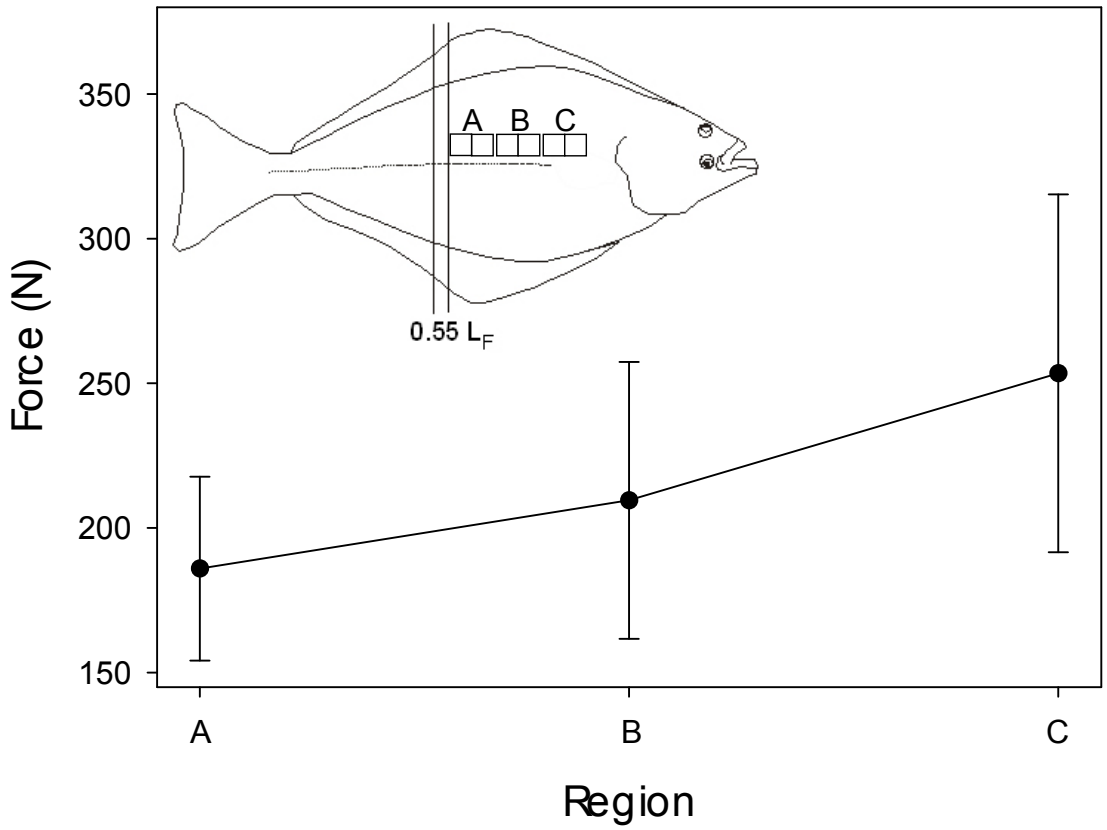

Figure 4.3. Validation of the variance in texture over the fillet in front of the 0.55 L_F (region A, B & C) cut showed that both texture and variance increased in a posterior-anterior direction. The region having the least variation in texture was region closest to the 0.55 L_F cut (region A). The insert shows the three different regions (A, B & C) where duplicate texture measurements were preformed (mean ± SD).

4.4.2 Seasonal variation in texture and pH

Fillet texture was not significantly different between male and female fish (P=0.462, ANCOVA), and data were therefore combined (Table 4.3). There was a significant seasonal effect on texture during the trial (P<0.001, ANCOVA). Texture was similar in May and August 2004, but increased by 2-fold from 66.7 to 134.7 mJ between August 2004 and May 2005 (Table 4.3). Fast muscle pH did not show any significant variation with season or between sex (P>0.1, ANCOVA).

4.4.3 Effect of season on muscle collagen and pyridinoline cross-link concentration

No differences in the a-s HYP or a-i HYP concentrations were observed between male and female halibut (P>0.2, ANCOVA), and data were therefore combined for further analysis of seasonal effects. There was a significant seasonal effect on the concentration of a-s HYP (P<0.001, ANCOVA) (Table 4.3). The a-s HYP was similar between May and August 2004 (Table 4.3), but decreased 2-fold by mid-winter the following year. In May 2005 the a-s HYP concentration was not different from that of May 2004 (Table 4.3). The seasonal effect on a-i HYP fraction showed a similar trend to that of a-s HYP, but not as obvious, decreasing during the fall and increasing during the winter, having the highest value in May 2005 (P<0.01, ANCOVA) (Table 4.3).

The concentration of PYD showed a highly significant seasonal effect (P<0.001, ANCOVA). The concentration of PYD in May and August 2004 was similar, but increased by ~70% in May 2005 (Table 4.3). In the same period a drop in total protein content was observed,

Table 4.3. Biological data (fork length, body mass and sexual distribution), analyzed variables and their changes according to season. Shear work (mJ), *post-rigor* pH, muscle fibre density (FD), alkaline soluble (a-s) and insoluble (a-i) HYP, total HYP and hydroxylysyl pyridinoline (PYD) cross-links. Numbers in brackets are percentage of total HYP. Values represent mean ± SD.

	24.05.04	**20.08.04**	**26.11.04**	**18.02.05**	**05.05.05**
Sex (# fish)	13♀ / 7♂	9♀ / 11♂	11♀ / 9♂	10♀ / 10♂	13♀ / 7♂
Body mass	1255 ±275	1942 ± 314	2208 ± 344	2036 ± 438	2079 ± 538
Fork length	47.1 ± 3.0	53.6 ± 2.9	55.0 ± 2.4	55.6 ± 3.4	57.0 ± 2.9
Shear work (mJ)	82.7 ± 29.2	66.7 ± 8.9	76.0 ± 27.1	98.1 ± 27.4	134.6 ± 28.4
pH	6.48 ± 0.1	6.45 ± 0.1	6.33 ± 0.1	6.34 ± 0.1	6.47 ± 0.1
FD (fibres/mm^2)	293 ± 40	252 ± 46	229 ± 41	233 ± 34	277 ± 57
a-s HYP (µmol g^{-1} dry mass)	12.6 ± 2.6 (~51%)	12.5 ± 2.9 (~53%)	7.0 ± 3.8 (~39%)	6.3 ± 2.3 (~32%)	12.3 ± 4.5 (~45%)
a-i HYP (µmol g^{-1} dry mass)	12.2 ± 4.9 (~49%)	11.2 ± 3.1 (~47%)	10.9 ± 3.8 (~61%)	13.2 ± 4.5 (~68%)	14.9 ± 6.9 (~55%)
Total collagen	24.7 ± 6.0	23.7 ± 5.1	17.9 ± 6.3	19.5 ± 3.6	27.2 ± 9.4
PYD cross-links (pmol g^{-1} dry mass)	1029 ± 308	849 ± 138	934 ± 167	1071 ± 248	1458 ± 415
Ratio PYD / A-i HYP (~)	1:12000	1:13000	1:12000	1:12000	1:10000

being most obvious in males (~20%) ($P<0.005$, see Hagen et al., 2006). Thus, the protein fraction of the fast muscle was decreasing and becoming enriched for PYD during the winter ($r^2=0.64$, $P<0.001$, not shown). At the same time an increase in water was seen, with a difference between sexes during the late winter (see Hagen et al., 2006), due to protein depletion in males. Water content also showed a highly significant correlation to PYD ($r^2=0.61$, $P<0.001$, not shown).

4.4.4 Impact of pH, fibre density, water, collagen and pyridinoline cross-link on texture

To identify which independent variables affected texture, pH, fibre density, a-i HYP, a-s HYP, water and PYD were further investigated using MLR, with backward exclusion of independent variables. Since PYD was found to correlate with water and a-i HYP, two MLR with PYD excluding a-i HYP and water and one only without PYD was preformed. The outcome of the MLR showed that the independent variables being most important to texture were: PYD > water (%) > a-i HYP > fibre density, while pH and a-s HYP did not show any correlation to texture. The output of the MLR analysis was confirmed using a PLS. The results of the PLS gave a good overview of which of the variables that affected the texture of the flesh, and Principal Component 1 (PC1) and Principal Component 2 (PC2) explained 59% of the total variation (Fig. 4.4). A linear regression analysis was preformed to show the relationships between the dependent and the most important independent variables. The water content of the flesh correlated with the texture of females (Fig. 4.5 A) and males (Fig. 4.5 B), explaining 53% and 60% of the total variation respectively.

Muscle fibre density was found to be significantly different between males and females ($P<0.01$, n=98, ANCOVA, not shown). On a group level the impact of fibre density to texture changed during the season, from being not significant in August and November 2004 to

significant in May 2005, explaining 31% of the total variation (r^2=0.31, P<0.001, n=20) (not shown). When male and female where plotted separately, male displayed the strongest correlation (r^2=0.19, P<0.01, n=44), explaining ~19% of the variation (Fig. 4.6). The total HYP fraction displayed a weak correlation to fibre density (r^2=0.14, P<0.01, pooled data, n=98, not shown).

The a-i HYP fraction made a similar contribution to texture in both male and female fish, and the pooled data explained ~24% of the total variation (Fig. 4.7 A). On the other hand, a-s HYP did not correlate with texture and the total HYP fraction showed a weaker significant correlation (r^2=0.15, P<0.001, n=98, not shown) than the a-i HYP fraction. The single parameter showing the strongest correlation to texture was the concentration of PYD, explaining as much as 64% of the total variation (Fig. 4.7 B). All equations for the linear relationships are given in the respective figure legends. PYD concentration increased with the age of the fish and this was reflected as an increase in fillet firmness between May 2004 and 2005 (Table 4.3).

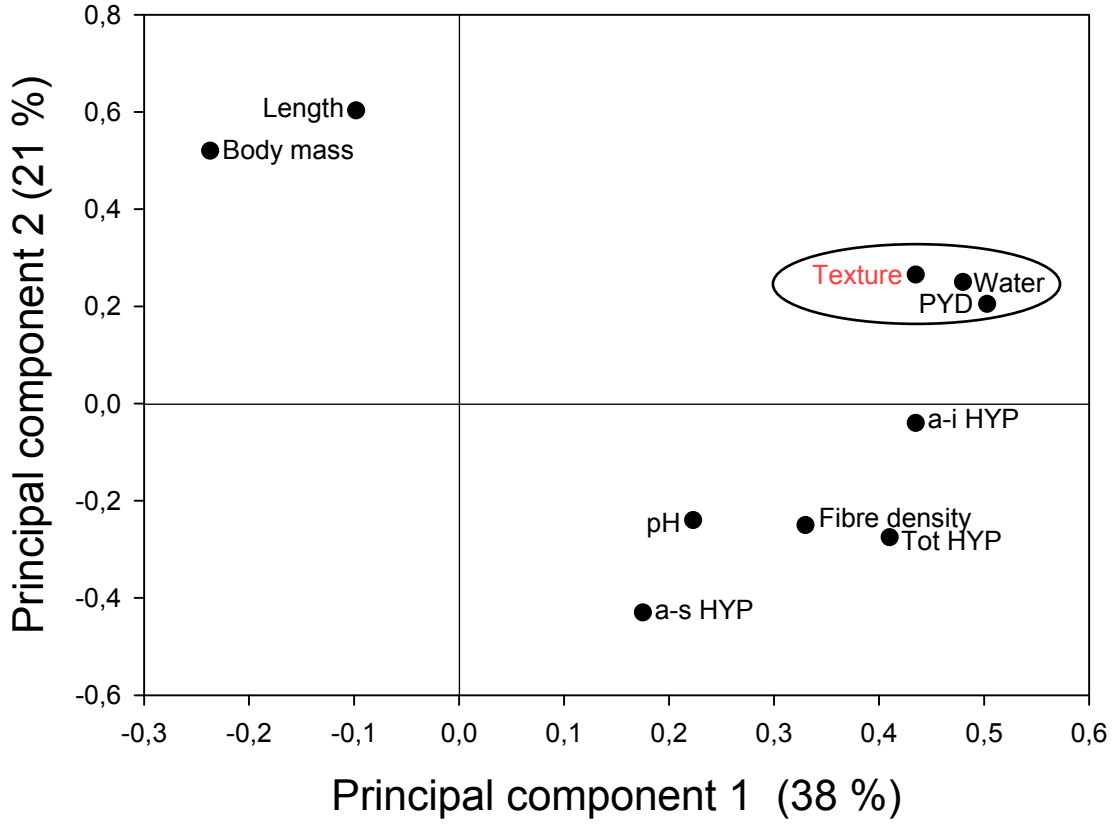

Figure 4.4. A PLS illustrating the relationship between the different variables. The variables that affected texture (in red) the most are circled. The PLS explained 59% (PC1 + PC2) of the total variation.

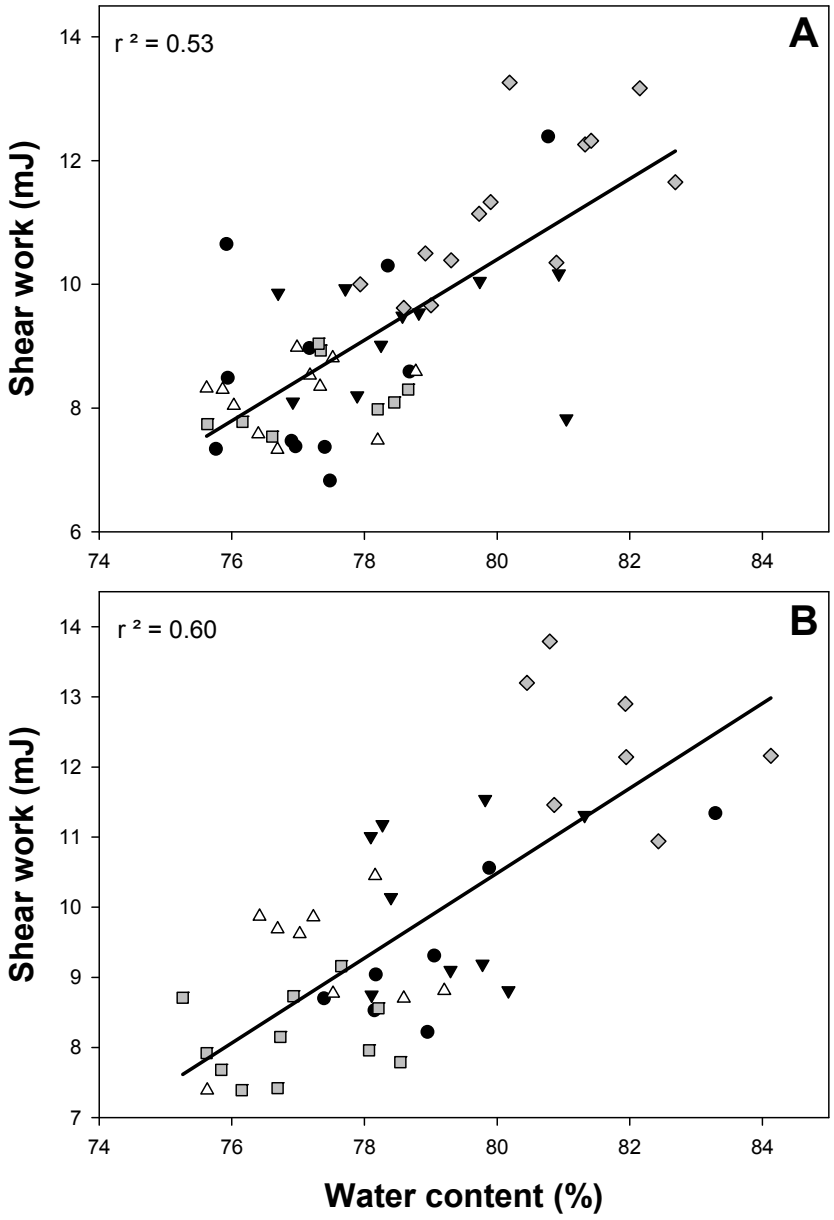

Figure 4.5. A: Linear regression analysis of water (%) and texture (square root transformation) in females (n = 54, $r^2 = 0.53$, $P < 0.001$). A first order linear regression was fitted to the data, shear work = 0.653 (water) – 41.804. B: Regression analysis of water (%) and texture in males (n = 44, $r^2 = 0.60$, $P < 0.001$). A first order linear regression was fitted to the data, shear work = 0.605 (water) – 37.924. Symbols indicate the different samples, 24.05.04 (●), 20.08.04 (■), 26.11.04 (Δ), 18.02.05 (▼), 05.05.05 (♦).

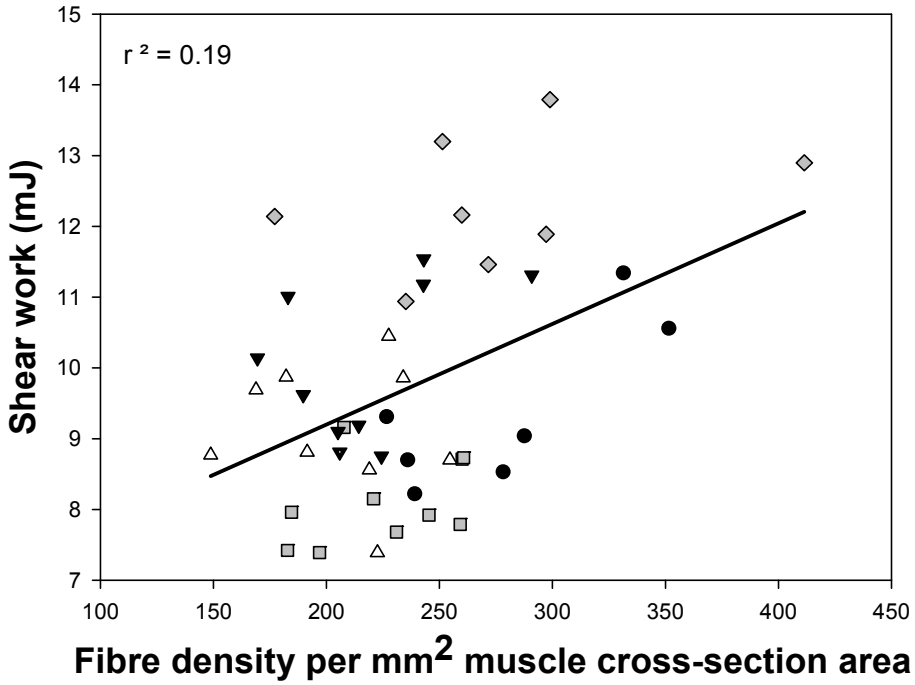

Figure 4.6. Regression analysis of fibre density (FD) and texture (square root transformation) in males (pooled data, n=44, r^2=0.19, P<0.01). A first order linear regression was fitted to the data, shear work = 0.014 (FD) + 6.356. Symbols indicate the different samples, 24.05.04 (●), 20.08.04 (■), 26.11.04 (Δ), 18.02.05 (▼), 05.05.05 (♦).

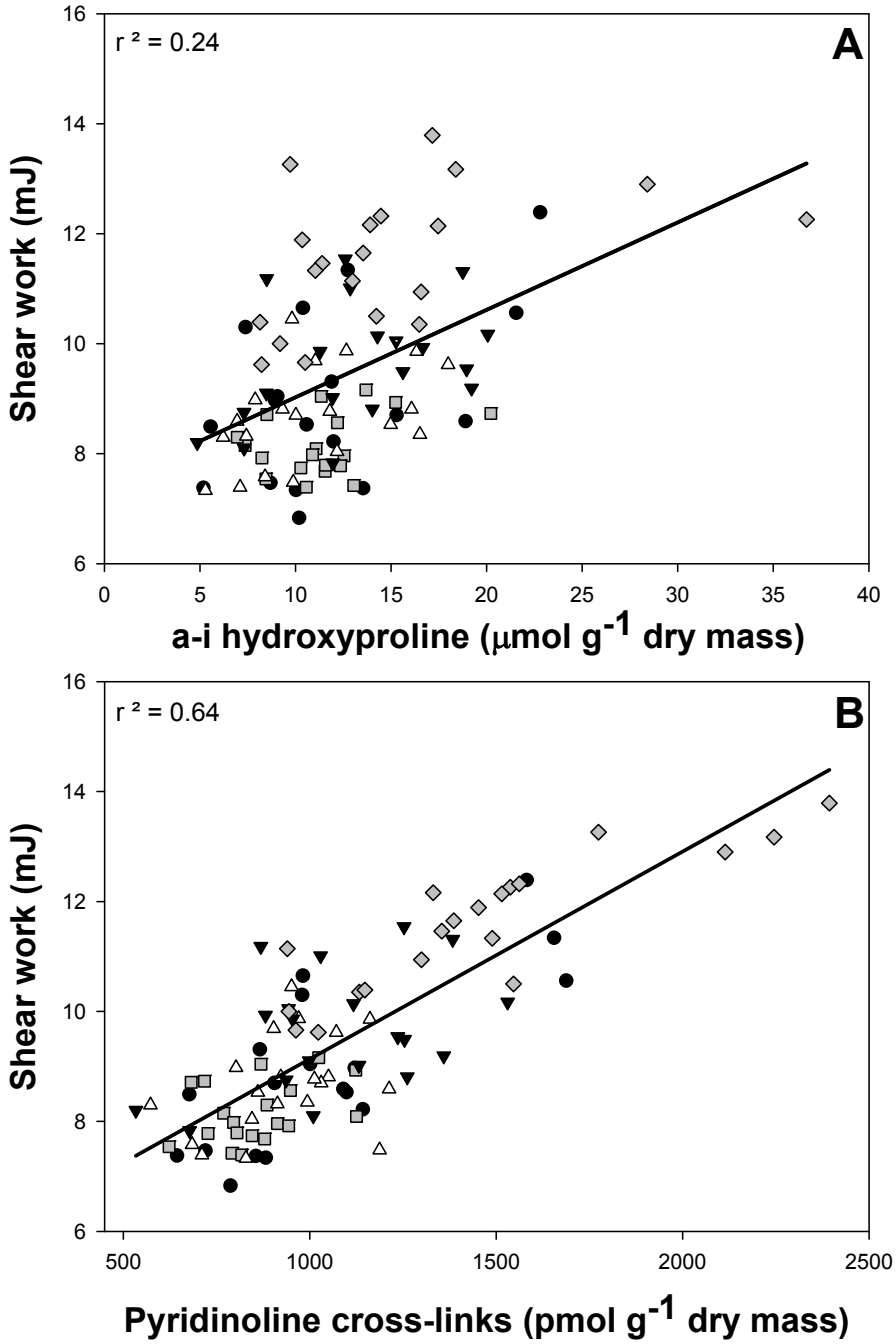

Figure 4.7. The relationship between **(A)** alkaline-insoluble collagen (n=98, r^2=0.24, P<0.001) and (B) hydroxylysyl pyridinoline (n=98, r^2=0.64, P<0.001) and fillet texture (square root transformation) for Atlantic halibut. A first order linear regression was fitted to the data, A: shear work = 0.159 (a-i HYP) + 7.432, B: shear work = 0.04 (PYD) + 5.361. Symbols indicate the different samples, 24.05.04 (●), 20.08.04 (■), 26.11.04 (Δ), 18.02.05 (▼), 05.05.05 (♦).

4.5 Discussion

This study has shown that mature hydroxylysyl pyridinoline (PYD) cross-links make a major and significant contribution to fillet firmness in the Atlantic halibut, explaining 64% of the total variation (Fig. 4.7 B). There are very few studies on collagen cross-links in fish muscle. In a recent study PYD concentration was shown to be positively correlated with firmness in fresh and smoked salmon, explaining 25% and 16% of the variation respectively (Li et al., 2005). It is likely that the a-s and a-i fractions contain native collagen and cross-linked collagen respectively, since almost 100% of PYD cross-links were recovered in the a-i fraction (Li et al., 2005). Johnston et al. (2006a) reported that a wild salmon population had significantly firmer flesh than a farmed population and yet had similar concentrations of PYD cross-links. This is probably because other mature and immature cross-links species contribute to texture together with the protein compartment (Johnston et al., 2000d; 2006a). Other immature cross-links include dihydroxy-, hydroxy- and lysinonorleusine and mature cross-links include deoxypyridinoline and pentosidine (Saito et al., 1997). It is assumed that one collagen molecule consists of ~200 HYP residues and one PYD is capable of connecting three collagen molecules (Li et al., 2005). Based on these assumptions, ~6% and ~3% of the a-i and total collagen were cross-linked in the final sample respectively, compared to just 1% in Atlantic salmon (Li et al., 2005). Thus compared to salmon (Johnston et al., 2006a; Li et al., 2005), halibut have a more densely cross-linked collagen and a firmer flesh (present study). No correlation between texture and the a-s HYP fraction which is thought to represent native non cross-linked collagen was found for either halibut (present study) or salmon (Li et al., 2005).

Farmed Atlantic halibut largely cease feeding in the winter due to low temperature (<6 ^{o}C) and short days resulting in a mobilisation of fast muscle proteins and an increase in water content (Hagen et al., 2006). This seasonal depletion is particularly severe for males of the body size used in the present study due to precocious sexual maturation which results in additional muscle protein mobilisation to build up the gonads (Hagen et al., 2006). In the present study, a significant seasonal effect in both a-s collagen ($P<0.001$) and a-i collagen ($P<0.01$) was found, but not as obvious in a-i as in the a-s collagen fraction. It is likely that the cross-linked collagen fraction is more resistant to proteolytic breakdown by collagenases (Goll et al., 1964). Red seabream showed no seasonal change in collagen concentration, but an increase with maturation and spawning (Touhata et al., 2000). This is in sharp contrast with the decreased collagen concentration observed during maturation and spawning in pacific herring (McBride et al., 1960), Ayu (Toyohara et al., 1997) and halibut (present study). In the present study all male halibut matured while females did not (Hagen et al., 2006). During the maturation period females outgrew males, resulting in them being significantly larger in May 2005 (Hagen et al., 2006). Since both sexes showed the same changes in a-i collagen concentration it is likely that seasonal factors rather than maturation are responsible. PYD is known to increase with age in both mammals (Bailey et al., 1998; Bailey 2001) and fish (Montero and Borderías, 1990). The reason why the contribution of water content to texture showed a small difference between sexes is that males displayed a more pronounced increase in water content than females during the winter (Hagen et al., 2006). PYD cross-links are more concentrated in the fish muscle with increased water content (r^2=0.61, pooled data). However, the difference in PYD to a-i ratio was not significant.

A weak significant correlation was found between total collagen and muscle fibre density, as reported in sea bass (Periago et al., 2005). This probably reflects the higher surface to volume

ratio of endomysium as muscle fibre density increases. However, the relative contribution of the endomysium collagen fraction is probably relatively small since most of the collagen in fish muscle is located in or associated with the myosepta.

The contribution of other variables to the texture of halibut flesh was explored using MLR and PLS. Muscle fibre structure and texture are known to correlate in both raw (Johnston et al., 2000d; Periago et al., 2005) and cooked fish (Hatae et al., 1990; Hurling et al., 1996) using taste panels and instrumental methods. In the present study a linear regression analysis revealed that fast muscle fibre density in males (Fig. 4.6, r^2=0.19, n=44) correlated better with texture than females, perhaps reflecting the sexual dimorphism of muscle fibre recruitment patterns in this species (Hagen et al., 2006). During the season and between sampling points the contribution of fibre density to texture varied from not being significant (August and November 2004) to having a larger effect in May 2005 (r^2=0.31, n=20), implying that muscle fibre density is just one of the factors affecting texture.

pH has been reported to influence the texture of fish muscle, particularly in the relation to the phenomena referred to as gaping (splits and tears in the connective tissue) (Ofstad et al., 2006), although its impact on technological characteristics of the flesh have been debated (Bjørnevik et al., 2003; Espe, 2004; Kiessling et al., 2004; Roth el al., 2005). Based on the present findings it is concluded that *post-rigor* pH is not a good predictor of texture in raw Atlantic halibut flesh.

In conclusion, collagen cross-linking constitutes a major and muscle fibre density a minor factor explaining fillet firmness in Atlantic halibut as measured with an instrumental texture analyser. Significant seasonal effects were observed in both collagen cross-links and muscle

fibre density which impacted on texture, and their relative importance varied during the year. During the winter when growth ceased, the a-i collagen fraction was enriched for PYD cross-links leading to a firmer texture. In practical terms, harvesting during the spring would result in fish with the firmest texture. However, the farmer would loose biomass due to maturation and muscle depletion during the winter months (Hagen et al., 2006) and therefore we recommend harvesting in the fall or early winter when nutritional state (Hagen et al., 2006) and texture are good.

Chapter 5

The influence of cathepsins and collagenase on protein and water holding capacity in muscle of commercially farmed Atlantic halibut (*Hippoglossus hippoglossus*).

5.1 Abstract

Atlantic halibut (*Hippoglossus hippoglossus*) were commercially farmed in Helgeland, Norway (May 2004-May 2005). The average weight (Mb) of fish increased over the 12 month production cycle by ~73% for females and by ~50% for males, although during the winter months (November–early May) Mb was unchanged in females and declined by 18% in males due to sexual maturation and sperm release. Periods of zero or negative growth were associated with up to 5.7% (females) and 17.9% (males) decline in fast muscle protein content. The activities of cathepsins B, B + L, H and D showed a reciprocal relationship, and were highly correlated with the changes in protein content. Water holding capacity, measured as liquid loss increased from 3-5% in November to 11-13% in May. Two general additive models (GAM) showed that cathepsin B + L, cathepsin D and collagenase explained 73.1% of the total variance in protein content, while cathepsin H was the largest contributor to liquid loss explaining 48.8% of the total variance. The results indicate that to obtain the best flesh quality Atlantic halibut should be harvested in the fall or early winter when the liquid loss and cathepsin activities are low and less likely to cause problems during secondary processing and storage.

5.2 Introduction

Fish contain 60-80% water depending on species and various biological and environmental factors (Love, 1970). Water holding capacity (WHC) is therefore not surprisingly an important quality parameter for both industry and consumers (Fennema, 1990; Schäfer et al., 2002). Muscle proteins have a central role in the binding of water, originating in the unequal distribution of positive and negative charges of the water molecules (Wismer-Pedersen, 1987). The interactions between water and proteins can be divided into three different categories based upon binding. These are; (A) primary hydration shell (bound water), (B) secondary hydration shell (loosely bound water) and (C) unperturbed water (free water) (Wismer-Pedersen, 1987). Variation in the WHC of the flesh is due to how well the physically bound water is locked up in the muscle structure (Wismer-Pedersen, 1987). When harvesting commercially produced fish WHC should be taken into consideration since drip loss can be considerable. In aquaculture problems of drip loss can potentially be minimised by manipulating conditions (e.g. feed, light regimes and potentially temperature) as well as harvest time to obtain fish of the best quality. Several small scale experiments have been performed to investigate the WHC of current Norwegian aquaculture species, including salmon (Ofstad et al., 1993, 1995), cod (Ofstad et al., 1993; Duun and Rustad 2007; Olsson et al., 2007; Skipnes et al., 2007) and halibut (Olsson et al., 2003a, 2003b, 2003c; Haugen et al., 2006), but in commercially produced fish the optimal timing of harvesting in respect of WHC has not yet been addressed. Several parameters have been associated with poor WHC, such as ionic strength, pH, temperature (Ofstad et al., 1995; Olsson et al., 2003a), detachment of sarcolemma, gaps in the extracellular matrix, widening of the intermyofibrillar space and transversal shrinkage of the muscle fibres (Olsson et al., 2003c). The presence of certain bacteria during cold storage is also known to affect the WHC of the flesh (Olsson et al.,

2003c). The majority of the water in the flesh is bound to the myofibrils and the WHC of the muscle is linked to the spacing of the myofibrils (Offer and Trinick, 1983). Any proteases capable of degrading muscle proteins could potentially affect the WHC of the muscle.

Cathepsins are one class of proteolytic enzyme that have been extensively studied in fish, aspects such as *post-mortem* autolysis (Aoki and Ueno, 1997; Delbarre-Ladrat et al., 2004; Chéret et al., 2007), ice storage (Hultmann and Rustad, 2004), effect of smoking temperature (Hultmann et al., 2004), effect of season and fishing ground on by-products (Sovik and Rustad, 2006) and degradation of proteins and softening of muscle tissue which leads to reduced quality during starvation or maturation (Konagaya, 1982; Konagaya, 1985; Yamashita and Konagaya, 1990a). The cathepsins (B, B + L and H) belong to the cysteine peptidase family, a group of enzymes that are associated with the lysosomes in many tissues, including muscle. Cathepsin D on the other hand, belongs to the aspartic peptidase family (see review of Sentandreu et al., 2002; Bechet et al., 2005). Cathepsins show a range of substrate specificities. For example, myosin heavy chain (Schwartz and Bird, 1977), troponin I, troponin T and tropomyosin (Noda et al., 1981) are all degraded by cathepsin B. In addition, cathepsin B (B1) is also thought to degrade native collagen (Burleigh et al., 1974). Cathepsin L is capable of degrading most myofibrillar structural proteins, such as myosin, actin, troponin I and T except troponin C and tropomyosin (Matsukura et al., 1981; Aoki and Ueno, 1997), while cathepsin H is known to degrade troponin T (Katunuma et al., 1983). In addition, a light microscopic study proved evidence that cathepsin L is involved in the degradation of the M-protein in the M-line and structural components of the Z-line (Matsukura et al., 1984). The regulations of collagenases are complex, but they are synthesized and secreted in a number of cells including muscle fibres and fibroblasts (Singh et al., 2000; Balcerzak et al., 2001). The matrix metalloproteases peptidase family, including collagenase has specific functions with particular specificity towards collagen substrates (Sentandreu et al., 2002).

The main objective of the present study was to investigate the activity of cathepsine B, B + L, D, H and collagenase over an annual production cycle in commercially produced Atlantic halibut and determine their impact on protein content and liquid loss. Based on the results an optimal timing of harvesting to minimise water drip loss and maximise flesh quality is suggested.

5.3 Material and methods

5.3.1 Fish farming

For details about fish husbandry see section 3.3.1.

5.3.2 Sample preparation

For details about sample location see section 4.3.3.

The fish were rinsed and filleted (dorsal and ventral side) anterior to 0.55 L_F, and minced for 3x5 s using a food processor and a fraction was removed and used for WHC analysis (see below). The remaining mince was homogenised for an additional minute and one gram was accurately measured in 15 tubes per fish and stored in a -40 ^{o}C freezer until enzyme analysis was preformed.

5.3.3 Water holding capacity

The WHC was measured according to Ofstad et al. (1993). Briefly, the mince was kept on ice at all times and 15 g was accurately measured in duplicate into a cup with a fine netting bottom. The cup was placed on top of a collection vessel and centrifuged at 210 g for 15 minutes at 10 ºC. WHC was measured as liquid loss and expressed as percentage weight released after the following formula:

$$Liquid\ loss\ (\%) = \frac{Liquid\ loss\ (g)}{15\ g}\ x\ 100$$

Due to the low fat content of the samples (see Hagen at el., 2006), the potential "fat fraction" of the liquid *post* centrifugation was ignored.

5.3.4 Enzyme kinetics

5.3.4.1 Cathepsin B, B + L and H activity assay

The 1 g of muscle measured into tubes with screw chaps were homogenised in 5 ml of cold extraction buffer (50 mM sodium acetate, 0.1 M NaCl, 1 mM EDTA (Ethylenediaminetetraacetic acid) and 0.2% (w/v) Chaps [3-[(3-Cholamidopropyl)-dimetyl ammonio]-1-propan-sulfonat, Fluka/VWR, Oslo, Norway), pH 5.0) for one minute at 22000 rpm using a Polytron (mod. PT 1200 CL, Kinematica AG/Anders Phil AS, Dale i Sunnfjord, Norway). The extracts were stored on ice for 10 minutes and 1.5 ml of the homogenised sample were removed and centrifuged at 20000 g for 30 minutes at 4 ºC. The supernatants were removed and stored at -80 ºC until analysis.

The optimal pH of the assay buffer for cathepsin B, B + L and H was validated and determined by using 5 fish from the initial sampling point (24.05.04).

Cathepsin B, B + L and H activity were measured according to Barrett and Kirschke (1981). The substrates Z-Arg-Arg-MCA (cathepsin B), Z-Phe-Arg-MCA (cathepsin B + L) and Arg-MCA (cathepsin H) (Sigma, Oslo, Norway) were used for each of the enzyme assays. Cathepsin B and L was assayed together because there was no specific substrate available for cathepsin L only at the time of these studies. Enzyme activation was performed by incubating 25 µl enzyme extract (kept on ice at all times) with 975 µl assay buffer (see Table 5.1) for 2 minutes at 30 ºC, before adding 1 ml of 10 µM freshly made substrate. The incubation was performed in quartz cuvettes in the automated "peltier element" (with stirring) inside the

fluorescent spectrophotometer (Eclips, Varian/Holger, Oslo). Samples were measured in triplicates and the excitation (λ_{ex}) and emission (λ_{em}) wavelengths were set to 380 and 460 nm respectively. One unit of enzyme activity was defined as the amount that hydrolysed 1 mmol substrate per minute at 30 ºC.

Table 5.1. Composition and optimum pH of cathepsin B, B + L and H assay buffers.

Cathepsin B (pH 6.0)	Cathepsin B + L (pH 6.0)	Cathepsin H (pH 6.6)
200 mM Na-phosphate	200 mM NaOAc	200 mM Na-phosphate
2 mM Na_2EDTA	2 mM Na_2EDTA	2 mM Na_2EDTA
0.05% Chaps	0.05% Chaps	0.05% Chaps
4 mM DTT *	4 mM DTT *	4 mM DTT *

DTT was added freshly just before use.

5.3.4.2 Cathepsin D activity assay

The preparation of the enzyme extract was similar to the cathepsin B, B + L and H assay except that the extraction buffer comprised of 1mM EDTA and 0.2% (v/v) Tween 20 (Sigma, Oslo, Norway), in PBS buffer. The activity was measured using a 2.5% bovine (w/v) haemoglobin (Sigma, Oslo, Norway) substrate (Mycek, 1970). The haemoglobin was dissolved in distilled water (2.5 g/100 ml) and left over night in a refrigerator (no stirring). The reaction mixture consisted of 100 µl 2.5% haemoglobin, 50 µl 0.2 M citrate buffer (assay buffer), pH 2.8 and 25 µl enzyme extract (kept on ice at all times) and was incubated at 37 ºC in a water-bath for 60 minutes. 50 µl 15% (v/v) TCA (Trichloroacetic acid, Sigma, Oslo, Norway) was added to terminate the reaction

and the assay mixture was centrifuged at 20000 g for 5 min at 20 ºC. The supernatant was neutralised by adding 2% (v/v) of 10 M NaOH, and the TCA-soluble peptides in the supernatant were determined by the QuantiPro^{TM} BCA assay kit (Sigma, Oslo, Norway). The QuantiPro^{TM} BCA assay kit consists of two reagents. 1 ml $CuSO_4$ (reagent B) and 50 ml BCA stock (reagent A) was mixed together and finally 100 µl 10N NaOH was added to neutralize TCA in the samples. For the control reaction pepstatin (Sigma) was added before adding the enzyme extract, inhibiting the enzyme reaction. Samples were measured in triplicates in 4 ml disposable cuvettes (PS, VWR, Oslo, Norway) using a spectrophotometer (Biomate 3, Thermo spectronic, USA) at 320 nm wavelength. One unit activity was defined as the amount of cathepsin D digesting 1 mg haemoglobin (with BSA (Bovine serum albumin (Sigma, Oslo, Norway) as standard) in 60 min at 37 ºC. The protein concentration in the enzyme extract was determined using the Bradford method (Bradford, 1976). To make the Bradford reagent, 100 mg Coomassie Brilliant Blue (Sigma, Oslo, Norway) was dissolved in 50 ml 95% ethanol. 100 ml of 85% phosphoric acid was added to the solution and then distilled water up to a final volume of 1 l. The protein concentration was assayed after a 50-fold dilution. A 7 point standard curve (0-20 µg/ml) was used to quantify the protein content of the enzyme extract measured at 595 nm.

5.3.4.3 Collagenase activity assay

The enzyme extraction step was similar to that of cathepsin D except that a different extraction buffer was used, consisting of 50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$ and 0.2% (v/v) Tween-20 (pH 7.4). Collagenase activity was determined based on the method of Wünsch and Heidrich (1963), using a synthetic substrate (4-phenylazobenzyloxy-carbolyl-pro-leu-gly-D-arg) (Sigma, Oslo, Norway). To each 1 ml assay buffer consisting of 0.1 M Tris-HCl (pH 7.1), 20 µl of 1 M $CaCl_2$ was added together with the synthetic substrate. For

each sample 400 µl assay buffer was mixed with 50 µl enzyme extract (kept on ice at all times) and incubated in a water-bath at 37 ºC. After 15 min 100 µl 25 mM citric acid and 1 ml of ethyl acetate was added to terminate the reaction. The reaction mixture was centrifuged and the supernatant removed into a new tube containing 100 mg anhydrous $NaSO_4$. The optical density of the supernatant was measured in triplicates in disposable solvent resistant 1.5 semi-micro cuvettes (PMMA, VWR, Oslo, Norway), at 562 nm wavelength using a spectrophotometer (Biomate 3, Thermo Spectronic, USA). For the blank reaction 100 µl 25 mM citric acid was added before the enzyme extract and incubated together for 15 min. One unit of activity was defined as the amount of enzyme required to liberate 0.01 µmole of substrate in 15 min at pH 7.1 at 37 ºC, during the linear phase (<20 minutes) of the substrate breakdown (Fig. 5.1). The extinction coefficient of 0.01 µmole substrate in 5 ml ethyl acetate is 0.042 (according to Sigma). The protein concentration in the enzyme extract was determined using the Bradford method after a 50-fold dilution (Bradford 1976).

All samples identified as outliers (having a large SD) after analysis were re-analysed immediately, because freezing and re-thawing of samples will affect the enzyme activity.

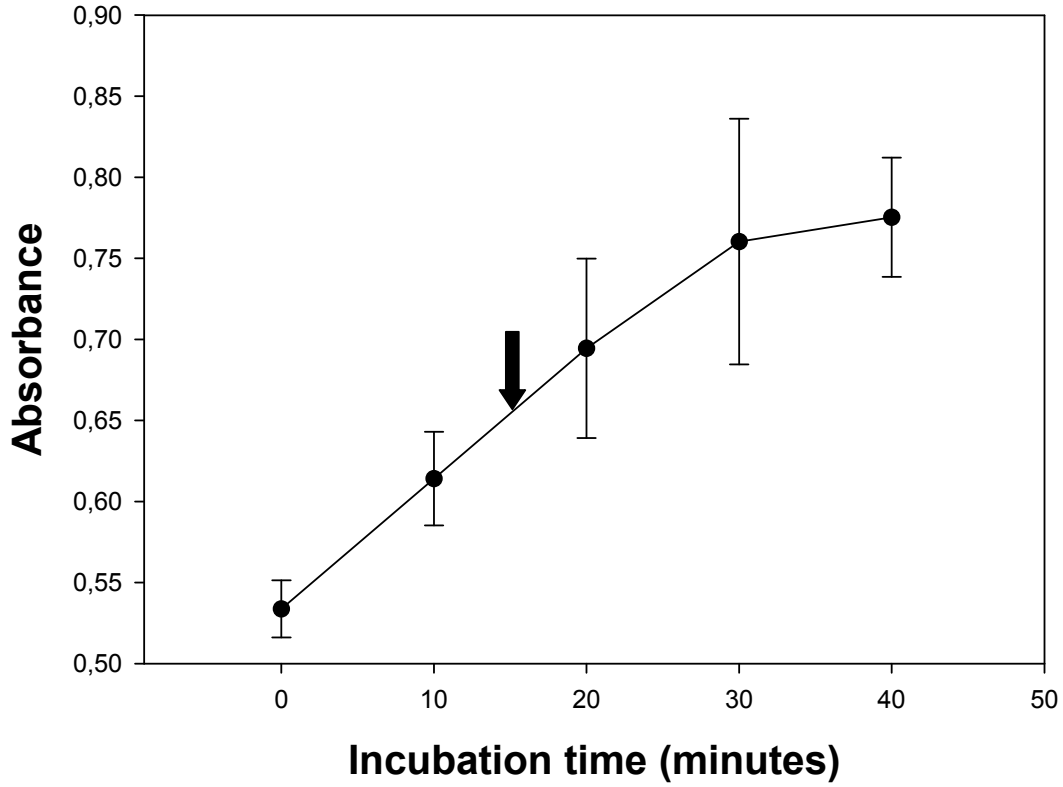

Figure 5.1. Incubation time in relation to the breakdown of collagenase substrate, 4-phenylazobenzyloxy-carbolyl-pro-leu-gly-D-arg (n=5). The arrow indicates the termination point of the incubation (15 minutes), which is within the linear phase. Mean ± SD.

5.3.5 Statistics

Statistical tests were performed in the statistical packages Minitab (Release 14.20, Minitab Inc., State College PA, USA) and *R* (R Development Core Team, 2007). The variables were considered separated by sex using a general linear model (GLM):

$$Dependent\ variable = sample\ point + sex(males\ or\ females) + fish\ length \quad (A)$$

with *sex* and *sample point* as factors and *fish length* as a covariate. The dependent variables were transformed because of heterogeneity in the residuals of a model based on the raw data. Cathepsin B + L and cathepsin H were square root transformed and Cathepsin D was log transformed to comply with the model assumptions. A matrix scatter plot was constructed of all factors to identify underlying connections between factors that could affect the outcome of the chosen general additive model (GAM, see below).

To investigate if enzyme type, protein or liquid loss differed between sex for the different sampling points the following GLM was used:

$$Dependent\ variable = sample\ point + sex + sample\ point \times sex \quad (B)$$

where *sample point* and *sex* are considered as factors and × represents an interaction. Since the data for males and females overlap in all of the sample periods except in the last, a significant interaction between sex and sampling point will indicate that a difference between sex was present in the last sampling point.

The effect of enzyme level on protein content and liquid loss was considered using two GAM's of initial form:

$$Protein = sex + s(Cathepsin\ B) + s(Cathepsin\ B + L) + s(Cathepsin\ D) + s(Cathepsin\ H) + s(Collagenase) \quad (C)$$

$$Liquid\ loss = sex + s(Cathepsin\ B) + s(Cathepsin\ B + L) + s(Cathepsin\ D) + s(Cathepsin\ H) + s(Collagenase) \quad (D)$$

using the library package mgcv (Wood, 2006) in *R*. *s* represents a thin plate smooth function of each variable with a maximum of six degrees of freedom. Model selection was by generalised cross validation (gcv, Wood, 2006). The error structure was considered to be Gamma and an identity link function was used. Because of a risk of over fitting (unnecessarily including explanatory variables or using smooth functions with too many degrees of freedom) when using GAMs (Kim and Gu, 2004), an additional penalty was added to the smoothing (Wood, 2006).

In addition to the GAM used for analysing the impact of enzyme activity on protein content, the relationships were also investigated using a polynomial (quadratic) regression.

5.4 Results

5.4.1 Validation of pH optimum for cathepsin B, B + L and H

The optimum pH of the assay buffer was validated and the pH giving the highest value was used as standard for the individual assays. The pH that gave the highest activity was 6.0, 6.0 and 6.6 for cathepsin B, B + L and H respectively (indicated with an arrow in Fig. 5.2 A-C).

5.4.2 Seasonal variation and influence of enzymes on protein and liquid loss

Over the annual production cycle Mb increased by ~73 and 50% for female and male fish respectively ($P<0.01$), and the pattern of weight gain differed between the sexes (Table 5.2). Fish length showed a similar pattern to weight (Table 5.2). In female fish, Mb was unchanged over the winter period (26th November-5th May) (Table 5.2), corresponding to the period of short days when water temperature fell below the threshold for feeding (~6 ^{o}C) (Hagen et al., 2006). Male fish exhibited a decline in Mb of 19.8% over the same period (Table 5.2) corresponding to the period of sexual maturation. Since sperm had been released prior to the last sample this represented a loss of somatic tissue. The pattern of protein content change observed in the fast muscle is consistent with this tissue being a significant net contributor of amino acids during seasonal depletion in both sexes and following sexual maturation in males (Fig. 5.3). The maximum decrease in protein was up to 5.7% in females and 17.9% in males (Fig. 5.3). A GLM (model A) revealed a significant effect of sample time on protein content and a significant interaction between sex and sample period ($P<0.05$, model B). In the last sample, the average protein content of fast muscle was 15.4% lower in male than female fish.

The activities of cathepsins B, B + L, H and D exhibited an approximately reciprocal pattern to that observed for fast muscle protein content over the annual cycle (Fig. 5.4 & 5.5).

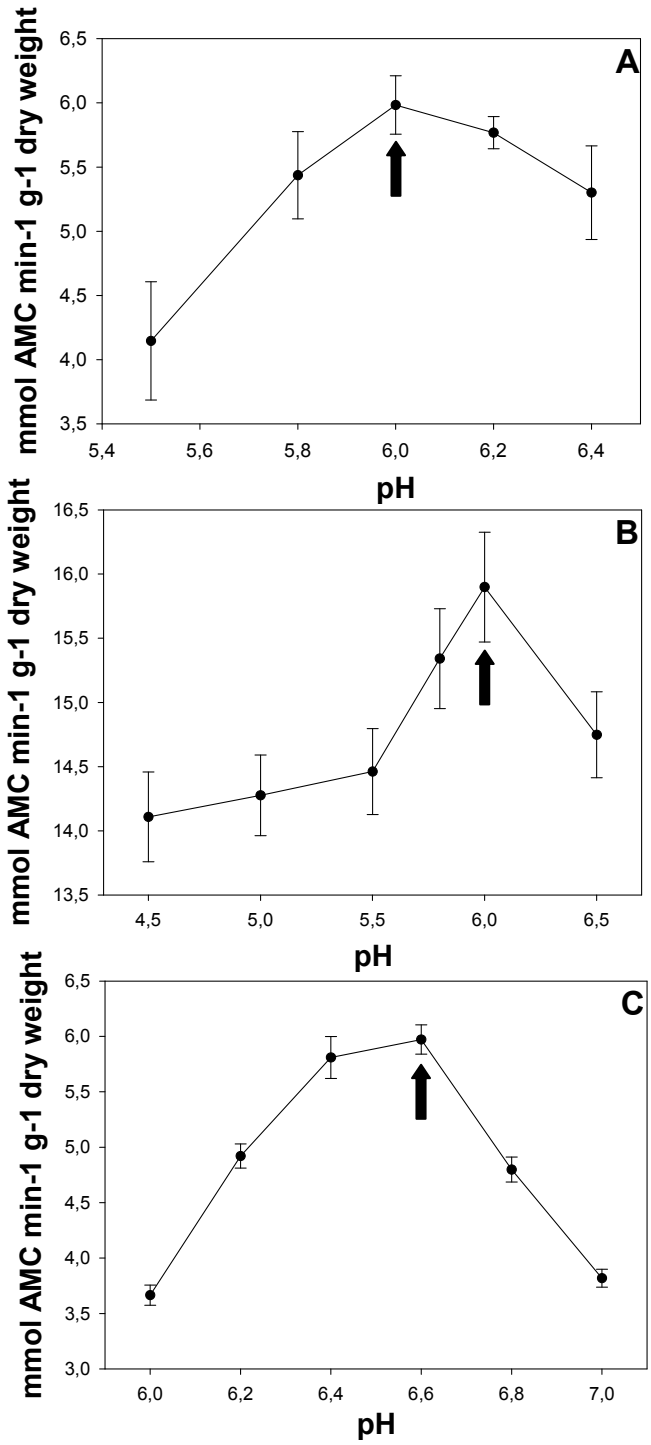

Figure 5.2. pH validation for the cathepsin B (A), cathepsin B + L (B) and cathepsin H (C) assay buffer. The optimal pH is 6.0, 6.0 and 6.6 for Cathepsin B, B + L and H respectively (indicated with an arrow). AMC: 7-Amino-4-methylcoumarin. Mean ± SE, n = 5.

Table 5.2. Fork length (L_F), body mass (g), pH and liquid loss of Atlantic halibut. Not determined (ND). Mean ± SE.

	Sex	24.05.04	20.08.04	26.11.04	18.02.05	05.05.05
Body mass (g) [a]	♀	1323.7 ± 278 (n=13)	1906.1 ± 351 (n=9)	2290.1 ± 236 (n=11)	2233 ± 457 (n=10)	2288 ± 404 (n=13)
	♂	1126.4 ± 214 (n=7)	1969.7 ± 287 (n=11)	2106.7 ± 144 (n=9)	1839 ± 430 (n=10)	1689.3 ± 332 (n=7)
L_F (cm) [a]	♀	47.4 ± 3.2	53.8 ± 3.4	55.9 ± 1.5	57.32 ± 3.2	58 ± 1.8
	♂	46.6 ± 2.8	53.5 ± 2.8	54.6 ± 3.1	53.8 ± 2.8	54 ± 2.7
pH [b]	♀	6.45 ±0.1	6.45 ± 0.1	6.33 ± 0.1	6.37 ± 0.1	6.47 ± 0.1
	♂	6.52 ± 0.2	6.45 ± 0.1	6.34 ± 0.1	6.31 ± 0.1	6.47 ± 0.2
Liquid loss (%)	♀	ND	ND	3.40 ± 0.9	8.97± 0.5	12.81± 0.7
	♂	ND	ND	4.59 ± 0.9	11.59 ± 0.7	10.77 ± 1.6

[a] = see Hagen et al. (2006). [b] = see Hagen et al. (2007).

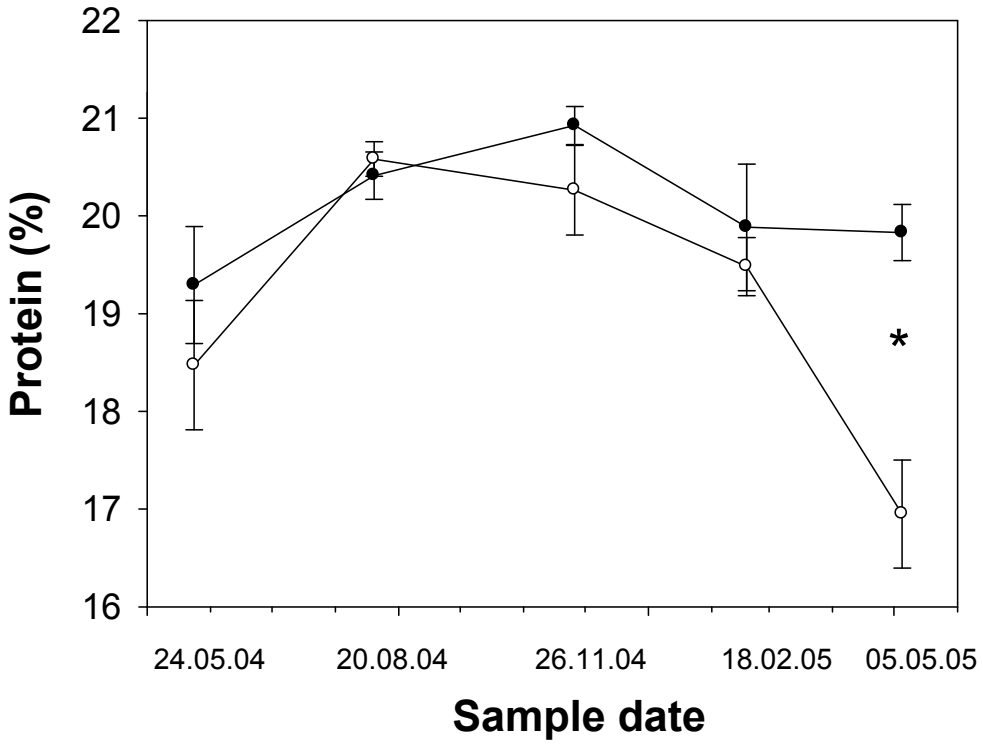

Figure 5.3. Seasonal changes in protein content of male (open symbols, n=44) and females (closed symbols, n=54). Asterisks (*) indicates significant differences between sex ($P<0.05$). Mean ± SE.

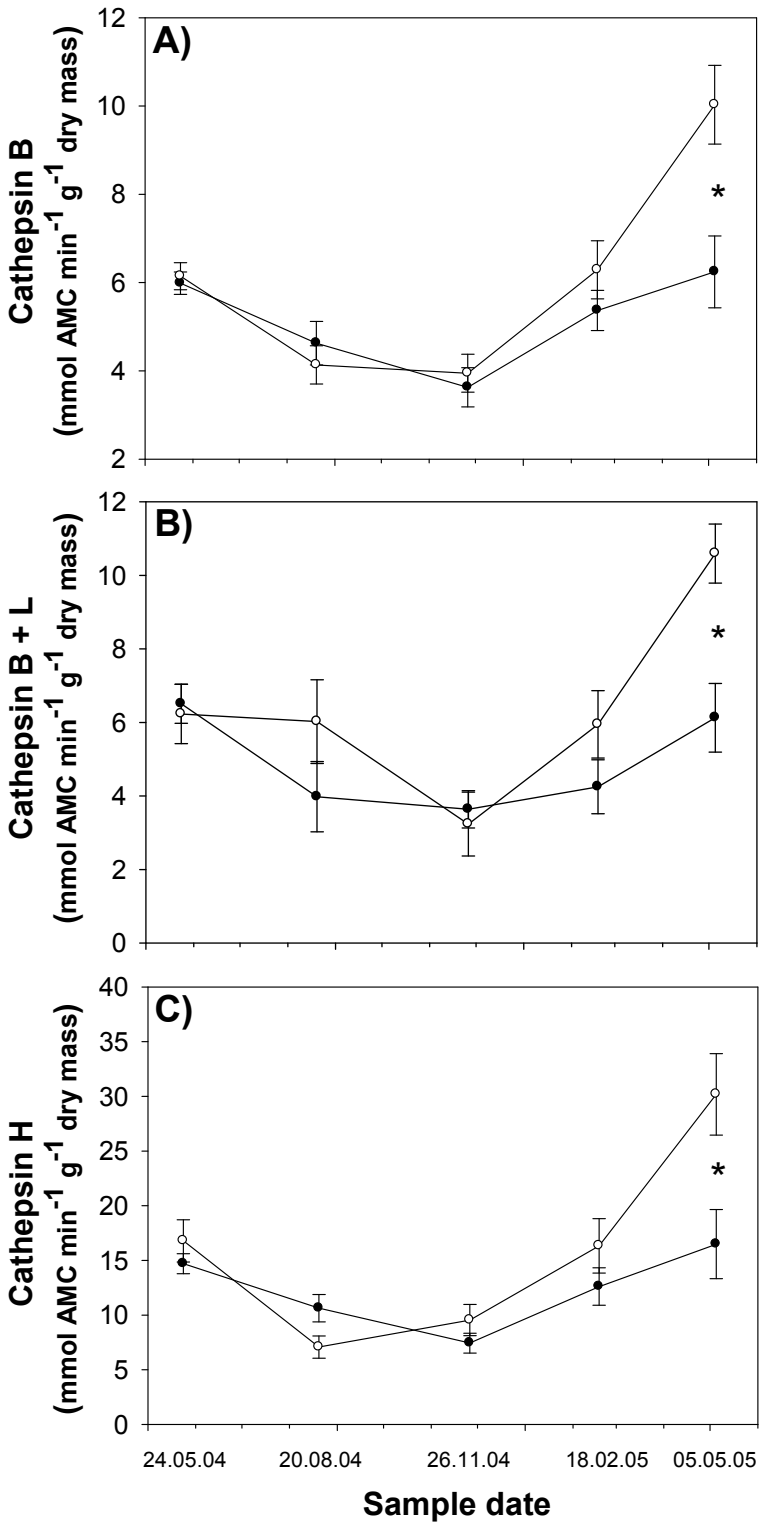

Figure 5.4. Seasonal changes in cathepsin B (A), cathepsin B + L (B) and cathepsin H (C) activity of males (open symbols, n=44) and females (filled symbols, n=54). Asterisks (*) indicates significant differences between sex ($P<0.05$). Mean ± SE.

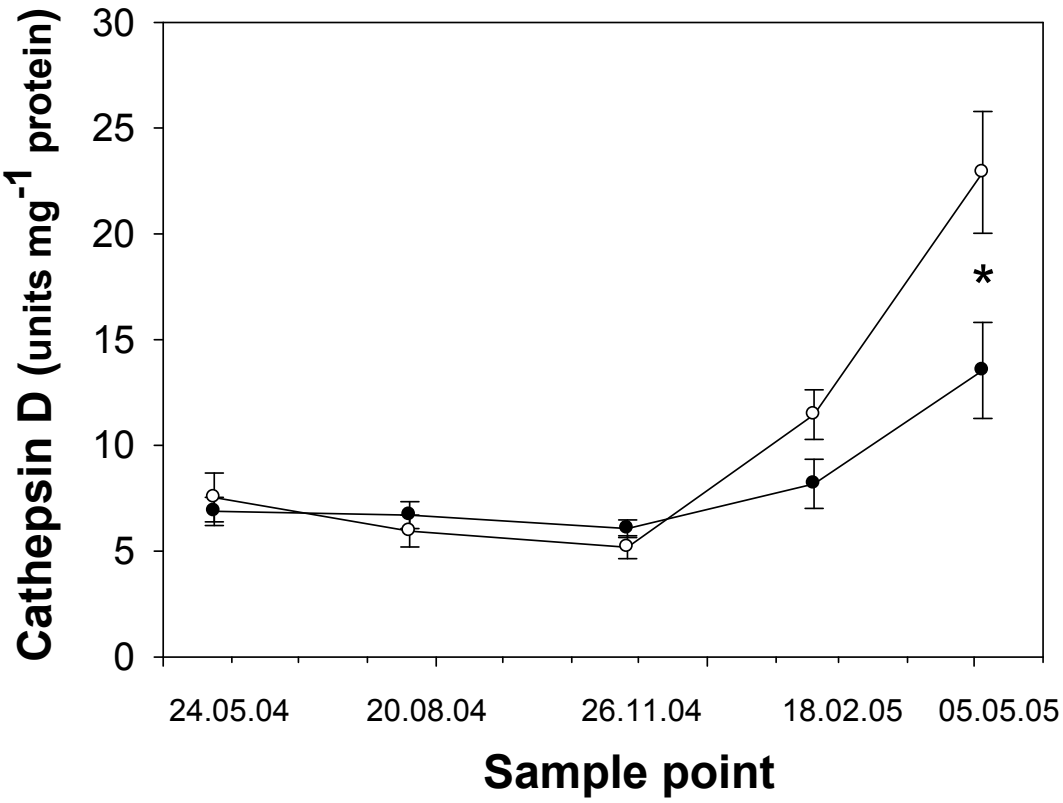

Figure 5.5. Seasonal changes in cathepsin D activity of males (open symbol, n=44) and female (closed symbol, n=54). Asterisks (*) indicates significant differences between sex ($P<0.05$). Mean ± SE.

A GLM showed *sample point* (P<0.05, both sexes, model A) and the *sample point x sex* interactions (P<0.05, model B) were significant for each cathepsin. In the last sample cathepsin B, B + L, D and H activities (indicated with asterisks) were 60.6, 60.2, 69.2 and 83.2% higher respectively in male than female fish (Fig. 5.4 & 5.5, P<0.05, model B). Regression analysis was used to further investigate the relationship between cathepsin activities and protein content (Fig. 5.7). The relatively high R-sqr values obtained (0.49-0.57, P<0.001) suggest that cathepsins are likely candidates involved in the degradation of muscle protein (Fig. 5.7). A GLM (model C) of the form *Protein = s(Cathepsin B + L) + s(Cathepsin D) + s(Collagenase)* explained 73.1% of the total variance (*p*-values were <0.01, <0.001 and <0.01 respectively). However, such analysis should be treated with caution because cathepsin activities were highly correlated (Fig. 5.8), perhaps reflecting co-ordinated changes in enzyme concentration with changes in lysosome abundance. Collagenase activity in fast muscle was independent of season in female fish, but was significantly lower in August than other months for male fish (Fig. 5.6, P=0.004, model A). In May 2005, the collagenase activity in male fish had increased to approximately the same levels as in May the previous year.

The water holding capacity of the myotomal muscle, measured as liquid loss, showed a significant effect of sample time and a significant *sample point x sex* interaction (P=0.045). Liquid loss was 3.4 ± 0.9 and 4.6 ± 0.9% in November compared to 9.0 ± 0.5 and 11.6 ± 0.7% (mean ± SE) in February for male and female fish respectively (Table 5.2). The outcome of the GAM (model D) was *Liquid loss = s(Cathepsin H)*, which explained 48.8% of the variance (Fig. 5.9, P<0.01). It is likely that cathepsin activities are correlated to water holding capacity because of effects on protein content and distribution, although other factors are undoubtedly also important. pH did not show any sex or seasonal effect (Hagen et al., 2007). In contrast to a previous study no correlation between *post-mortem* pH and WHC was found, perhaps due to the lack of samples with a pH < 6.3 (Olsson et al., 2003a).

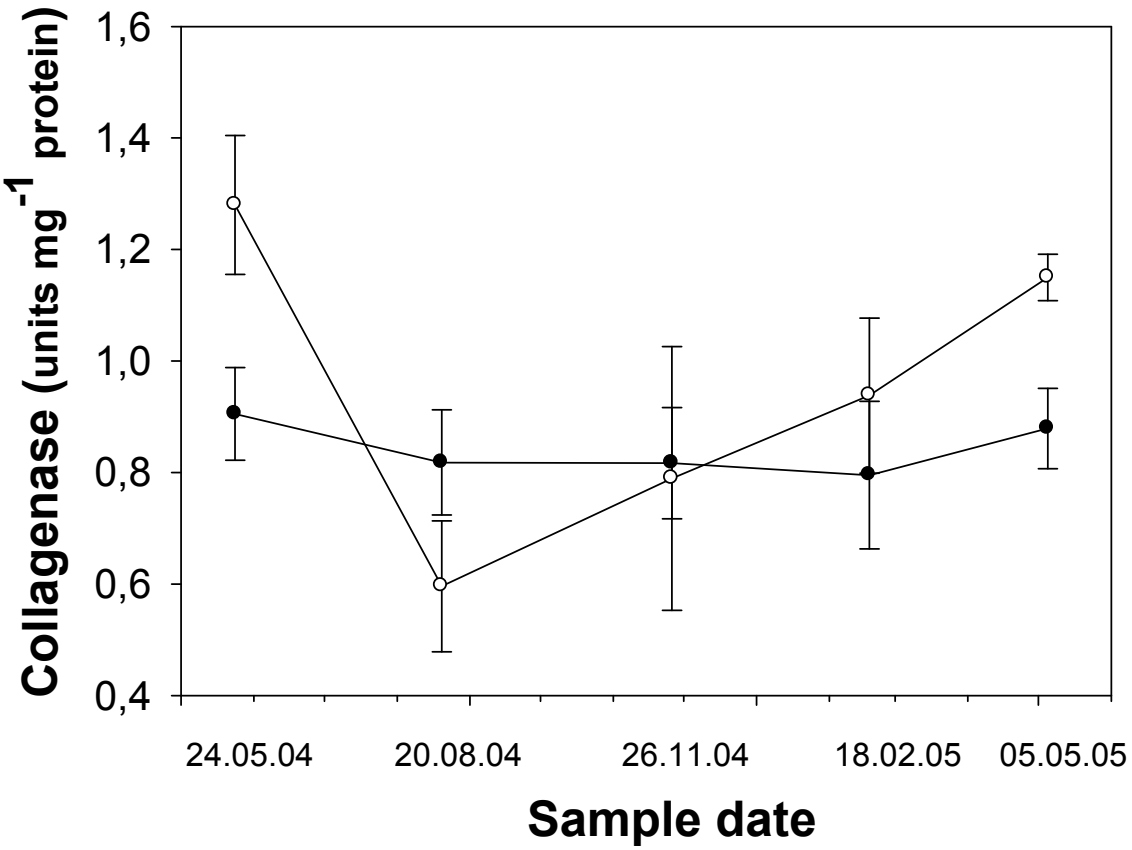

Figure 5.6. Seasonal changes in collagenase activity level for males (open symbols, n=44) and female (closed symbols, n=54). Mean ± SE.

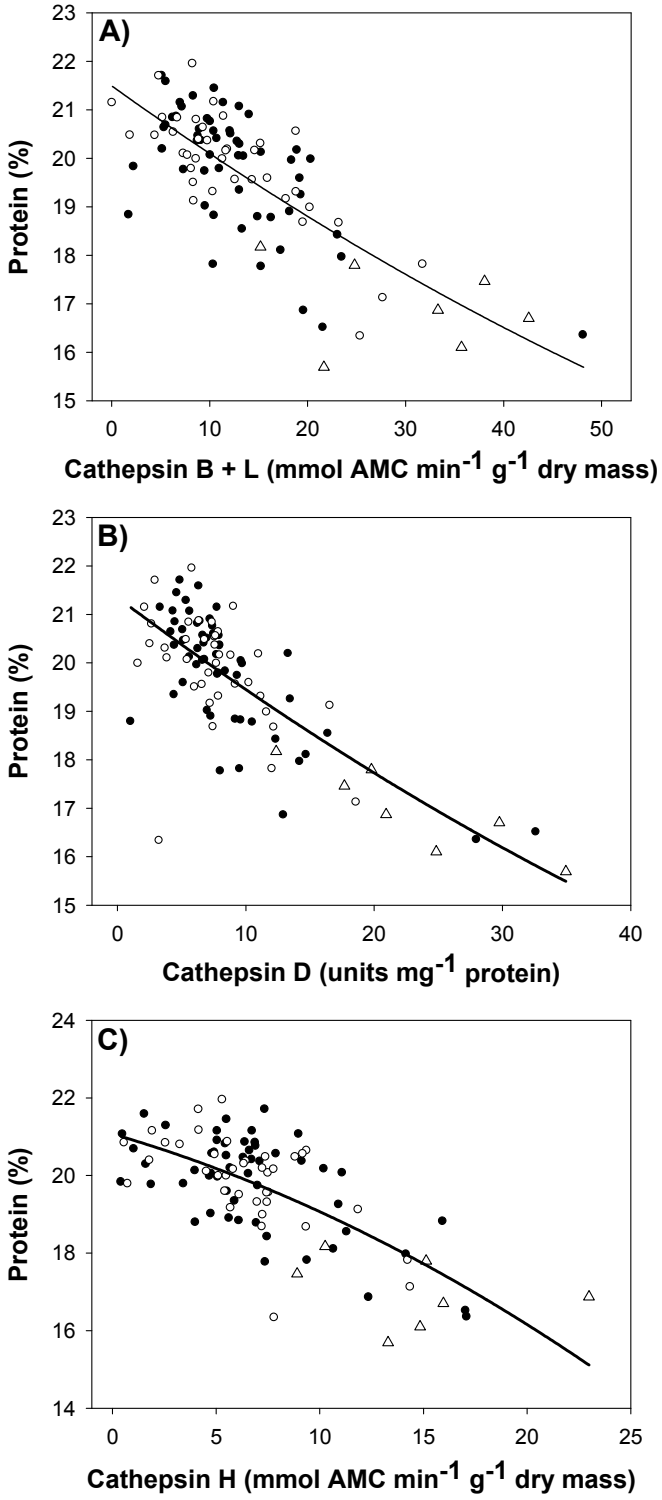

Figure 5.7. Polynomial regressions between protein content and the activity of cathepsin B + L (A), cathepsin D (B) and cathepsin H (C): r^2 values were 0.57, 0.56 and 0.49 respectively. Open symbols illustrates males (n=44) and closed symbols females (n=54). Triangles illustrate males in the last sampling point (note the high activity level and low protein content).

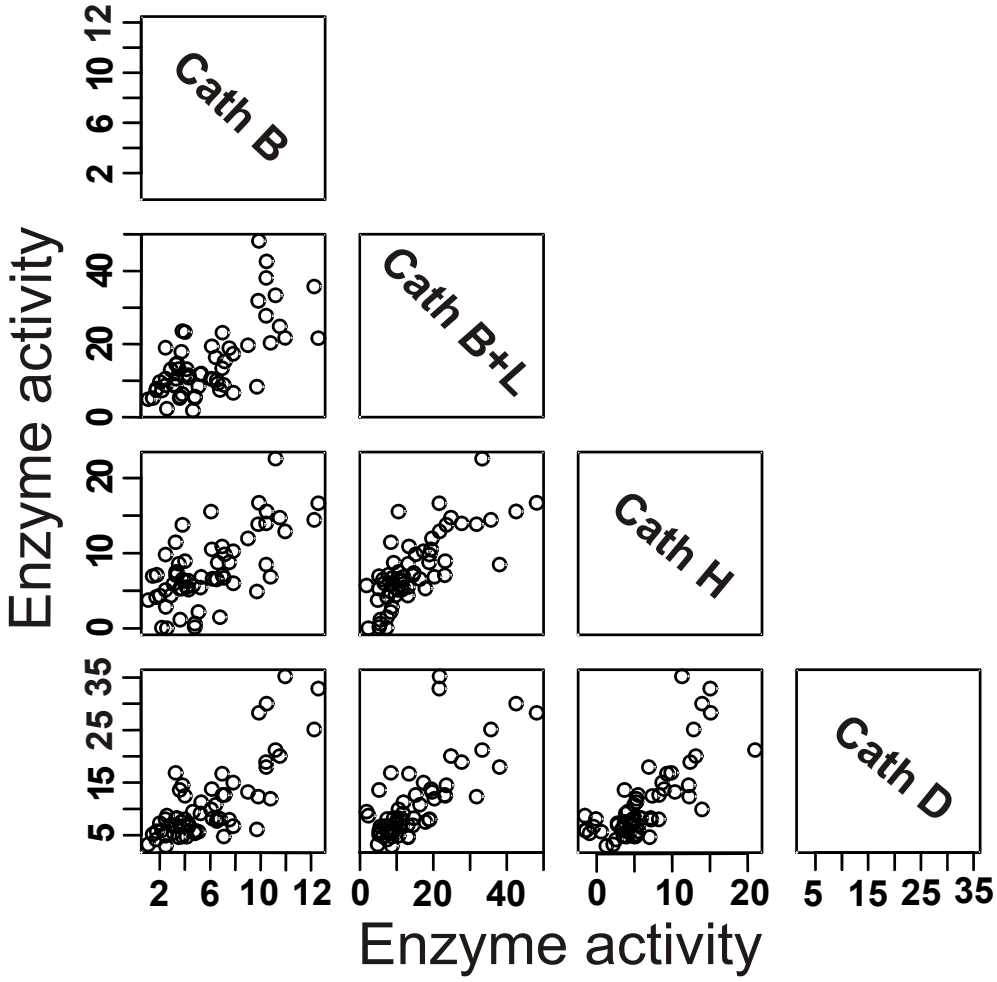

Figure 5.8. Matrix scatter plots illustrating the connection between the cathepsin B, B + L, H, (mmol AMC min^{-1} g^{-1} dry mass) and cathepsin D (units mg^{-1}protein).

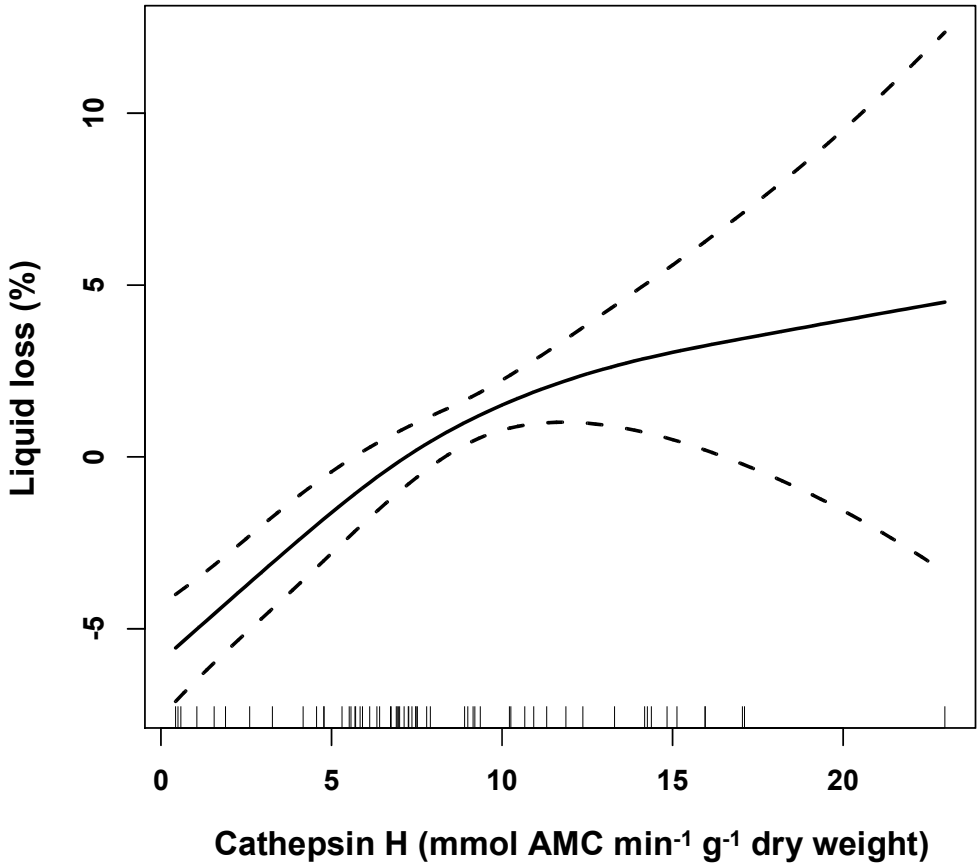

Figure 5.9. Graphical illustration of the outcome of the general additive model *Liquid loss = sex + s(Cathepsin B) + s(Cathepsin B + L)+ s(Cathepsin D) +s (Cathepsin H) +s(Collagenase)* (model D) (n=98). The relationship between liquid loss (%) and cathepsin H explained 48.8% of the variance. Note the increase in the 95% confidence interval (dotted lines) at high enzyme activity. Zero on the Y-axis represents the average response, while the ticks on the x-axis illustrate the distribution of the data points. Enzyme activity units are mmol 7-Amino-4-methylcoumarin min^{-1} g^{-1} dry mass.

5.5 Discussion

One of the largest challenges in the grow-out phase of halibut farming is the precocious maturation of males and poor growth during the winter (both sexes) due to low water temperatures (<6 ºC) which suppress feeding (Hagen et al., 2006). In the present study, the protein content of fast myotomal muscle paralleled seasonal changes in Mb suggesting this tissue acts as a reservoir from which amino acids are mobilised for maintenance and the generation of testis in males. The utilization of muscle proteins during periods of limited food supply or starvation is a well documented phenomenon in fish species from temperate latitudes (Beardall and Johnston, 1983, 1985a, 1985b; Konagaya, 1985; Yamashita and Konagaya, 1990a). Depletion of fast muscle protein results in a reduction in the diameter of myofibrils and myofibers leading to a rise in water content (Beardall and Johnston, 1983, 1985a, 1985b), changes that are reversed with re-feeding (Beardall and Johnston, 1983, 1985a, 1985b). The present study provides strong evidence for the involvement of cathepsins in this process (Fig. 5.7). In agreement with the present results, Beardall and Johnston (1985a) reported fast muscle lysosomal enzyme activities increased by 70-100% during starvation in saithe (*Pollachius virens* L.). Similarly, Chum salmon (*Oncorhynchus keta*) showed high proteolytic activity of cathepsin L during the spawning migration and this enzyme was believed to be the most important contributor to the observed softening of the flesh (Yamashita and Konagaya, 1990a). Further, cathepsin activities of fast and slow muscle peak during the spawning season of sardine (*Sardine pilchardus*) (Gómez-Guillén and Batisa, 1997) and ayu (*Plecoglossus altivelis*) (Yamashita et al., 1990). In sea bass the activity of cathepsin B and B + L was 29.7 and 4.7 times higher than that of bovine muscle suggesting that these enzymes played a principal role in the *post mortem* degradation (Chéret et al., 2007). However, the biological factors resulting in increased cathepsin activities are

unknown, but could be due to an increased activation and secretion of cathepsin proenzymes stored within the lysosomes, increased transcription, reduced amounts of inhibitors in the muscle or a combination of these factors. For example, Nomata et al. (1985) reported a decrease in specific inhibitors of calpain and trypsin activity in muscle from mature chum salmon (*Oncorhynchus keta*) compared to immature fish.

Myosin, actin and tropomyosin make a significant contribution to the WHC in meat (Hamm, 1986; Wismer-Pedersen, 1987). It seams likely that degradation of myofibrillar proteins will alter the WHC and possibly result in leaking of fluid from the muscle tissue, especially during processing. Of the enzymes investigated, cathepsin B + L, D and H are known to degenerate a wide range of structural muscle proteins (Schwartz and Bird, 1977, Matsukura et al., 1981; Noda et al., 1981; Katunuma et al., 1983; Aoki and Ueno, 1997) and are therefore potential candidates to influence the protein content and hence liquid loss of fish muscle (Fig. 5.7 & 5.9). The relationship between liquid loss and cathepsin H activity is non-linear and approaches asymptotic values (Fig. 5.9). Although cathepsin H showed the strongest correlation with liquid loss the interactions between variables complicates the analysis (Fig. 5.8). It is most likely that there are synergic effects of several proteolytic enzymes on muscle protein breakdown (Fig. 5.7) (Delbarre-Ladrat et al., 2004; Chéret et al., 2007) in combination with other factors eventually affecting liquid loss (Ofstad et al., 1995; Olsson et al., 2003a, 2003b, 2007). Other candidate proteolytic enzymes involved are the calpain family, which are also known to mobilise myofibrillar proteins in fish (Sentandreu et al., 2002; Delbarre-Ladrat et al., 2004; Chéret et al., 2007).

It is concluded that the seasonal changes in liquid loss observed in commercially farmed Atlantic halibut (Table 5.2) are in broad agreement with published results from small-scale experiments (Olsson et al., 2003a; Haugen et al., 2006), indicating that it is a robust finding. Further, it is documented that cathepsins have a significant impact on the protein content and

hence liquid loss in Atlantic halibut muscle, and are therefore key enzymes in catabolic processes. The present results support previous conclusions regarding the timing of the harvesting of Atlantic halibut (Hagen et al., 2007). If halibut are harvested during the spring it is likely to reduce the shelf life of the product due to the high cathepsin activity (Fig. 5.4 & 5.5), which will increase the rate of muscle disintegration during storage (Ladrat et al., 2000).

Chapter 6

Muscle expression of growth-related genes during fasting and refeeding in juvenile Atlantic halibut, *Hippoglossus hippoglossus*.

6.1 Abstract

The aim of this study was to establish a fasting-refeeding protocol to investigate the expression of growth-related genes during the transition between catabolic and anabolic states in Atlantic halibut (*Hippoglossus hippoglossus*). Juveniles of ~950 g were maintained at ambient temperature (5-8 ^{o}C) until the 1st of May, then fasted for two months and refed for two months at 7.7-8.0 ^{o}C under continuous low light. Fast epaxial myotomal muscle was sampled at -64 days (d), -38 d, 0 d (start of refeeding), 3 d, 7 d, 14 d, 30 d and 60 d. Average body mass (Mb) was unchanged over the fasting period but increased by 24.4% following 60 d refeeding. qPCR was used to analyse the stability of expression of five potential reference genes (*Eef2*, *Fau, 18S rRNA, Actb* and *Tubb2*) with *GeNorm* and *Normfinder*. Expression of the growth-related genes, *cathepsin B (ctsb), cathepsin D (ctsd), insulin-like growth factor-I and II (IGF-I* and *II)* and *insulin-like growth factor-I receptor 1a* (*IGF-IRa)* was normalised using the geometric average of the two most stable house keeping genes, *Fau* and *18S rRNA*. *IGF-I* mRNA showed a transient 2.6-fold increase in abundance with refeeding at 7d whilst transcripts for *IGF-II* and *IGF-IRa* were elevated during fasting and decreased 3.8-fold and 3-fold between the 0 d and 3 d samples respectively. *Ctsb* expression increased between -64 d and 0 d and then decreased ~10-fold by 14 d refeeding. In contrast, *ctsd* was relatively unaffected by the fasting-refeeding cycle, showing a modest (~35%) transient decrease in expression between the 0d and 30d refeeding samples. It was concluded that the experimental protocol adopted and housekeeping genes identified were suitable for investigating the catabolic-anabolic transition in halibut skeletal muscle.

6.2 Introduction

Teleost fish from temperate latitudes often experience seasonal cycles of low temperature coupled with restricted food supply in the winter, leading to reduced protein synthesis, metabolic depression (Johnston, 1981; Love, 1988) and slower growth (Maclean and Metcalfe 2001; Purchase and Brown 2001; Hagen et al., 2006). Laboratory experiments have shown that the restoration of favourable conditions following growth restriction leads to a period of compensatory growth relative to continuously fed groups, largely as a result of increased appetite and feeding activity (Jobling and Koskela, 1996; Nicieza and Metcalfe, 1997; Ali et al., 2003). The degree of compensatory growth observed varies with developmental stage (Nicieza and Metcalfe, 1997), the length of the deprivation/refeeding period (Hayward et al., 1997; Nicieza and Metcalfe, 1997), temperature (Nicieza and Metcalfe, 1997; Purchase and Brown, 2001) and species (Ali et al., 2003; Hayward et al., 1997). The extent to which metabolic depression occurs with starvation or food restriction is a significant factor in inter-specific variation in fasting-refeeding responses, because of the sparing effects on energy stores (Carter et al., 1998; Johnston, 1981). The winter months with their unfavourable growing conditions coincide with a dramatic increase in the mass of the gonads in sexually competent individuals involving considerable metabolic reorganisation (Hemre et al., 2002; Roth et al., 2007a; Solberg and Willumsen, 2008). The fast skeletal muscle acts as a reservoir of amino acids for gonadal protein synthesis (Bradford, 1993; Love, 1988; Mommsen, 2004) and energy production in many teleost species (Desrosiers et al., 2008; Finn et al., 1995). Seasonal cycles of food restriction and sexual maturation are consequently associated with seasonal changes of muscle protein content and activity of proteolytic enzymes (Yamashita and Konagaya 1990b; Toyohara et al., 1991; Gómez-Guillen and Batista, 1997; Hagen et al., 2008b).

There has been considerable interest in harnessing the compensatory growth phenomena to increase growth (Hayward et al., 1997; Zhu et al., 2005), improve food conversion efficiency (Jobling et al., 1994; Zhu et al., 2001) and modify muscle composition (Heide et al., 2006) in aquaculture. In the case of Atlantic halibut (*Hippoglossus hippoglossus*), compensatory growth is an unavoidable component of farming since short winter days and temperatures below 6 ºC result in a dramatic reduction in feeding activity (Hagen el al., 2006). Superimposed on this winter growth restriction are problems associated with the precocious maturation of males leading to cessation of muscle fibre recruitment, mobilisation of muscle protein and an increase in cathepsin B (Ctsb) and D (Cstd) activities (Hagen et al., 2006; Hagen et al., 2008b). Ctsb and Ctsd have been implicated in the *post-mortem* degradation and softening of fish flesh and are therefore of considerable technological interest (Yamashita and Konagaya 1990b; Delbarre-Ladrat et al., 2004; Mommsen, 2004). However, it is not known whether changes in cathepsin activity reflect an increased activation and secretion of its zymogen, decreased activity of enzyme inhibitors (Nomata et al., 1985; Toyohara et al., 1991) and/or changes at a transcriptional level.

The fasting-refeeding paradigm has been widely used to investigate the molecular mechanisms underlying the transition from a catabolic to an anabolic state in fish (Chauvigné et al., 2003; Uchida et al., 2003; Pierce et al., 2005; Fox et al., 2006; Picha et al., 2006; Montserrat et al., 2007a,b; Terova et al., 2007). For example, in juvenile rainbow trout (*Oncorhynchus mykiss*) fasted for 10 weeks (6-11.5 ºC), refeeding resulted in a 15-fold increase in *insulin-like growth factor-I* (*IGF-I*) mRNA 12d following refeeding, and a much smaller increase in the less abundant *IGF-II* mRNA (Chauvigné et al., 2003). In the same study, *IGF receptor 1a* (*IGF-RIa*) mRNA was found to decrease significantly with refeeding whereas *IGF-RIb* transcript abundance was unchanged (Chauvigné et al., 2003). In juvenile channel catfish (*Ictalurus punctatus*), *IGF-II* mRNA was more abundant than *IGF-I* mRNA

and highly correlated with family growth rate (Peterson et al., 2004), indicating some inter-specific variation in the relative abundance of genes regulating growth.

The aims of the present study were to establish a fasting-refeeding protocol in Atlantic halibut and to identify stable housekeeping genes to allow quantification of transcript abundance of some key genes involved in muscle wasting (*ctsb*, *ctsb*) and growth regulation (*IGF-I*, *IGF-II* and *IGF-R1a*). Since *IGF-I* and *IGF-II*, *ctsb* and *ctsd* have not been previously characterised in Atlantic halibut partial cDNAs for these genes were cloned.

6.3 Material and methods

6.3.1 Fish maintenance and sample preparation

Seventy male Atlantic halibut from the 2004 generation (hatched and farmed at Mørkvedbukta Research Station, MRS) were chosen due to their abundance and the fish were held indoors in two square tanks (2x2 m, 40 cm water depth) under constant light (similar to dawn) in seawater pumped from 250 m depth at MRS (Bodø University College, Bodø, Norway). Prior to the experiment all fish were tagged intraperitoneally with passive integrated transponder (PIT) -tags and weighed to allow individual growth rates to be calculated. The experimental period lasted from the 3rd of May until the 31st of Aug 2007 and samples were taken at 8 time points with respect to the start of the refeeding period (0 d). Muscle samples were taken at -64 d, -38 d, 0 d (fasting period) and 3 d, 7 d, 14 d, 30 d and 60 d (refeeding period). Fish were fed a mixture of 3-9 mm commercial pellets (Europa Marine S Halibut, Skretting, Norway) that were provided via an automated feeding system in combination with manual inspection and hand feeding. All fish were anaesthetised with MS-222 (Argent Chemical Laboratories, Washington, USA) and weighed at -64 d, -38 d and 0 d to enable growth rate estimation during the fasting and refeeding periods. At each sampling point, six fish were randomly netted from the tank and sacrificed with a sharp blow to the head. Samples of fast myotomal muscle were dissected from the dorsal right midsection of the fish, snap-frozen in liquid nitrogen and stored at -80 °C until processing. Samples for muscle water content analysis were collected in duplicate (5 g) and analysed as described in section 3.3.3.

6.3.2 RNA extraction and cDNA synthesis

Duplicate ~100 mg samples of fast muscle were placed into Lysing Matrix D tubes (QBiogene/Medinor, Oslo, Norway) containing QIAzol (Qiagen, Nydalen, Sweden) and homogenised for 40 s at 6000 rpm using the MagNA Lyser instrument (Roche, Mannheim, Germany). Total RNA was extracted according to the manufacturer's instructions and treated with the gDNA wipe-out buffer (Qiagen) to remove any potential genomic DNA contamination. Assessment of RNA quality was performed by electrophoresis on a 1.2% (w/v) agarose gel containing SYBR safe DNA gel stain (Invitrogen, Oregon, USA) (Fig. 6.1 A), whereas RNA purity and quantity were determined using a Nanodrop spectrophotometer (Nanodrop Technologies/Saven Werner, Kristiansand, Norway). All RNA preparations had absorbance ratios (260/280 nm) greater than 1.9, an indication of high purity RNA. cDNA was produced with the QuantiTect reverse transcription kit (Qiagen) following the recommended protocol and the single-stranded cDNA products were re-quantified using the Nanodrop spectrophotometer. Prior to use as template in the qPCR reactions, the cDNA was diluted 8-fold in deionised water.

6.3.3 Cloning of *IGF-I, IGF-II, ctsb* and *ctsd*

Degenerate primers for *IGF-I* and *IGF-II* were designed by searching for orthologous genes from other fish species using the NCBI database (http://www.ncbi.nlm.nih.gov/). From the ClustalW alignments obtained with Bioedit (Ibis Bioscience, California, USA), degenerate primers were selected within the most conserved regions of the aligned sequences. *Netprimer* (http://www.premierbiosoft.com/netprimer/) was used to estimate the melting temperature and G/C content of the primers, as well as to investigate the presence of potential hair pins, primer

dimers and cross dimers. The degenerate primers used are listed in Table 6.1. Each of the 25 µl reactions in PCR tubes consisted of 1 µl 10 x diluted halibut cDNA as template, 1 µl of each primer at 50 µM, 0.5 µl 10 mM dNTP mix, 0.125 µl GoTaq (Promega, Southampton, UK), 5 µl GoTaq buffer and 16.4 µl nuclease free water. The PCR tubes were placed in a Techne Touchgene Gradient PCR (Techne, Cambridge, UK) and after an initial denaturation step of 10 min at 95 ºC, 40 cycles of amplification were performed according to the following thermocycling profile: denaturation for 30 s at 95 ºC, annealing for 30 s at 50-60 ºC with 2 ºC intervals and extension for 30 s at 72 ºC. Following electrophoresis, the PCR products of interest were excised from the gel (Fig. 6.1 B&C), purified, cloned and sequenced as previously reported (Fernandes et al., 2005). Sequencing products were read with an ABI3730 DNA Analyser (Applied Biosystems, Foster City, USA) at the Oxford sequencing facility (Oxford, UK). Full-length cDNA sequences for *ctsb* and *ctsd* were obtained from high quality expressed sequence tag (EST) clusters identified in Atlantic halibut cDNA libraries (Bai et al., 2007) using 5' and 3'- rapid amplification of cDNA ends (RACE), according to the manufacturer's instructions. RACE products were cloned and sequenced as above.

6.3.4 Design of real-time PCR primers

The halibut sequences of *IGF-I* (EU682415) and *IGF-II* (EU682416) were used to design gene-specific primers for qPCR. The gene-specific primers for *ctsb* and *ctsd* were based on the full-length cDNA sequences available at NCBI (GenBank accession numbers DQ993253 and DQ912663, respectively). The gene-specific primers for *Eef2*, *Fau* and *Tubb2* were obtained from high-quality ESTs derived from three Atlantic halibut cDNA libraries (Bai et al., 2007). The genomic sequences of orthologous genes from *Danio rerio*, *Gasterosteus aculeatus*, *Oryzias latipes*, *Takifugu rubripes* and *Tetraodon nigroviridis* were retrieved from

Ensembl (http://www.ensembl.org/). Intron/exon borders within the Atlantic halibut ESTs, *IGF-I, IGF-II, ctsb* and *ctsd* sequences were then predicted using the Spidey software (http://www.ncbi.nlm.nih.gov/spidey/) by aligning the sequences with the corresponding genomic sequences from the other teleost species. Whenever possible, primers were designed across the most conserved splice junctions. All gene specific primers crossed at least one intron/exon border containing both donor and acceptor sites, in order to avoid amplification of any contaminating genomic DNA. Primer pairs for qPCR amplification were designed manually and screened for hairpins, homo- and cross-dimers using Netprimer (http://www.premierbiosoft.com/netprimer/). The *18S rRNA and IGF-IRI* gene-specific primers from Atlantic halibut have been recently reported (Hildahl et al., 2007). All primers used are listed in Table 6.1.

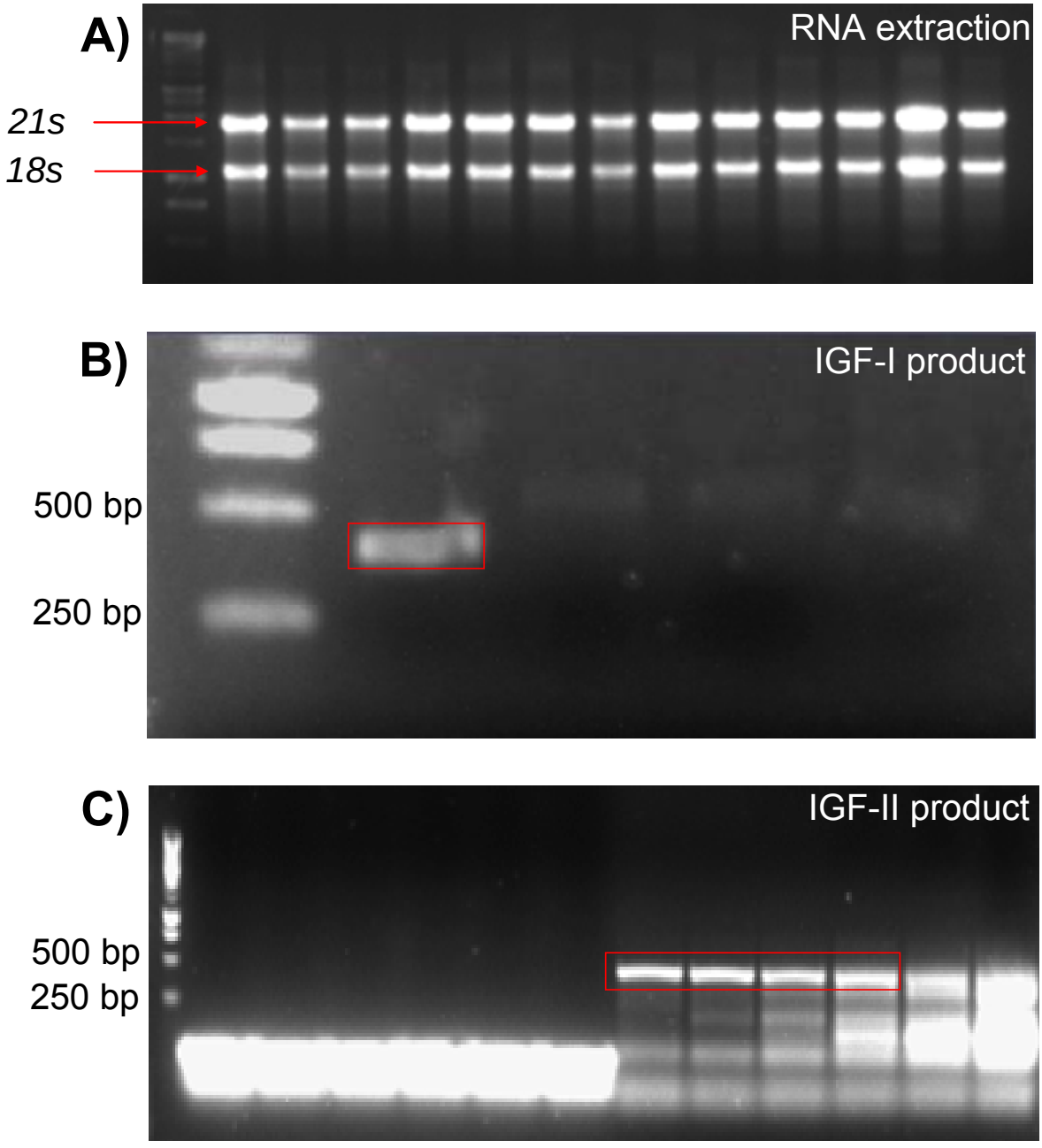

Figure 6.1. A: RNA validation on agarose gel shows the *21s* and *18s* band. B: Band of expected size obtained with degenerate primers for *IGF-I* was cut from the gel (indicated with red box) and purified post cloning. C: Band of expected size obtained with degenerate primers for *IGF-II* (50-60 ºC) was cut (indicated with red box) from the gel and purified post cloning.

6.3.5 Quantitative real-time PCR analysis

The qPCR gene amplifications were performed with a LightCycler® 480 thermocycler (Roche, Oslo, Norway) using the LightCycler® 480 SYBR Green I Master (Roche), which contains ROX as reference dye. Each 10 ml reaction in a 96-well plate comprised 4 ml of 8-fold diluted cDNA template, 1 ml of each primer pair at 2.5/5 μM (see Table 6.1 for details) and 5 ml of QuantiTect SYBR Green containing ROX as reference dye (Qiagen). Plates were sealed with adhesive optical film (Roche), and after an initial denaturation step of 15 min at 95 °C, 45 cycles of amplification were performed according to the following thermocycling profile: denaturation for 15 s at 94 °C, annealing for 20 s at 60 °C (see Table 6.1 for details) and extension for 20 s at 72 °C. Fluorescence data were acquired during this last step. A dissociation curve with a temperature gradient from 65 to 97 ºC was used to investigate the specificity of each qPCR reaction and the presence of PCR artefacts. C_T (threshold cycle) values were determined by the fit-points method. All samples were run in duplicate along with minus reverse transcriptase, no template and positive plate controls. The mean gene expression profiles of the two cDNA samples sampled from each fish were obtained. Five-point standard curves of a 4-fold dilution series (1:1-1:256) were made from pooled cDNA and the PCR efficiencies were calculated using the LightCycler® 480 software.

The suitability of the potential reference genes (*Eef2*, *Fau, 18S rRNA, Actb* and *Tubb2*) was assessed with GeNorm (Vandesompele et al., 2002) and Normfinder (Andersen et al., 2004). Normalisation factors were calculated by GeNorm (Vandesompele et al., 2002) as the geometric average of the two most stable reference genes. After converting the C_T values into quantities using the standard curves, gene expression levels were normalised by dividing the raw data by the appropriate normalisation factors.

Table 6.1. Details of degenerated and qPCR primer used in the experiment. Abbreviations are as follows: PC, primer concentration: AT, annealing temperature; E (%), PCR reaction efficiency.

Gene	Type	PC / AT	Fwd sequence (5' → 3')	Rev sequence (5' → 3')	E (%)	Size (bp)
IGF-I	Degenerated	50 µM, 50-60 ºC	CGCAATGGAACAAAGTCG	YTGRAAGCAGCAYTCGTC		399
IGF-II	Degenerated	50 µM, 50-60 ºC	CRCWCASTTTGYCACACCTGC	CCTSAGRATGGCSGGGAC		486
IGF-I	qPCR	2.5 µM / 63 ºC	CAGGAGATACAGCACATCGCAC	GGCTTTGACTTGCCGAGAC	97.1	98
IGF-II	qPCR	2.5 µM / 60 ºC	GCTTCTATTTCAGTAGGCCAACC	GACACATCCCTTTCGGACTTG	100	148
*IGF-IRa**	qPCR	2.5 µM / 60 ºC	TGTCTGATGTCTGGTCGTTTGG	GGTAGGGCTGCTCCGCTAAG	88.9	66
Ctsb	qPCR	2.5 µM / 60 ºC	ATCCTGCTGGGCATTTGGC	ACAGGCGTCACAACAGGTCAG	86.1	118
Ctsd	qPCR	2.5 µM / 61 ºC	ATGAAGGAGTTCCAGAAGGGTTC	GCGTCGTTCTACATCTACTTGGC	97.9	182
*Tubb2***	qPCR	5 µM / 60 ºC	CTACAATGAGGCTTCAGGTGG	TCCCTCTGTGTAGTGACCCTTG	90.2	134
*Fau***	qPCR	5 µM / 60 ºC	GACACCCAAGGTTGAAAAGCAG	GGCATTGAAGCATTTAGGAGTTG	92.7	149
*18S rRNA**	qPCR	5 µM / 60 ºC	GCATGCCGGAGTCTCGTT	TGCATGGCCGTTCTTAGTTG	92.3	140[1]
Actb	qPCR	5 µM / 60 ºC	GAGAAGATGACTCAGATCATGTTCG	CCAGCCAGGTCCAGACGG	89.0	154
*Eef2***	qPCR	5 µM / 60 ºC	ATGGAGTCATTTGGTTTCACAGC	GAGACCCTTGCGTTTGCG	90.8	121

* See Hildahl et al. (2007). ** See Fernandes et al. (2008). [1] Estimated by agarose gel electrophoresis.

6.3.6 Statistical analysis

Statistical analysis was done using SPSS v. 14.0.1 (SPSS Inc., Chicago, IL, USA). After a square root transformation of *IGF-IRa* and *IGF-II* expression data all variables complied with the assumptions for a one-way ANOVA. When significant differences were identified, supplementary Tukey's *post*-hoc tests were performed to investigate pair wise differences. Statistical significance was established at $P<0.05$.

6.4 Results

6.4.1 Cloning and sequencing of *IGF-I, IGF-II, ctsb* and *ctsd*

Two partial sequences of *IGF-I* and *IGF-II* of 399 and 487 bp, respectively, were obtained by homology cloning (GenBank accession numbers EU682415 and EU682416). In addition two full sequences of *ctsb* (DQ993253) and *ctsd* (DQ912663) of 1791 bp and 1847 bp, respectively, were also obtained by cloning. CLUSTALW alignments with orthologous sequences showed that both halibut IGF-I and IGF-II are conserved amongst different teleosts species and between fish and other taxa (Fig. 6.2 A&B). As expected, IGF-I was more conserved between fish species, compared to mammals and birds (Table 6.2). Similarly, the amino acid identity with halibut IGF-II ranged from 44.4% in *Mus musculus* to 96.2% in *Paralichthys olivaceus* (Table 6.2). In general, the degree of conservation was higher for IGF-I than for IGF-II in all taxa examined. Sequence identities both for IGF-I and IGF-II were particularly striking amongst flatfish species. For example, the deduced amino acid sequences of IGF-I and IGF-II in Atlantic halibut showed 97.8 and 96.2% identity to those of *Paralichthys olivaceus* and 73.4 and 65.6% identity to the *Anguilla japonica* sequences, respectively. In contrast, the IGF-I and IGF-II protein sequences shared 56.3 and 44.4% identity to *Mus musculus* and 55.3 and 46.2% identity to *Homo sapiens* (Table 6.2).

CLUSTALW alignments of Ctsb and Ctsd with other orthologous protein sequences showed that both were conserved between taxa. The domains of Ctsb and Ctsd, outlined as boxes in Fig. 6.3 A&B, were particularly conserved. In general, Ctsb proteins in different species were more similar than the Ctsd orthologues. Identity amongst fish species ranged from 69.7 to 79% and 58.1 to 58.3% for Ctsb and Ctsd, respectively (Fig. 6.3 A&B, Table 6.3).

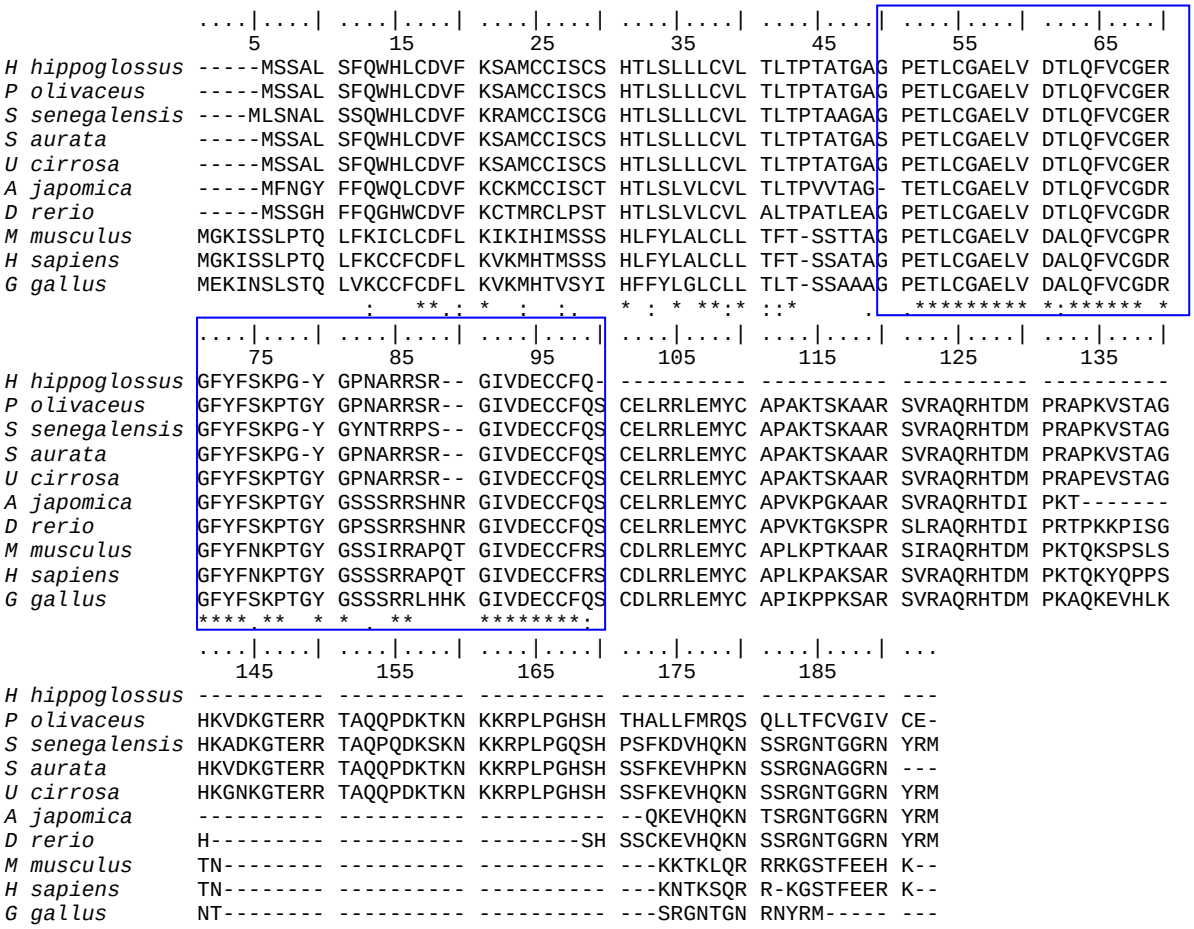

Figure 6.2A. Figure legend on next page.

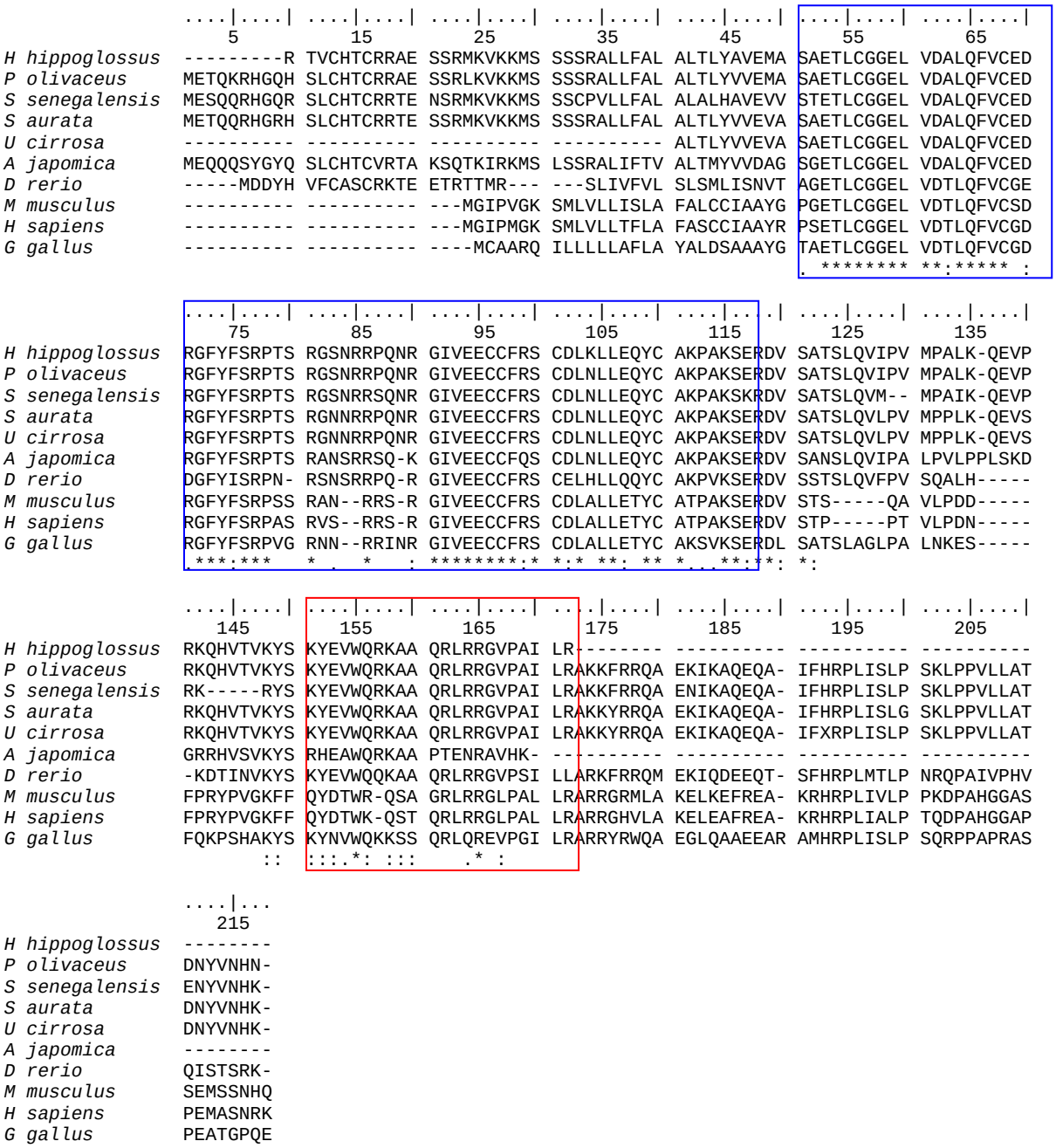

Figure 6.2B. Deduced amino acid sequence alignment comparing IGF-I (A) and IGF-II (B) sequences of Atlantic halibut with orthologous from other vertebrate species. The coloured boxes indicates the Insuline/IGF/Relaxin superfamily (in blue) domain for IGF-I (A) and IGF-II E-peptide domain (in blue) and IGF domain (in red) for IGF-II (B). Symbols at the bottom line of each alignment stand for identical (*), conserved substitution (:) and semi conserved substitution (.). Accession numbers are given in Table 6.2.

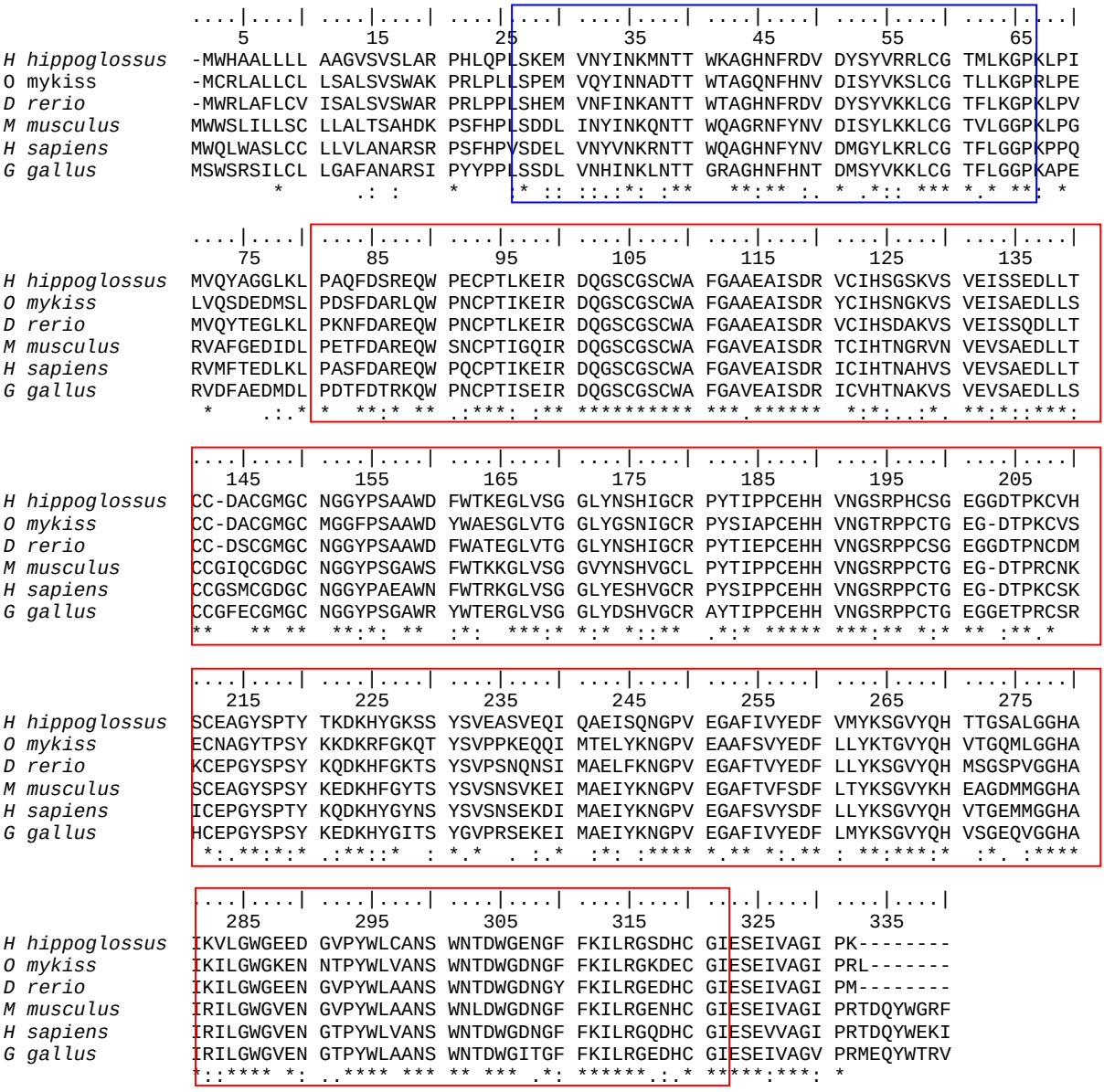

Figure 6.3A. Figure legend on next page.

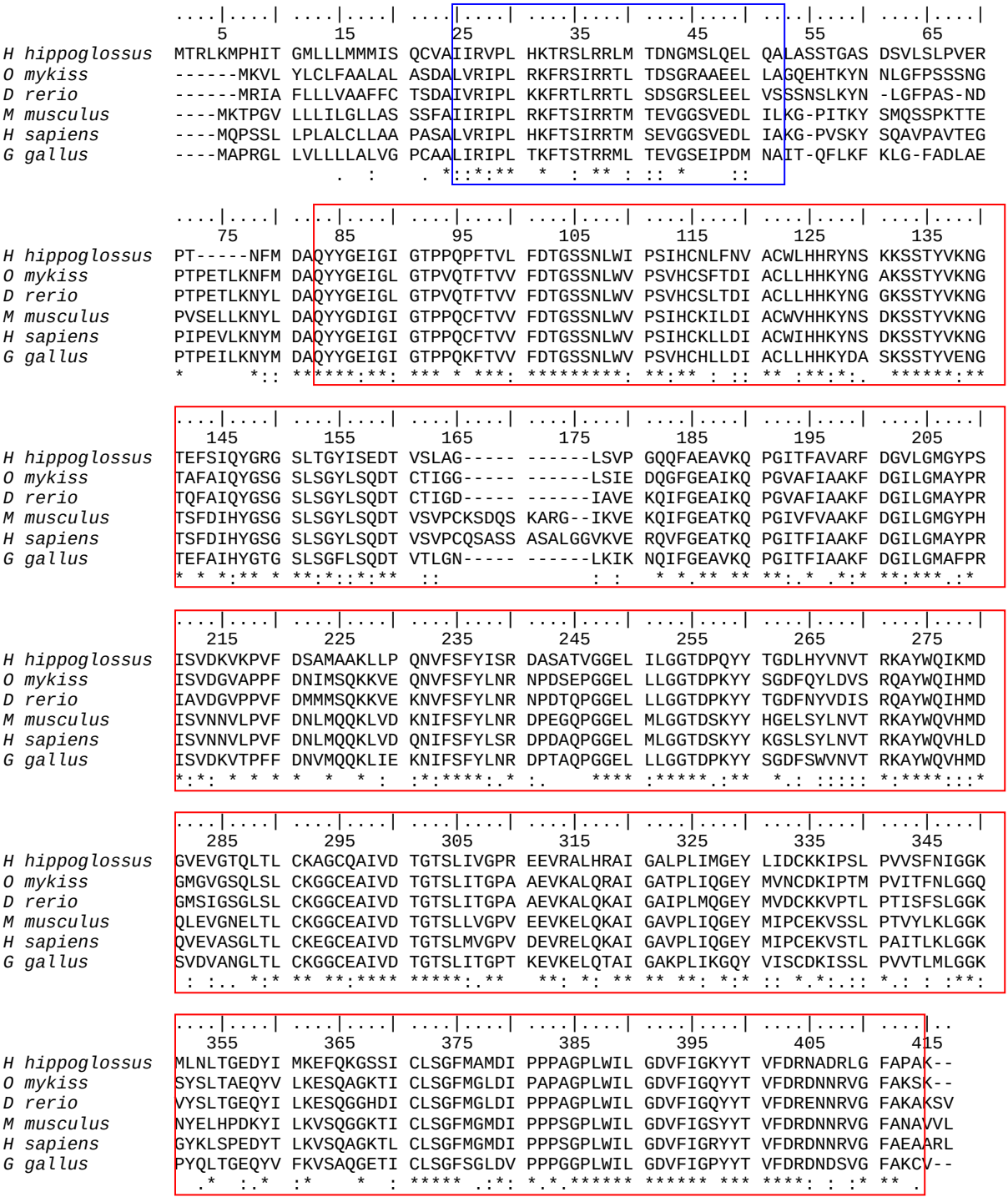

Figure 6.3B. CLUSTALW alignment of the deduced Ctsb (A) and Ctsd (B) amino acid sequences of Atlantic halibut and orthologous of other vertebrate species. The coloured boxes indicates the different domains, which are peptidase family C1 propeptide (in blue) and C1 peptidase superfamily, cathepsin B group (in red) for Ctsb (A). For Ctsd (B) the domains are A1 propeptide (in blue) and Aspartyl protease superfamily (in red). Symbols at the bottom line of each alignment stand for identical (*), conserved substitution (:) and semi conserved substitution (.). Accession numbers are given in Table 6.3.

Table 6.2. Comparison of the partial deduced amino acid sequence of Atlantic halibut with orthologous IGF-I and IGF-II sequences from other fish and vertebrate species.

Species	**Genebank accession no**	**Aa identity (%)**	**Aa identity (%)**
	IGF-I / IGF-II	***IGF-I***	***IGF-II***
Paralichthys olivaceus	AAB64052 / AAC61761	97.8	96.2
Solea senegalensis	BAF02628 / ABF02629	89.0	84.5
Sparus aurata	ABQ52656 / ABQ52655	98.9	93.2
Umbrina cirrosa	AAY21628 / AAX37666	97.8	76.5
Anguilla japonica	BAF74503 / BAF74506	73.4	65.6
Danio rerio	AAK58584 / NP_571508	72.3	58.6
Mus musculus	NP_001104753 / NP_000603	56.3	44.4
Homo sapiens	NM000618.2 / NM 000612.2	55.3	46.2
Gallus gallus	NP_001004384 / NP_001025513	57.4	50.0

Table 6.3. Comparison of the partial deduced amino acid sequence of Atlantic halibut with orthologous Ctsb and Ctsd sequences from other fish and vertebrate species.

Species	**Genebank accession no**	**Aa identity (%)**	**Aa identity (%)**
	ctsb / ctsd	***ctsb***	***ctsd***
Oncorhyncus mykiss	NP_001117776 / NP_001118183	69.7	58.1
Danio rerio	NP_998501 / AAL61540	79.0	58.3
Mus musculus	NP_031824 / NP_034113	65.0	57.1
Homo sapiens	AAH10240 / AAP36305	67.3	57.3
Gallus gallus	NP_990702 / NP_990508	65.2	57.9

6.4.2 Fasting-refeeding experiment

The experiment started in May at the end of the natural fasting period during the winter. Halibut show reduced appetite below 6 ^{o}C and it is likely that the fish underwent natural fasting, due to a period of reduced appetite in the winter, until -64 d when the water temperature reached this threshold (see Fig. 6.4 dashed line). All fish were feeding by -64 d when food was withdrawn. During the 124 d of the experiment the water temperature remained within the range 7.5 to 8.0 ^{o}C (see Fig. 6.4 dashed line). The Mb of the fish did not change significantly during the fasting period ($P>0.05$, ANOVA). At the end of refeeding the average weight had increased from 950 ± 189 g to 2005 ± 129 g (Fig. 6.4, $P<0.001$, ANOVA, mean ± SE). The % weight increase, calculated from PIT-tagged individuals, was 24.4% (Fig. 6.4, $P<0.001$, ANOVA). During the refeeding period the fish had good appetite and an average specific daily growth rate of 0.44%.

Muscle water content remained stable from -64 d to -38 d fasting (76.6 ± 0.2%, mean ± SE), but then increased to 77.7 ± 0.5% (mean ± SE) at 14 d ($P<0.05$, ANOVA, not shown). At 60 d the muscle water content had returned to initial levels (-64 d).

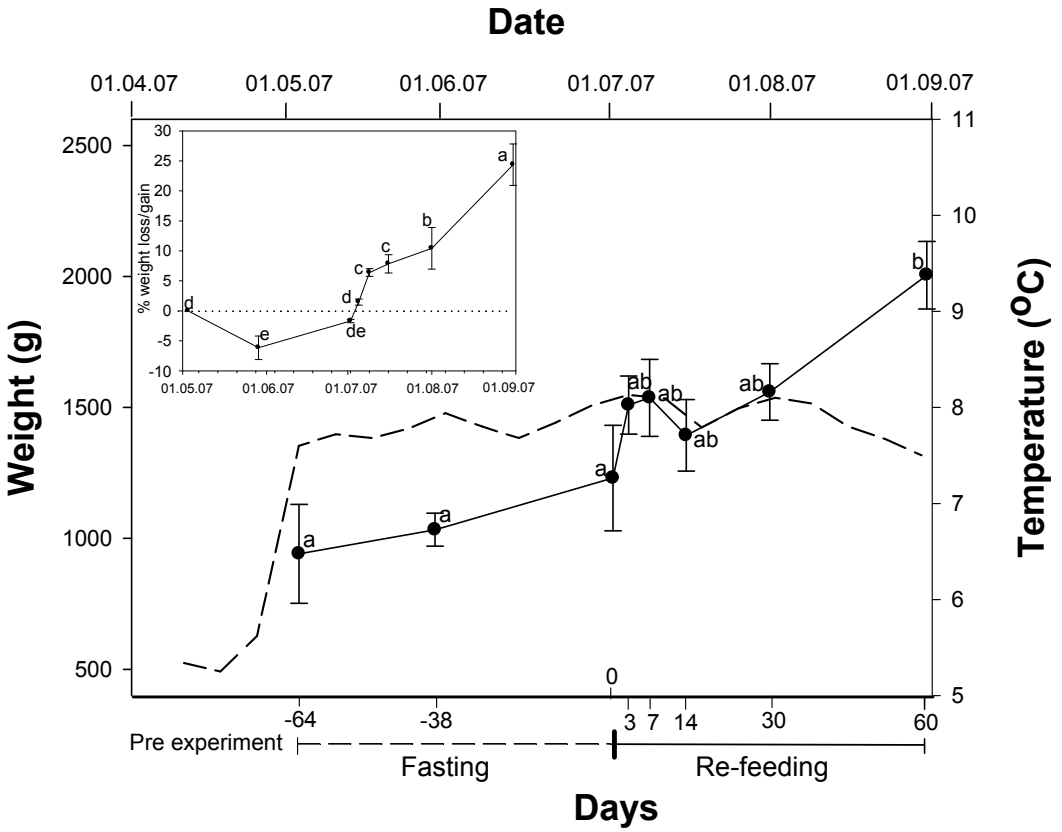

Figure 6.4. Body mass and temperature (dashed line) changes during the different sample points of the fasting and refeeding experiment. Insert show % weight change at the different sample points. Different letters indicates significant difference between sample points (mean ± SE, n=6).

6.4.3 Gene expression

Of the investigated candidate housekeeping genes (*Eef2*, *Fau, 18S rRNA, Actb* and Tubb2, see Table 6.1), *Fau* and *18S rRNA* were selected by both Normfinder and GeNorm as the most stable and suitable gene pair, and were therefore used to normalise the target gene expressions. Normalisation factors were calculated by GeNorm (Vandesompele et al., 2002) as the geometric average of these reference genes (*18S rRNA* and *Fau*) (Fig. 6.5).

During the fasting period *ctsb* transcript levels increased, reaching a plateau between -38 d to 0 d ($P>0.05$). At 7 and 14 d, *ctsb* mRNA was reduced by ~2.4- and 9.7-fold respectively relative to 0d (Fig. 6.6 A, $P<0.001$, ANOVA) and remained at this level until 60d. In contrast, *ctsd* transcripts were relatively unaffected by the fasting-refeeding cycle, showing a modest (~35%) transient decrease in expression between the 0 d and 30 d refeeding (Fig. 6.6 B, $P<0.05$).

IGF-I transcript levels were similar in the -64 d, -38 d, 0 d and 3 d samples and then showed a transient 2.6-fold increase at 7 d refeeding (Fig. 6.7 A). In the 30 d and 60 d samples *IGF-I* mRNA levels were approximately 25-50% higher than fasting levels. *IGF-IRa* transcript amount were relatively stable in the -64 d, -38 d, and 0 d samples, but decreased by 3.8-fold at 3 d refeeding (Fig. 6.7 B, $P<0.001$). During the rest of the refeeding period transcript levels remained relatively constant except for a small transient increase between 7 d and 30 d ($P<0.05$). *IGF-II* mRNA increased ~94% during fasting from -64 d to 0 d (Fig. 6.7 C, $P<0.001$), but showed a rapid decrease to initial levels within 7 d of refeeding. The *IGF-I* / *IGF-II* ratio was approximately 1 during the fasting period and increased 2.2-fold in the 7d sample ($P<0.05$).

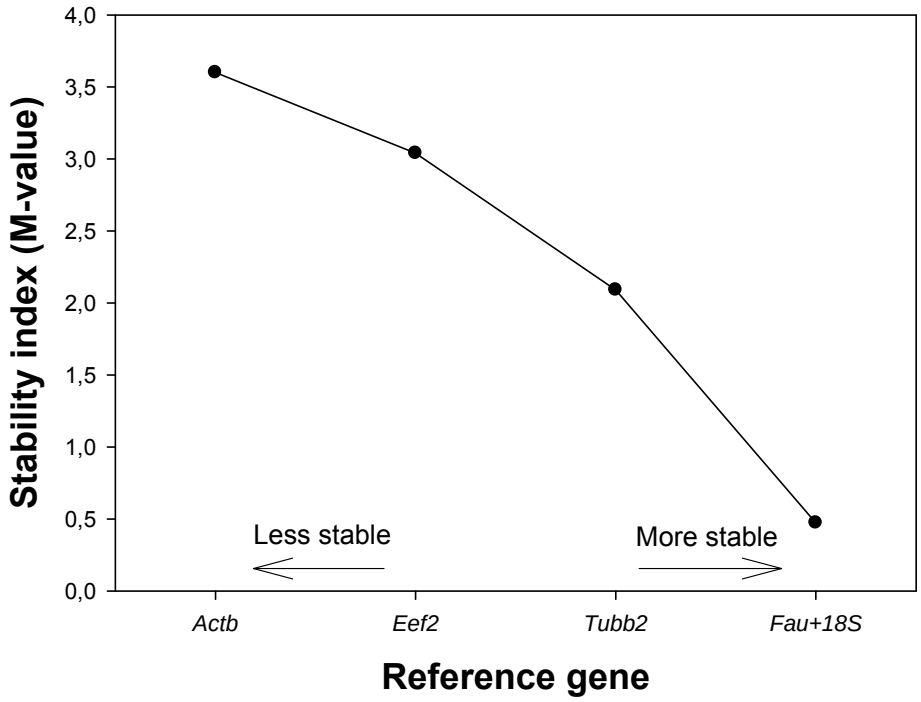

Figure 6.5. Ranking of reference genes according to their average expression stability during fasting and refeeding of Atlantic halibut, calculated by GeNorm. The expression stability is inversely correlated to their stability index. *Fau* and *18S* were identified as the most suitable gene pair for data normalization.

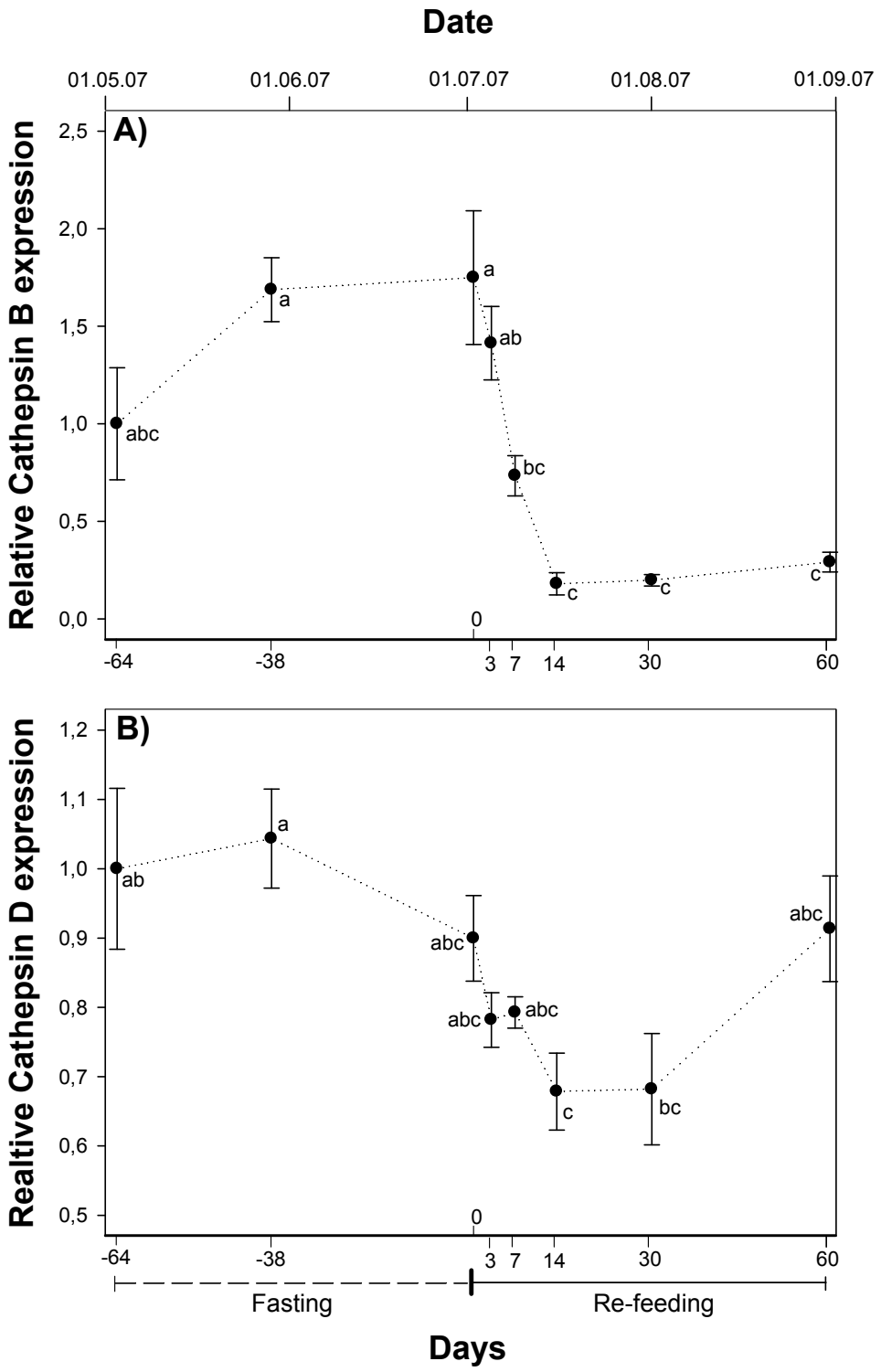

Figure 6.6. Relative gene expression of *ctsb* (A) and *ctsd* (B) during the experiment. The normalisation factors were calculated by *GeNorm* as the geometric average of *18S* and *Fau*, being identified as the most suitable reference genes. Different letters indicates significant difference between sample points (mean ± SE, n=6).

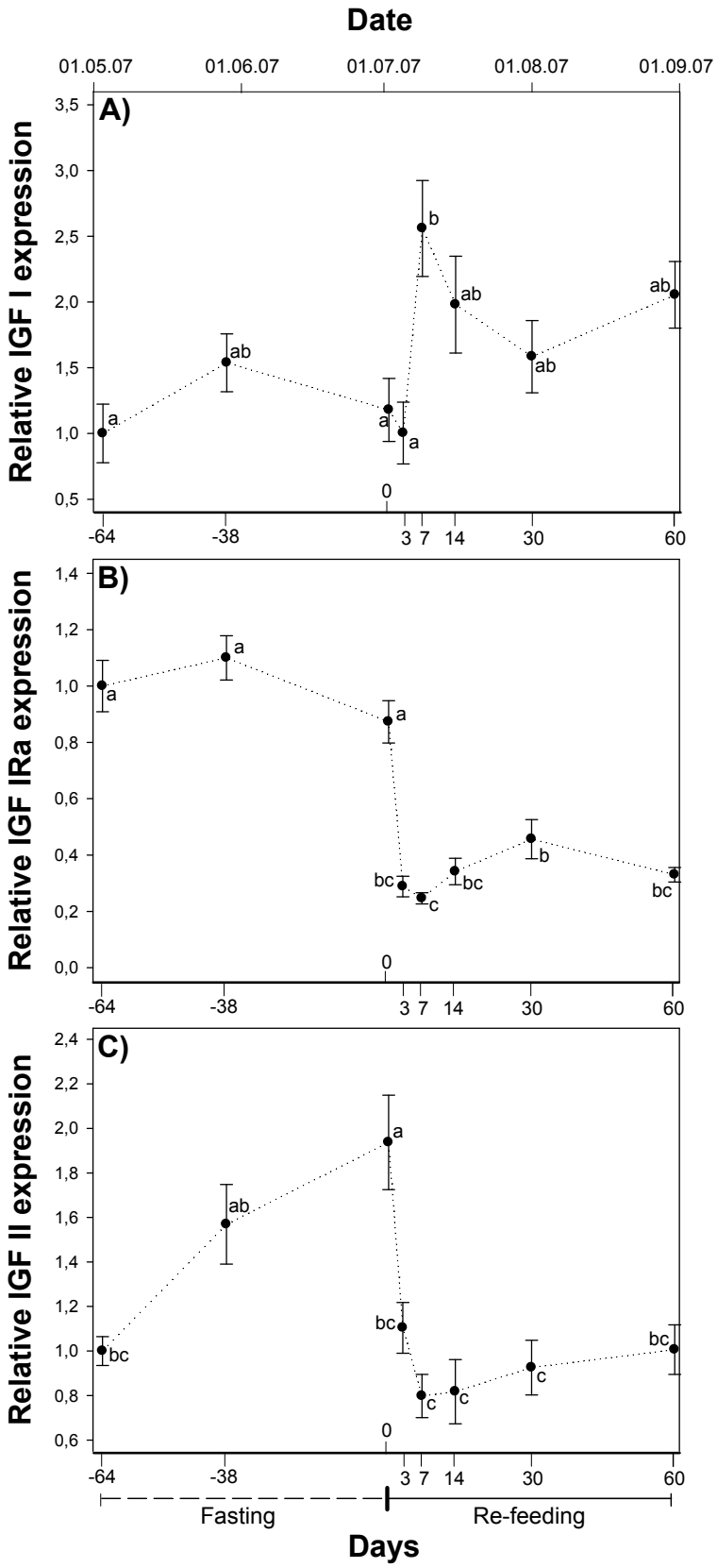

Figure 6.7. Relative gene expression of *IGF-I* (A), *IGF-IRa* (B) and *IGF-II* (C) during the experiment. The normalisation factors were calculated by *GeNorm* as the geometric average of *18S* and *Fau*, being identified as the most suitable reference genes. Different letters indicates significant difference between sample points (mean ± SE, n=6).

6.5 Discussion

Fasting and refeeding experiments can be used to investigate the transition from catabolic to anabolic metabolism and compensatory catch-up growth in teleosts (Blasco et al., 1992; Heide et al., 2006). The state of sexual maturity of the fish and the time of year the experiments are conducted are expected to be critical factors determining the responses observed, due to the seasonal switching of energy allocation between growth and reproduction and natural cycles of food restriction associated with low temperature and/or decreased prey availability respectively (Jobling, 1981, 1993; Johnston and Battram, 1993). The present study took advantage of the natural endogenous rhythm of winter fasting in the Atlantic halibut which reduces feeding activity below 6 ^{o}C, temperatures which are experienced for several months each year in northern parts of Norway during the juvenile stage when the fish are living in shallow costal water (Haug 1990). In our experiments the fish had been repeatedly fed during the winter period and then fasted for 2 months prior to refeeding (Fig. 6.4). It is known that the duration of the increase in metabolic rate that follows feeding (specific dynamic action, SDA) is strongly temperature dependent (Johnston and Battram, 1993; Mallekh and Lagardère 2002; Lou and Xie, 2008). For example, in relatively sedentary demersal species the duration of the SDA (corrected for Mb and energy intake) following a single satiating meal varied from around 57 h in the tropical fish *Cirrhitichys bleekeri* to 208 h in the Antarctic species, *Notothenia corriceps* (Johnston and Battram, 1993). In Atlantic halibut a 2-month fasting period was not sufficient to produce a significant decrease in Mb (Fig. 6.4). Flatfish (*Pleuronectes flesus*) respond to prolonged fasting with reduced protein turnover (Carter et al., 1998) and up to a 40% reduction in metabolic rate in *Pleuronectes platessa* (Johnston, 1981), resulting in a sparing effect on body energy reserves. In contrast, for more active warm water species such as the Mediterranean sea bream (*Sparus aurata*),

which have higher metabolic rates, as little as one week is sufficient to produce a significant reduction in Mb with fasting (Montserrat et al., 2007a). We found that although Atlantic halibut was very resistant to fasting, the gene expression changes observed upon refeeding were on a similar timescale (3-14 d) to those reported in warm water fish (Chauvigné et al., 2003; Montserrat et al., 2007a).

Real-time PCR is considered to be the most accurate method of quantifying gene expression, but it is essential that appropriate reference genes are used to obtain a meaningful interpretation of the results (Bustin et al., 2005). The selection of suitable reference genes for qPCR studies in Atlantic halibut is currently very limited. Of the five reference genes investigated in the present study, *β-actin* and *elongation factor 2* were identified as the least appropriate reference genes having a GeNorm stability index (M value) of 3.6 and 3.0 respectively (Fig. 6.5). In two recent fasting and refeeding trials on sea bass (*Dicentrarchus labrax*), *β-actin* was chosen as a single reference gene (Ayson et al., 2007; Terova et al., 2007). However, our results indicate that *β-actin* and genes involved in metabolism (e.g., protein synthesis) should be avoided in fasting/refeeding protocols due to the treatment effect upon these genes. Similarly, in a survey of 535 human housekeeping genes, *β-actin* was found to vary by 7 to 22-fold (Warrington et al., 2000).

At least 13 different cathepsins have been identified, of which six (A, B, C, D, H and L) are present in skeletal muscle tissue (Goll et al., 1983). Cathepsins are of major importance during muscle breakdown and the softening of fish flesh (Yamashita and Konagaya, 1990b; Kubota et al., 2003; Ladrat et al., 2003; Hultmann and Rustad, 2004; Chéret et al., 2007; Hagen et al., 2008b). Ctsb and Ctsd have been shown to degrade a wide range of structural proteins in fish muscle (Burleigh et al., 1974; Noda et al., 1981), with increased activity during fasting and have also been implicated in *post-mortem* muscle degradation and softening (Beardall and Johnston 1985a; Yamashita and Konagaya, 1990b; Gómez-Guillen

and Batista, 1997; Delbarre-Ladrat et al., 2004; Mommsen, 2004; Hagen et al., 2008b). In a recent study of Atlantic halibut over an annual cycle we found that Ctsb and Ctsd activities were inversely correlated with fast muscle protein content, and were higher in male than female fish in the winter/spring period, reflecting sexual maturation and muscle protein mobilisation in males (Hagen et al., 2008b). The mechanism underlying increased cathepsin activity during winter fasting and/or spawning (Beardall and Johnston, 1985a; Gómez-Guillen and Batista, 1997; Hagen et al., 2008b) could theoretically be due to an increased activation and secretion of proenzymes stored within the lysosomes, decreased activity of enzyme inhibitors (Nomata et al., 1985; Toyohara et al., 1991) and/or increased gene expression. The present study has shown that higher enzyme activities of *ctsb* and *ctsd* in fasted than refed individuals result, at least in part, from an increase in mRNA levels (Fig. 6.6).

Both circulating IGF-I and tissue-specific mRNA expression are correlated with nutritional state and growth rate (Duan, 1998; Chauvigné et al., 2003; Beckman et al., 2004b; Cruz et al., 2006; Li et al., 2006; Imsland et al., 2007; Luckenbach et al., 2007). For example, IGF-I plasma concentrations in turbot (*Scophthalamus maximus)* were found to be ~3 times higher in fast- than slow-growing individuals (Imsland et al., 2007). Further, the level of *IGF-I* mRNA in muscle of fasted rainbow trout muscle (*Oncorhynchus mykiss*) (Chauvigné et al., 2003; Montserrat et al., 2007b) and sea bass (*Dicentrarchus labrax*) (Terova et al., 2007) increased significantly after one to two weeks of refeeding. In the present experiment, *IGF-I* transcripts were more abundant in fed than fasted individuals with peak expression 7 d refeeding (Fig. 6.7 A), and it is likely that *IGF-I* expression may contribute to muscle compensatory growth as reported in other similar studies (Chauvigné et al., 2003; Montserrat et al., 2007a,b; Terova et al., 2007).

The *IGF-IRa* expression profile during the present fasting and refeeding protocol (Fig. 6.7 B) is similar to that observed in rainbow trout (*Oncorhynchus mykiss*) muscle and liver for one of

the *IGF-IRa* isoforms (Chauvigné et al., 2003; Montserrat et al., 2007b). Montserrat et al. (2007b) concluded that the two isoforms in rainbow trout responded differently to feeding, suggesting that they have a complementary role in muscle growth due to their distinct regulation (Chauvigné et al., 2003). This could also be the case for halibut, but additional experiments are required to investigate this possibility.

In fish little is known about the nutritional effect on *IGF-II* and its involvement in growth metabolism in fish muscle. Florini et al. (1991) have reported that the autocrine action of IGF-II is essential for myoblast differentiation in cell lines. In mammals, mTOR has been identified as a regulator of muscle size, and the response to mechanical stimulation depends on the mTOR pathway (Hornberger et al., 2006; Pallafachina et al., 2002). Moreover, it has been demonstrated that mTOR might also be involved in the nutritional regulation of *IGF-II* in skeletal muscle including transcript levels (Erbay et al., 2003). The results of the present study showed that *IGF-II* transcript levels decrease with refeeding (Fig. 6.7 C), similar to the observations in *Oncorhynchus mykiss* (Montserrat et al., 2007b) and *Salmo salar* (Bower et al., unpublished). In contrast, an earlier study of *Oncorhynchus mykiss* (Chauvigné et al., 2003) and *Dicentrarchus labrax* (Terova et al., 2007) showed the opposite trend in *IGF-II* expression, a steady increase in expression during four weeks of refeeding. The reason for these differences within and between species is not easily explainable, but could be due to differences in developmental stage and/or due to the different treatments prior to feeding (Bower et al., unpublished results).

The complexity of interactions between components (receptors, different splice variants and IGF binding proteins that control the availability and half-life of IGFs), multiple tissue expression, mTOR regulation and diverse biological functions (growth, osmoregulation and maturation), make the IGF-system one of the most studied and complex endocrine systems (see review of Wood et al., 2005). To add another dimension to this already complex IGF-

system, mRNAs from the IGF axis could also be regulated by microRNAs (Bagga et al., 2005; Chan and Slack, 2006).

To be able to achieve a more detailed understanding of the different constituents of the IGF system and their interactions in relation to muscle growth, it would be necessary to perform a more comprehensive gene expression analysis distinguishing between different splicing variants, IGF binding proteins, growth hormones, insulin and other muscle regulatory genes. Nevertheless, the present fasting and refeeding protocol has proven useful for investigating gene transcript levels during a catabolic-anabolic transition in halibut muscle.

Chapter 7

General discussion

The overall aim of this research project was to investigate flesh quality and muscle growth in the Atlantic halibut, *Hippoglossus hippoglossus*. A few small scale experiments have been conducted in the past to investigate muscle growth and quality, but never in large scale commercial farming. We have successfully established several quantitative methods to investigate muscle fibre growth dynamics, gene expression (*IGF*s, *ctsb* and *ctsd*), connective tissue content (collagen and hydroxylysyl pyridinoline), proteolytic activity (cathepsins and collagenase) and general quality parameters. This research has generated a number of novel results that lead to one submitted and four published full research papers. The overall goal and sub-goals for this thesis have therefore been successfully fulfilled.

7.1 Muscle growth

The environmental impact on muscle growth in teleosts is considerable, especially that of temperature and light (Johnston, 2001; Johnston et al., 2003a, 2003c; Johnston, 2006; Katersky and Carter, 2007; López-Albors et al., 2008). The optimal temperature for growth is found to decline 1 to 2 ºC for every 50-fold increase in size for channel catfish (*Ictalurus punctatus*) (Cuenco et al., 1985), which seems similar to that in Atlantic halibut. The optimal temperature for growth halibut has been estimated as 14, 11.4, and 9.7 ºC for fish in the size range of 10-60 g, 100-500 g and 3-5 kg, respectively, but the optimal temperature for efficient growth was 5.5 ºC during the on-growing stage (3-5 kg) (Björnsson and Tryggvadótir, 1996). Continuous light treatment has proven to be very successful in improving the growth of several fish species, such as farmed Atlantic salmon (*Salmo salar*) (Krakenes et al., 1991; Johnston et al., 2003c), red sea bream (*Pargus major*) (Biswas et al., 2006), barramundi (*Lates calcarifer*), largemouth bass (*Micropterus salmoides*) (Petit et al., 2003) and turbot (*Scophthalmus maximus*) (Imsland et al., 1995), but the usefulness of light manipulation in

halibut farming has been moderate (Aune et al., 1997; Norberg et al., 2001; Imsland et al., 2005; Haugen, 2006). Nevertheless, a recent light manipulation experiment of Atlantic halibut has proven promising. By exposing the fish to simulated natural light while kept in tanks, continuous light from February to November 2005 after being transferred to sea cages, and then simulated natural light until the termination of the experiment (Oct. 2007, ~5.2 kg), the fish had outgrown the control group (kept at simulated natural light) by 23% or ~1.2 kg (Imsland et al., 2008). The presence of sexually mature fish was also reduced in the light treated group, which is an additional bonus in respect of quality (see section 7.2).

In common with other fish species living in sub-arctic regions, the Atlantic halibut makes the most out of the marked seasonal changes in temperature and light by boosting somatic growth between May and October. In fact, this is the only period of the year in northern Norway (May - November) in which the relative slow growing Atlantic halibut recruits muscle fibres and increases its biomass (Hagen et al., 2006). Similar to the Argentine hake (Calvo, 1989), the Atlantic halibut show sexual dimorphism in muscle fibre growth characteristics (Hagen et al., 2006; Hagen et al., 2008a). Females have a higher hyperplasia rate (Hagen et al., 2006), which lasts until 177.5 cm (81 cm in males), allowing females to reach a higher final fast fibre number (1.73×10^6 in females vs. 8.96×10^5 in males) and a larger ultimate size compared to males (Hagen et al., 2006; Hagen et al., 2008a).

In Hagen et al. (2008a) fish up to ~100 kg were analysed, but due to the rarity of female fish >45 kg relatively few were studied and it is likely that the Gompertz curve fitted may have overestimated the body size at which fast fibre recruitment ceased (Hagen et al., 2008a). Nevertheless, the largest female individuals did not have fast fibres <40-50 μm which suggests that muscle fibre recruitment had not taken place recently. In the case of Atlantic halibut it is very likely that the hyperplastic growth phase would extend into adulthood, as reported in other species achieving a relative large ultimate size (Greer-Walker, 1970;

Weatherley et al., 1979; Stickland 1983; Zimmermann and Lowery, 1999; Johnston et al., 2003a).

Haugen et al. (2006) suggested that halibut recruits muscle fibres during the winter season as a growth spurt, whereas temperatures and light increases in the spring/summer. This is in sharp contrast to our results (Hagen et al., 2006) and it could be linked to the difference in geographical location of the farm site (north vs. south of Norway), which represents a large difference in experimental setup (particularly photoperiod) or it could be a method related issue. During the winter, commercially farmed Atlantic halibut show a growth stagnation resulting in a temporary halt in muscle fibre recruitment, due to low light conditions, a drop in water temperatures (<6 ºC) and maturation of males (Hagen et al., 2006). A halt in muscle fibre recruitment during the winter is in agreement with the results reported in Atlantic salmon (*Salmo salar*) (Johnston et al., 2003c).

Growth zones are not frequently observed in juveniles or adult fish, but are essential during the larval stage and are referred to as germinal zones (Rowlerson and Veggetti, 2001; Rescan, 2005; Johnston, 2006). Based on the differences in muscle cell cross-sectional area (mm^2) between sampling locations, it was speculated that growth zones existed in Atlantic halibut (Haugen, 2006). Within the size range of fish (~2g - 100kg) we have investigated (Hagen et al., 2006; Hagen et al., 2008a), growth zones could not be documented for this species, but newly recruited fibres were observed scattered throughout the dorsal and ventral muscle compartment (especially in female halibut), which is typical of mosaic hyperplasia (Rowlerson and Veggetti, 2001).

The maximum fast muscle fibre number (FN_{max}) from a group of Atlantic salmon (*Salmo salar*, originated from 360 families) exposed to continuous light treatment was estimated to be

~8.8 x 105 fibres (Johnston et al., 2003c). In comparison to Atlantic halibut female, which achieve a final size at least ten times that of salmon, FN_{max} is only 2-fold greater (1.73 x 10^6). The main reason for this apparently inconsistent correlation between final size and FN_{max} in these two species is that salmon is not capable of growing fast fibres larger than 240 μm diameter (FD_{max}) (Johnston et al., 2000a), while halibut can grow fibres of at least 360 μm (Hagen et al., 2008a). However, FD_{max} of Atlantic halibut is not established because the present results indicated that FD_{max} was not reached in the largest individuals investigated (~100 kg, Hagen at al., 2008a). For example, in a 96 kg halibut FD_{max} was estimated to be ~330 μm based on the 97th percentile, but only 4% of the fast fibres had exceeded 300 μm illustrating the large hypertropic growth potential of this species.

During the last two decades an increasing number of genes, particularly transcription and growth factors, have been associated with muscle growth in fish, but little is known from Atlantic halibut. One group of transcription factors which has received a lot of attention are the myogenic regulatory factors (MRF), consisting of *MyoD*, *Myf5*, *Myogenin* and *Myf6* which are responsible for committing mesodermal cells to the muscle lineage as well as initiating and maintaining the muscle differentiation programme (reviewed by Rescan, 2001). These transcription factors share two conserved regions, a basic motif which mediates DNA binding and a helix-loop-helix domain important for the dimerization with the ubiquitously expressed E proteins (Lassar et al., 1991). Galloway et al. (2006) investigated the expression profile of *MyoD2* and *myogenin* in developing Atlantic halibut embryos reared at different temperatures using in situ hybridisation and reverse transcription - PCR. The temporal and spatial expression of *MyoD2* and *myogenin* was not influenced by temperatures, in agreement with others (Hall et al., 2003; Cole et al., 2004), but the *in situ* hybridisation showed an interesting asymmetric *MyoD2* expression pattern in the fast muscle precursor cells, until 20-

30 somites were formed. However, this is in contrast to the temperature dependent MRF expression reported from *Takifugu rubripes*, where *myogenin* expression was 2-fold higher at 21 ºC compared to 18 or 15 ºC (Fernandes et al., 2006). The expression had a rostral-caudal progression and became more symmetric after the 30 somite stage, but faded during development and was only being expressed in the caudal half in newly hatched larvae, if expressed at all (Galloway et al., 2006). The functional significance for the bilateral *MyoD2* expression is unknown, but one hypothesis is that this MyoD2 has adopted a new function related to the development of external asymmetry in halibut (Galloway et al., 2006). Another possibility is that this asymmetry in expression is related to the development of a greater muscle mass of the dorsal side of halibut (Galloway et al., 2006), as presented in this thesis (Hagen et al., 2008a), even though asymmetry is not present until metamorphosis (Galloway et al., 1999b, 2006).

Myostatin is a negative regulator of growth (McPherron et al., 1997) and knockdown experiments of myostatin in zebrafish (*Danio rerio*) have proven to up-regulate transcription of *IGF-II*, *muscle-specific creatine kinase*, *MyoD* and *myogenin* (Amali et al., 2004). In chapter 6 we have shown that IGF-I is a likely candidate involved in muscle growth regulation, which is in agreement with others (Chauvigné et al., 2003; Montserrat et al., 2007a,b; Terova et al., 2007). However, the role of IGF-II in halibut is less clear and additional experiments are required to investigate the effect of this growth factor on growth. This thesis (chapter 6) focused only on male halibut, which are known to have a poorer growth rate than females. The underlying molecular mechanisms behind the sexual dimorphism in growth are unknown, but the IGF-system could potentially be differentially expressed between males and females of this species.

7.2 Flesh quality

The quality and sensory parameters of fish flesh are affected by a wide range of quality traits, namely texture, chemical composition, water holding capacity and colour.

In Hagen et al. (2006) we reported a large and critical drop in protein content (increase in water content) for males during the spring as a result of poor appetite and sexual maturation. Females also showed a significant seasonal drop in protein content during the same period, but much less pronounced than in males, for which maturation was superimposed on the seasonal effect. The biomass of males was reduced by ~18% and the quality of the fish was significantly reduced during this period. A similar decrease in protein content has recently been reported from farmed cod (*Gadus morhua*) during maturation, where the gonadal somatic index (GSI) increased up to 33 and 45% in males and females, respectively (Solberg and Willumsen, 2008). This is also the case for wild cod where both mature and immature fish show a significant increase in muscle water content during the winter (Love, 1988), suggesting the involvement of proteolytic enzyme systems.

In a recent halibut study, the texture, colour and slaughter yield of mature (two years older than the immature fish) and immature males of similar size were compared. The results showed that the quality of immature fish was superior to that of mature fish, having a 5.8% higher fillet yield (Roth et al., 2007a). The shear force and fracturability increased with maturation in agreement with our results (Hagen et al., 2007), and the breaking force was found to be significantly different between the two groups. The increased texture with maturation was partially explained by difference in growth rate and muscle fibre structure, as suggested by Haugen et al. (2006), but the importance of connective tissue and collagen cross-links was not taken into consideration (see Lee et al., 2005; Johnston et al. 2006a;

Hagen et al., 2007). In Hagen et al. (2007) we showed the importance of collagen and especially its mature hydroxylysyl pyridinoline cross-links (PYD) for the texture of halibut muscle, perhaps being the most significant results presented in this thesis. Muscle fibre density only showed a weak significant correlation to instumental texture measurements in agreement with previous reports (Johnston et al., 2000d; Periago et al. 2005). Since the PYD compartment of muscle increases with age (Montero and Borderías, 1990; Bailey et al., 1998; Bailey 2001), it could be the reason why the muscle of older mature fish had a significantly higher breaking strength compared to immature fish (see Roth et al., 2007a). Further, the increase of the PYD compartments during the winter enriched the alkaline insoluble collagen compartment leading to 2-fold increase in fillet firmness (Hagen et al., 2007), possibly explain the increase in texture observed during maturation by Roth et al. (2007a). Seasonal changes in texture are not unique for halibut and similar results have been reported for other aquaculture species such as Atlantic salmon (*Salmo salar*) (Espe et al., 2004; Roth et al., 2005) and red seabream (*Chrysophrys major*) (Touhata et al., 1998). In salmon, texture showed a significant variation between anterior and posterior sampling sites and ice storage conditions, having the firmest texture in the winter (Espe et al., 2004). In addition, the texture measurements from anterior and posterior sampling sites differed with season. While the anterior part was firmest in February, the posterior part had a firmer texture in June, underlining the complexity of evaluating texture in fish muscle. The texture of red seabream showed parallel changes with GSI, being strongly affected (increasing) by sexual maturation in males (Touhata et al., 1998). In contrast to halibut, both male and female ayu (*Plecoglossus altivelis*) got a thinner and weaker connective tissue during the spawning period (Ito et al., 1992), which could be explained by the increased serum levels of collagen (hydroxyprolin) during spawning indicating enzymatic breakdown (Toyohara et al., 1997).

Fish collagens are less thermostable than their mammalian counterparts and start to denature into gelatine at 30 ºC (Yunoki et al., 2003), and do not contribute to texture in cooked fish. In contrast, muscle fibre structure is more likely to impact texture of cooked fish (Hurling et al., 1996). Nevertheless, even if connective tissue is of little importance for the texture of cooked fish, raw fish texture is a very important quality parameter when the fish is evaluated by the consumer (Botta, 1991).

During periods of food depletion, starvation and/or maturation the fish supports its basal metabolism by degrading structural proteins, carbohydrates and lipids with the activation a variety of distinct enzymes within the fast muscle and the liver (Moon and Johnston, 1980; Beardall and Johnston, 1985a; Yamashita et al., 1990; Toyohara et al., 1991; Gómez-Guillen and Batista, 1997; Kubota et al., 2000; Sovik and Rustad, 2005). In halibut we have found that cathepsins (particularly cathepsin B + L, D and H) are good candidates contributing in to the observed fast muscle protein degradation, affected by both season (females and males) and spawning (males) (Hagen et al., 2008b). Further, we have also documented that the action of cathepsin H significantly altered liquid loss in halibut muscle. The effect of cathepsin H on liquid loss is most likely to be a secondary effect of protein degradation since proteins are the main site of water binding in muscle due to the electrostatic attraction between charged protein residues and water molecules (Wismer-Pedersen, 1987). The effect of enzyme activity on water holding capacity (liquid loss) has not been investigated in fish before, but studies from mammals have suggested that enzyme activity might be of importance (Bee et al., 2007; Bond and Warner, 2007), since the degradation/denaturation of proteins will affect their structure and influence water binding (van Laack and Solomon, 1994).

Cathepsin L has been identified as the main enzyme contributing to the observed muscle softening in Chum salmon (*Oncorhynchus keta*) (Yamashita and Konogaya 1990a, 1991) and mackerel (*Scomber japonicus*) (Aoki and Ueno, 1997). In Hagen et al. (2008b) cathepsins B and L were assayed together using the same substrate (Z-Phe-Arg-MCA) and it was therefore not possible to determine the individual effect of cathepsin L. However, cathepsin B was assayed individually, but did not correlate to protein content as well as the cathepsins B + L assay did, suggesting that cathepsin L might be more important to protein breakdown than what the cathepsin B + L assay indicated. Nevertheless, it is important to keep in mind that enzymes with similar substrate specificity are most likely to work synergistically, leading to protein degradation and reduction of flesh quality (Ladrat et al., 2004; Chéret et al., 2007).

To the best of our knowledge, cathepsin mRNA transcripts in fish muscle have not been previously investigated. We have shown that the transcript levels of *ctsb* and *ctsd* remained relatively high during fasting and were significantly down-regulated with refeeding (chapter 6). The reason why transcript levels did not increase significantly with fasting (64 d) could be due to the already high transcript levels present pre-experiment, caused by the natural fasting period in the winter due to suboptimal temperatures. In Hagen et al. (2008b) we showed that cathepsin activity peaked in May, the same time as the fasting refeeding experiment was initiated. However, a correlation between transcript level and the biological active protein should not be taken for granted. In mammals, variation in cathepsin mRNA level did not systematically correlate with similar modifications in lysosomal cathepsin enzyme activity (Tournu et al., 1998; Combaret et al., 2003). In support of our results, the expression of *calpain, calpastatin, proteasome* and *ctsd* was up-regulated after 8 days of fasting in rabbit, being most pronounced for *ctsd*, indicating that this gene might have a central role in muscle degradation (Ilian and Forsberg, 1994).

Based on the results reported in this thesis we have good evidence for suggesting that farmed Atlantic halibut reared under similar conditions should be harvested in the fall or early winter when the nutritional value (Hagen et al., 2006), texture (Hagen et al., 2007) and liquid loss (Hagen et al. 2008b) are favourable, increasing consumer acceptability and reducing any downgrading losses during secondary processing (e.g. drip loss). However, to establish if a low liquid loss, firm texture and a good nutritional value of halibut are synonymous of optimal eating quality, sensory tests need to be conducted.

7.3 Future perspectives

If possible, commercial halibut farming should be avoided in coastal areas where water temperature drops below 6 ºC for prolonged periods during the winter. However, this is most likely not achievable since northern regions (Hagen et al., 2006), as well as southern regions of Norway (Haugen et al., 2006) do experience temperatures below this threshold value during the winter season. Nevertheless, the seasonal changes in temperature should be investigated before farming is initiated.

As shown in this thesis, halibut, and especially males suffer from poor growth due to sub optimal temperatures and maturation which causes a drop in appetite, a halt in female biomass gain and reduction of male biomass. One way of overcoming the most serious growth stagnation in halibut farming would be to only produce female halibut fish. However, to date this has not been successfully accomplished since the tools required for this task are not available. The use of hormones has proven useful and as much as 74% females has been recorded (Hendry et al., 2003). However, from an ethical point of view, such strategies have low consumer acceptance in Europe and should therefore be avoided.

An ongoing "all female halibut production" project which is a collaboration between three Norwegian institutes and the University of Stirling is trying to increase the knowledge of genetic and endocrine sex control mechanisms in Atlantic halibut and try to develop an efficient protocol for the production of all-female halibut without the use of hormones. If this project succeeds it will not only improve the growth of farmed halibut, but farmers will not suffer from quality problems related to early maturation of males (Hagen et al., 2006; Roth et al., 2007a).

In the light of our findings (Hagen et al., 2007) future textural studies of raw fish flesh should include or at least discuss the importance of the PYD cross-links and their impact on texture. Further, increased research focus should be directed towards the possibility of stimulating and potentially altering the collagen matrix and its cross-link content as a means of improving flesh texture in aquaculture species. Experiments could be performed with different feeding regimes, feed composition and feed additives (e.g. collagen boosting) to investigate this possibility. Preliminary results have shown that the texture of Atlantic salmon (*Salmo salar*) can be improved by adding collagen hydrolysate to the feed mixture. The observed increase in firmness could be due to increased uptake and deposition of hydroxyproline and proline (building blocks in collagen) in the collagen compartments (personal communication, Dr. Sissel Albrektsen, NOFIMA). To the best of my knowledge, Atlantic halibut is not subjected to textural problems such as soft texture or gaping, but the effect of "collagen boosting" would be interest for species suffering from these problems, particularly Atlantic salmon and cod (*Gadus morhua*) (Ofstad et al., 1996b; Espe et al., 2004).

Recent advances within the field of molecular biology have generated a significant number of molecular tools that can prove useful for marker-assisted selection in fish breeding

programmes. This is especially the case for production traits that are difficult to measure, such as flesh quality. The enzyme lysyl oxidase is the only known enzyme being involved in the collagen cross-link formation, and the *lox* gene has the potential of being used as a molecular marker for texture traits in Atlantic halibut. One way of doing this would be to determine the genetic polymorphisms of the *lox* gene and investigate potential correlations between *lox* genetic variation, collagen crosslink content and fillet firmness of this species (see work of Consuegra and Johnston, 2008).

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