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RESPONSES OF MYOGENESIS AND METABOLISM TO TEMPERATURE AND GROWTH FACTORS IN FISH

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Abstract

Fish are becoming more important in the human diet as they represent a high protein source and a source of essential free fatty acids. Fish production from aquaculture has been improved by the selection of faster growing individuals, but fish continue to remain an expensive nutrient source. A better understanding of fish growth mechanisms and regulation will help to further improve aquaculture techniques. Moreover, studying those factors that stimulate cells implicated in fish muscle growth could provide useful knowledge and possible applications in mammalian muscle illness.

In contrast to mammals and birds, fish growth is generally indeterminate and most fish grow throughout their life by the formation of new muscle cells (hyperplasia) and/or by increasing muscle cell volume (hypertrophy). Proliferation and differentiation of muscle cells are regulated by transcription factors called myogenic regulatory factors (MRF). MyoD and myogenin are MRF involved in cell proliferation and differentiation in vertebrates.

Many factors affect fish growth including external factors such as temperature and food availability, and internal factors, principally hormones. The effects of temperature on fish growth and metabolism were assessed in Atlantic cod (*Gadus morhua*) and rainbow trout (*Oncorhynchus mykiss*), and the effects of hormones were investigated using Atlantic salmon (*Salmo salar*) made transgenic for growth hormone (GH) and rainbow trout fed β-agonists. In order to understand the mechanisms and regulation of myogenesis, morphometric parameters were recorded, metabolic enzymes were assayed in liver and skeletal muscle and myogenesis was measured in white and red muscles in each

experiment. Moreover, several growth factors and hormones were tested *in vitro* on myosatellite cell proliferation and differentiation.

The results demonstrate that metabolism and myogenesis are not affected by the same factors, and an increase in growth is not absolutely accompanied by an effect on tissue metabolism. High and constant water temperatures increased juvenile cod growth though there were no significant alterations in liver and white muscle metabolism. In contrast, factors such as photoperiod and internal cycles appear to be more important in the control of cod tissue metabolism. However, myogenesis was significantly decreased and metabolism increased in rainbow trout exposed to cold compared with high temperatures. Growth hormone, insulin-like-growth factor I (IGF-I) and fibroblast growth factor (FGF) were found to increase *in vitro* myosatellite cell proliferation in salmonids. My results also show that proliferation and differentiation of salmon muscle does not happen simultaneously but may alternate in red and white muscles.

These results taken together contribute to a better understanding of fish muscle growth and metabolism and will be used in future studies to optimize fish growth in aquaculture.

Résumé

Les poissons représentent une part importante des habitudes alimentaires humaines, grâce à leur haute teneur en protéines et en acides gras libres essentiels. La production aquacole à été fortement améliorée grâce à la sélection d'individus plus performants et de grande taille. Cependant le poisson reste un aliment cher. Une meilleure compréhension des mécanismes de croissance et de la régulation de la croissance aidera à améliorer les pratiques aquacoles. De plus, l'étude des facteurs qui stimulent les cellules impliquées dans la croissance musculaire des poissons procurera des informations essentielles dans le traitement des maladies musculaires.

Au contraire des mammifères et des oiseaux, la croissance halieutique est généralement indéterminée et la plupart des poissons grandissent toute leur vie soit par la formation de nouvelles fibres (hyperplasie), soit par l'augmentation de volume des cellules musculaires déjà existantes (hypertrophie). La prolifération et la différentiation des cellules musculaires sont régulées par des facteurs de transcription appelés facteurs de régulation myogénique (MRF). La MyoD et la myogénine sont des MRF impliqués dans la prolifération et la différentiation des cellules musculaires des vertébrés, incluant les poissons.

Beaucoup de facteurs influencent la croissance musculaire. Parmi eux, des facteurs externes tels que la température et la nourriture disponible, ainsi que des facteurs internes, principalement des hormones. L'effet des températures extérieures sur la croissance des poissons et le métabolisme ont étés testés chez la morue (*Gadus morhua*) et chez la truite arc en ciel (*Oncorhynchus mykiss*), L'effet des hormones a été testé chez le saumon Atlantique (*Salmo salar*) rendu transgénique pour l'hormone de croissance (GH) et la truite

arc en ciel nourrie avec des agonistes β_2-adrénergiques. Dans le but de comprendre les mécanismes et la régulation de la myogenèse, les paramètres morphologiques ont été notés, l'activité des enzymes métaboliques mesurée et les facteurs myogéniques mesurés dans le muscle rouge et le muscle blanc, récoltés dans chaque expérience. De plus, l'effet de plusieurs facteurs de croissance et hormones ont été testés *in vitro* sur la prolifération et la différentiation des cellules précurseurs de fibres musculaires.

Ces résultats montrent que le métabolisme et la myogenèse ne sont pas affectés par les mêmes paramètres, et qu'une augmentation de la croissance n'entraîne pas nécessairement un effet au niveau du métabolisme. Une température élevée et constante est plus favorable pour augmenter la croissance des morues juvéniles, bien qu'il n'y ait pas d'altération significative au niveau du métabolisme musculaire. Au contraire, les facteurs tels que la photopériode et le cycle physiologique interne des morues semblent plus importants pour contrôler le métabolisme des poissons. Chez la truite arc-en–ciel, une exposition à des températures froides induit une baisse de l'expression des facteurs myogéniques et une augmentation du métabolisme. L'hormone de croissance (GH), l'hormone de croissance insulinique (IGF-I) et le facteur de croissance fibroblastique (FGF) sont les meilleurs facteurs pour augmenter *in vitro* la prolifération des cellules myosatellites chez les salmonidés. Mes résultats ont aussi montré que la prolifération et la différentiation du muscle de saumon n'arrivent pas au même moment mais semblent alterner entre le muscle rouge et le muscle blanc. L'ensemble de ces résultats contribue à une meilleure compréhension de la croissance musculaire et du métabolisme chez les poissons et sera utilisé dans les recherches futures dans le but d'optimiser la croissance des poissons en conditions d'aquaculture.

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Abreviations

ABT	Ambient
ALT	Alanine amino transferase
AST	Aspartate amino transferase
BSA	Bovine serume albumin
CCO	Cytochrome C oxidase
CF	Condition factor
CLEN	Clenbuterol
CPT	carnitine palmitoyl transferases
CS	Citrate synthase
CST	Constant
FBS	Fetal bovin serum
FGFb	Basic flibroblast growth factor
G6PDH	Glucose-6-phosphate dehydrogenase
GDH	Glutamate dehydrogenase
GH	Growth hormone
GSI	Gonadal somatic index
HGF	Hepatocyte growth factor
HLH	Helix loop helix
HOAD	3-hydroxyacyl CoA dehydrogenase
HSI	Hepatosomatic index
HT	High temperature
IDH	Isocitrate dehydrogenase

IGF-I	Insulin-like-growth factor I
LDH	Lactate dehydrogenase
LT	Low temperature
MDH	Malate dehydrogenase
ME	Malic enzyme
MRF	Myogenic regulatory factor
MyC	Myosatellite cell
PCA	Principal Component Analysis
PEPCK	Phosphoenol pyruvate carboxykinase
PK	Pyruvate kinase
RACT	Ractopamine
RM	Red muscle
SGR	Specific growth rate
TAG	Tryglyceride
WM	White muscle
β_2-AA	β_2 adrenergic agonist
β_2-AR	β_2 adrenergic receptor

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CHAPTER I: GENERAL INTRODUCTION

I Rationale

This thesis is subdivided in two main objectives. The first is an analysis of factors that are implicated in fish muscle growth (myogenesis) and metabolism. The second is to provide information on fish muscle growth processes and mechanisms to better understand them with the aim to assist the aquaculture industry.

Fish are becoming more important in the human diet as they represent a high protein source. Fish is generally associated with a healthier diet than meat as fish muscle contains high omega-3 fatty acid levels and the fish filet contains less fat than red meat (Fauconneau *et al.*, 1995; Larsen *et al.*, 2004). Unfortunately many fishing techniques, such as trawling, are very damaging to the environment and the ocean fauna. Moreover, over fishing or bad fishing practices are directly or indirectly responsible for a decrease in marine biodiversity (Aerni, 2004). Fish aquaculture may be a reasonable solution to the increased demand for food by our growing population and to protect marine diversity. The solution would be to improve fish aquaculture production and thus reduce costs for the consumer. Concurrent with improvements in production, a reduction in environmental pollution caused by aquaculture (among them are the discharges of polluted water from ponds, the destruction of wetlands and mangroves and fish escape to the ocean) is necessary. Unfortunately, even if very important, these latter issues will not be discussed in my thesis as I focused my work on fish muscle growth. Fish aquaculture has already been improved by the selection of faster growing individuals, but fish remain an expensive nutrient source. The aquaculture industry is interested in lowering costs by using less expensive diets (increased carbohydrate content, while keeping essential amino acids and free fatty acids needed for

growth; Bergot, 1979; Shiau and Peng, 1993), decreasing animal source of protein in the diet (economy and animal protection issues; Von Der Decken, 1993), faster growing fish (reduced time to market; Guillen *et al.*, 1999), and fish that grow throughout the year, even at cold winter temperatures. In this thesis I will analyse two factors known to increase muscle growth capacity in fish: temperature and hormones.

Another very important objective that motivated me to do this research is the fact that the scientific community knows very little about the way fish produce muscle. The study of fish myogenesis is a new topic which opens many opportunities for research. The very interesting point is that growth is a continuous process during the life-time of most fish species. Of course this idea of continuous growth is specific to fish, but it could be useful knowledge when considering muscle illnesses in humans, such as muscle atrophies and dystrophies. The cells responsible for muscle growth in vertebrates including fish, called myosatellite cells, were first described by Mauro in 1961. During myogenesis, myosatellite cells increase the size of existing muscle fibers by combining with existing muscle fibers or creating an entirely new muscle fiber (Asakura *et al.*, 2002). Myogenic satellite cells or myosatellite cells can also repair and regenerate adult skeletal muscle (Allen and Rankin, 1990). Myosatellite cells (MyC) are muscle stem cells that reside beneath the basal lamina of adult skeletal muscle juxtaposed against skeletal muscle fibers and are normally mitotically quiescent, but are activated to mediate post-natal growth and regeneration of muscle (Seale and Rudnicki, 2000). The factors responsible for MyC activation remain under-studied.

During my PhD research I used different fish species, cod, rainbow trout and Atlantic salmon, all characterized by their importance in aquaculture. I conducted experiments using either fish tissues, mainly red and white muscles, and fish muscle MyC.

I looked specifically at the formation of muscle fibers and the energy involved during fish muscle growth by looking at metabolic enzymes.

II General hypothesis

External factors (such as diet, water temperature) and internal factors (hormones) impact fish intermediary metabolism and muscle myosatellite cell numbers, recruitment, proliferation and differentiation and ultimately skeletal muscle growth.

III Objective

Demonstrate the effects of temperature on metabolism and myogenesis in cod and rainbow trout, the effects of β_2-adrenergic-agonist feeding on rainbow trout and the impact of the GH transgene on Atlantic salmon myogenesis and metabolism.

IV State of the art

IV.1 Vertebrate skeletal muscle and muscle contraction

Three muscle types exist in vertebrates: smooth muscle (intestines, blood vessel walls, etc.), cardiac muscle and skeletal muscle. Skeletal muscles connect to bones and are used to generate movement. Skeletal muscle is a structurally unique tissue consisting predominantly of highly specialized plurinucleated myofibers (Zammit and Beauchamp, 2001). This extreme specialization is achieved through terminal differentiation. The primary function of adult skeletal muscle is to generate force, which is achieved by the

synchronous contraction of bundles of myofibers. Individual myofibers are highly specialized cells consisting predominantly of an organized contractile protein apparatus (myosin and actin) that is regulated by calcium cycling from the sarcoplasmic reticulum to the cytoplasm. In vertebrates and also in fish, each muscle fiber consists of multiple myofibrils. In each myofibril, actin thin filaments and myosin thick filaments are organized into linear chains of highly ordered structures called sarcomeres. When a nerve impulse reaches a skeletal muscle fiber, it causes a change in electric potential across the plasma membrane. Skeletal muscle fibers can rapidly convert this electrical signal (called depolarization) into a rise in cytosolic Ca^{2+} from the sarcoplasmic reticulum, which then initiates contraction (Lieber and Trobe, 2002). Myosin is a motor protein that interacts with actin filaments and couples the hydrolysis of ATP. In skeletal muscle, contraction is regulated by four proteins associated with the actin filament: tropomyosin and troponins C, I and T. The cytolosic Ca^{2+} concentration influences the position of these proteins on the thin filaments, which in turn controls myosin-actin interaction (Lieber and Trobe, 2002). During contraction, each sarcomere shortens by as much as 30 percent as the thin actin filaments slide past the thick filaments. ATP-dependent interactions between myosin heads and actin and the subsequent conformational change in the heads generate the sliding force that pulls the thin filaments towards the central zone of each sarcomere. As long as the Ca^{2+} that initiates muscle contraction is sufficiently high and ATP is present in muscle, the myosin cross bridges will cycle continuously.

Since the cost of such a high degree of specialization is the loss of the ability to proliferate, the capacity to generate new myonuclei for myofiber repair, growth or replacement in the adult is dependent upon the presence of a reserve population of undifferentiated cells (Zammit and Beauchamp, 2001; Hall *et al.*, 2003; Collins *et al.*,

2005). Skeletal muscle cells can be replenished from a pool of undifferentiated cells called skeletal muscle stem cells or myosatellite cells (MyC). These cells are fundamental to post-natal muscle homeostasis in response to turnover, growth demands or damage. It was shown that in mammals, fast myofibers contain approximately 300 myonuclei and have 5 to 12 MyC per fiber. In contrast there are approximatively 450 myonuclei in slow muscle fibers, with an associated population of 30 MyC (Zammit and Beauchamp, 2001)

IV.2 Fish skeletal muscle composition and comparison with mammals

Fish skeletal muscle represents more than 50% of the total body mass of the animal (Mommsen and Moon, 2001). In mammals there are 2 types of skeletal muscle. Type I which correspond to red muscle in fish and type II which is similar to white muscle in fish. Type II can be subdivided in type IIa (intermediary fibers) and type IIb (fast glycolytic fibers) (Imai *et al.*, 1999). In mammals, skeletal muscles consist of a mixture of the different fiber types. However in fish, fiber types are found in distinct compartments. The main part of the fish myotome is composed of white anaerobic fibers arranged in a V-shape pattern and superficial red aerobic fibers (Koumans and Akster, 1995). However, the muscle contraction apparatus is similar between fish and other vertebrates. A zone of intermediate (pink) fibers separates the red fibers from the white fibers, but will not be described as they have a low abundance and their importance in fish growth from an aquaculture perspective is unlikely to be significant. Another peculiarity of fish muscle is the possibility to form new muscle fibers for growth processes after birth, or hyperplasic growth (see below).

The white muscle comprises more than 90% of the myotome in most fish species and is reserved for high speed cruising and fast-starts associated with predation and escape

behaviors (Johnston, 2001). It consists of fast fibers (Altringham and Johnston, 1981) that depend on anaerobic metabolic pathways for energy generation (Johnston, 2001). Red muscle fibers are situated along the lateral line of the fish superficial to the white fibers with an insertion in the region of the horizontal septum. Red muscle fibers are responsible for slow, sustained swimming (Johnston *et al.*, 1977). The red muscle is composed of slow fibers containing a high volume of mitochondria (20 to 50%) and is well supplied with capillaries (Johnston, 1982). Mitochondria of red muscle are able to utilize both carbohydrate and lipids as fuel, as well as certain amino acids (Jones and Sidell, 1982; Moyes *et al.*, 1989).

IV.3 Muscle formation in embryonic and post-embryonic fish

The embryonic formation of muscle results from the proliferation of mononucleated mesodermal cells which differentiate into mononucleated myoblasts (myoblasts are MyC in a proliferation state). These myoblasts fuse together to form multinucleated myotubes and begin to express muscle-specific structural and contractile proteins (see Fig. I-1). In fish species, the origins of different muscle fiber types are spatially and temporally segregated (Koumans and Akster, 1995). Skeletal muscle of the trunk and tail of fish and other vertebrates derives from a specific embryological compartment, the myotome. Muscle precursors are called adaxial cells and are first located on both sides of the forming notochord (Currie and Ingham, 2001). Although muscle differentiation has been studied in only a few species of fish and interspecific variation exists, a general pattern can be described which consists of two main phases of muscle development. The first occurs in the embryo and in the yolk sac larva stage (the hatched larva that still depends on the yolk sac for nourishment). During this first phase, the fast muscle fibers (white) differentiate first,

and with them horizontal and vertical myosepta. Then, adaxial cells migrate radially from adjacent to the notochord to the lateral surface of the myotome where they differentiate into slow muscle fibers (red). During this phase muscle growth occurs by both hyperplasia (formation of new muscle fibers) and hypertrophy (increased size of existing fibers). The maturity of the red and white muscle zones at hatching varies. In zebrafish (Van Raamsdonk *et al.*, 1982), in several other Cyprinids (Talesara and Urfi, 1987; El-Fiky and Wieser, 1988), herring (*Clupea harengus*, Batty, 1984), guppies (*Poecilia reticulate*, Veggetti *et al.*, 1993) and sea bream (*Sparus auratus*, Mascarello et al., 1993), both zones are well developed at hatching. In rainbow trout (*Oncorhynchus mykiss*, Nag and Nursall, 1972), brown trout (*Salmo trutta*, Proctor *et al.*, 1980) and red sea bream (*Pagrus major*, Matsuoka and Iwai, 2005), the inner white muscle is well developed at hatching, but the superficial red layer still consists of undifferentiated myogenic cells. In the Mediteranean sea bass (*Dicentrarchus labrax*, Veggetti *et al.*, 1990), muscle fiber formation is still incomplete in the white fibers, and red fibers have only differentiated at the level of the horizontal septum. Subsequent to the formation of inner white and superficial red zones of muscle, the second stage of muscle development occurs in the free swimming larvae (yolk is depleted or almost depleted) when the adult red and pink fiber types form (Rescan *et al.*, 2001). Generally, in fish which grow to a large final size (aquaculture species such as trout, salmon, sea bream, carp, etc.), this phase is followed by a third round of hyperplasic growth resulting in a large increase in the total number of fibers, especially in the fast-white muscle layer, which acquires typical mosaic appearance (Weatherley *et al.*, 1980; Valente *et al.*, 1999). The relative timing of the main hyperplasic growth processes in relation to the life cycle varies between species. Finally, myoblasts on the surface of muscle fibers are activated in a process that continues throughout adult life (Johnston *et al.*, 1995; Koumans

and Akster, 1995; Rowlerson *et al.*, 1997) by hypertrophy until they reach a functional maximum diameter that is in the range 100-300 μm for fast white fibers depending on the fish species, but smaller for slow red fibers which are much more dependent upon the oxygen supply from adjacent capillaries (Egginton and Johnston, 1982; Johnston, 1982; Sänger and Stoiber, 2001). The ratio between the contributions of hypertrophic and hyperplasic growth decreases in fish muscle with age and size (Weatherley *et al.*, 1980; Stickland, 1983), as hypertrophic growth persists long after hyperplasic growth has ceased (Weatherley *et al.*, 1988; Koumans *et al.*, 1993; Rowlerson *et al.*, 1997).

Growth of fish muscle differs from mammals and birds after the larval stage. In mammals and birds the increase in the number of muscle fiber (hyperplasia) stops at birth or shortly thereafter (Campion, 1984) and further muscle growth is caused by outgrowth of existing fibers (hypertrophy). The hyperplasia that can occur in these animals after heavy exercise training is thought to be a form of regeneration (McCormick and Schultz, 1992).

IV.4 Fish growth

In contrast to mammals, fish growth is indeterminate which means that most fish grow throughout their life by the formation of new muscle cells (hyperplasia) or the increase of muscle cell volume (hypertrophy) (Johnston, 2001). In mammals, muscle fiber number is thought to be fixed at birth (Rowe and Goldspink, 1969), and increase in muscle size, is usually achieved only by hypertrophy. However, muscle in fish can still grow by addition of new fibers after hatching (Koumans and Akster, 1995). This peculiarity makes fish a unique and very good model to study growth processes in vertebrates. Many definitions of growth exist, among them that growth is defined as an increase in cell

number and/or cell size (Mommsen, 1998) and occurs when the animal has a positive energetic balance (Mommsen and Moon, 2001).

Like other vertebrates, fish have embryonic, juvenile and adult phases in their life cycle. Though the period of growth starts as soon as the young fish begins to feed, the specific growth rates vary in different phases of the life cycle (Dutta, 1994). Although many fish continue to grow after attaining sexual maturity, the growth pattern changes. Subsequent average growth rates are generally lower than those of immature fishes because a portion of food absorbed is diverted for use in cell turnover and the formation of reproductive gametes. Many large teleost species such as sturgeon (*Acipenser* Sp.) and cod (*Gadus morhua*) grow rapidly until they reach sexual maturity. Thereafter the growth continues throughout life but the rate become slower with advancing age. Specific growth rate (G) can be defined as $G = a \cdot W^b$, where "a" is the growth rate of a fish weighing one gram and "b", the weight or mass exponent, a negative number between -0.35 and -0.45 (Mommsen and Moon, 2001). Even under optimal conditions, fish growth rate is not constant and fast growth intervals alternate with lower growth rates (Noel and Lebail, 1997). Other phases governed by lunar, reproductive, or circannual cycles can be superimposed upon this internal growth periodicity (Horseman and Meier, 1978; Farbridge and Leatherland, 1992).

IV.5 Myosatellite cells (MyC)

MyC are stem cells specific to muscle tissue. Stem cells are undifferentiated precursor cells found in many tissues including bone marrow, blood, and brain. Several classes of stem cells exist. These include multipotent stem cells that can generate, after differentiation, any specific cells of the body when associated with the appropriate

conditions (growth factors and signals provided by the host tissue) (Seale and Rudnicki, 2000). For example, hematopoietic stem cells are multipotent. In addition to their ability to produce all blood lineages, hematopoietic stem cells also exhibit developmental plasticity when introduced into different tissues (Asakura *et al.*, 2002) and are often used as a comparator to other stem cell lines. Pluripotent stem cells lead to several types of cells; and, monopotent stem cells give rise to only one type of cell after differentiation. Muscle MyC were believed to be monopotent until Katagiri *et al.* (1994), Teboul *et al.* (1995) and Wada *et al.*(2002) were able to differentiate first the muscle cell line C2C12 and second muscle stem cells isolated from mouse gastrocnemius muscle, into osteoblast and adipose-like cells. However it is not clear if muscle MyC can generate bone and adipose tissue *in vivo* (Holterman and Rudnicki, 2005). It is now believed that most of the adult stem cell population including muscle stem cells can differentiate in a context-specific manner, presumably in response to growth factors and signals provided by their host tissues (Seale and Rudnicki, 2000) and are therefore at least pluripotent if not multipotent (Wada *et al.*, 2002). Moreover, it was demonstrated that muscle also contains another population of adult stem cells, called side population (SP) cells, isolated from MyC by FACS (Fluorescence-Activated Cell Sorting) using a Hoechst dye exclusion process, that are able to reconstitute the entire hematopoietic repertoire after intravenous injection into lethally irradiated mice (Jackson *et al.*, 1999; Kawada and Ogawa, 2001a,b). However, no data exist on the presence of muscle SP cells in fish. So far, histopathological and molecular biological studies (Bischoff, 1986; Garry *et al.*, 1997; Seale and Rudnicki, 2000; Collins *et al.*, 2005) indicate that muscle MyC are largely responsible for postnatal muscle growth, regeneration and repair, even if SP cells or other stem-like muscle-derived cells also contribute to muscle regeneration (Wada *et al.*, 2002; Qu-Petersen *et al.*, 2002).

Mauro (1961) first described MyC in mammalian and avian muscles. These cells are quiescent and interposed between the myofibers and the external lamina (Campion, 1984; Anderson, 2000), separated from the fiber by the cell membrane. The contribution of these cells to muscle hyperplasic and hypertrophic growth is now well described in vertebrates (Stockdale, 1992; Shultz, 1996). However, the factors involved in their recruitment are not fully understood (Quinn *et al.*, 1997). MyC originate from a pool of precursor cells produced at the end of embryonic development (Stockdale, 1992) (see IV-3). MyC have been extracted from white muscle of salmon (*Salmo sala;* Matschak and Stickland, 1995), trout (*Oncorhynchus mykiss;* Fauconneau and Paboeuf, 2000), carp (*Cyprinus carpio;* Koumans and Akster, 1995), catfish (*Ictalurus punctatus*; Cyrino and Mulvaney, 1999), zebrafish (*Danio rerio;* Sepich *et al.*, 1994), and cod (*Gadus morhua*; H. Levesque, *unpublished data*). MyC were observed *in situ* using transverse sections of fish muscle (Koumans and Akster, 1995; Stoiber and Sänger, 1996). These cells are small (5 μm), spindle shaped and characterized by a heterochromatic nucleus and a reduced cytoplasm (Feldman and Stockdale, 1991). MyC are myoblasts which remain non-proliferative until activation (Feldman and Stockdale, 1991).

Mammalian and avian MyC constitute 30% of the muscle nuclei in the neonate and decrease with age to 4% in the adult (Hawke and Garry, 2001). In fish, MyC also constitute 4 to 7% of the nuclei in the adult rainbow trout (Fauconneau and Paboeuf, 2001). In young carp (5 cm in length), MyC represent 6% of muscle nuclei, whereas in adult carp they account for only 1% of muscle nuclei (Koumans *et al.*, 1991). The ability of the fish MyC to enter the cell cycle is demonstrated by cytofluorometry (Alfei *et al.*, 1993), bromodeoxyuridine (BrdU) uptake and proliferating cell nuclear antigen (PCNA/cyclin) expression (Koumans *et al.*, 1991; Alfei *et al.*, 1994). After extraction, MyC express,

within a few hours, MyoD (Rescan *et al.*, 1994) and then myogenin (Rescan *et al.*, 1995), two key myogenic factors implicated in MyC proliferation and differentiation, respectively (see below). Pax7, another transcription factor, was also shown to be a specific determinant of MyC; in Pax7 null mice, MyC are absent (Marcelle *et al.*, 1995).

IV.5.1 Myosatellite cell populations:

Several populations of myogenic cells can be distinguished in mammals and birds. During development, embryonic myoblasts develop first in the somites and, after migration, also in the limb buds. Later, fetal myoblasts appear. These cells give rise to primary and secondary myotubes, respectively (Koumans and Akster, 1995). This leads to a temporary arrangement where small fibers surround larger fibers. In the adult, the two generations become indistinguishable in size. In large mammals, tertiary and possibly later generations of myotubes have been described (Wilson *et al.*, 1992). The embryonic myoblasts that give rise to adult MyC appear last. Embryonic, fetal and adult myoblasts appear to be different populations of cells, having different sizes, sizes of the formed myotubes in culture and isoforms of the myosin heavy chain (Stockdale, 1992). Previous studies using BrdU labeling *in vivo* in rat limb muscle reported that MyC could be differentiated into two populations (Shultz, 1996); 80% of the MyC have a cell cycle time of 32 h, while the remainder divided more slowly. This last group constitutes a reserve population of muscle stem cells. The possibility that MyC arise from two or more distinct developmental origins could explain the heterogeneity within MyC (Holterman and Rudnicki, 2005).

Stem cells other than MyC are also able to give rise to muscle tissue. Cells derived from the embryonic dorsal aorta (De Angelis *et al.*, 1999), bone marrow (Ferrari *et al.*,

1998; Gussoni *et al.*, 1999), the neural compartment (Clarke *et al.*, 1998), and various mesenchymal tissues (Young *et al.*, 2001) are capable of contributing to muscle growth and regeneration *in vivo* and *in vitro*, suggesting that stem cells from other sources could contribute to muscle regeneration (Qu-Petersen *et al.*, 2002). Finally the side population (SP) cells possess high hematopoietic progenitor activity (Seale and Rudnicki, 2000) and also may contribute to muscle regeneration (Gussoni *et al.*, 1999). It was suggested that in muscle, the SP cells could represent MyC progenitors. Qu-Petersen *et al.* (2002) also reported the presence of three subpopulations of myogenic cells in mouse skeletal muscle, with each population having unique abilities to proliferate and differentiate.

IV.5.2 Potential stimulators of myosatellite cells:

Under normal conditions, mammalian MyC are quiescent but can be activated following trauma (Holterman and Rudnicki, 2005). However, in fish with indeterminate growth, MyC will be continuously active throughout life in order to increase muscle mass, while a pool of undifferentiated MyC are kept within the muscle to serve as a reserve of cells for the future growth of the muscle (Zammit *et al.*, 2004). Many factors influence MyC activation and proliferation, but the actual mechanism(s) and sequence of stimuli are not known, even in mammals.

It is clear that HGF/SF (hepatic growth factor / scatter factor), the ligand for the c-met receptor tyrosine kinase, plays a crucial role in MyC activation. Bischoff (1986) demonstrated that an extract from crushed muscle was capable of activating quiescent MyC. Later, HGF was identified as this critical activating factor in the crushed muscle extract (Allen *et al.*, 1995). Furthermore, it was shown that HGF/SF was present in the basal lamina of skeletal muscle fibers, providing a reservoir of HGF within skeletal muscle

(Tatsumi *et al.*, 1998). More recently it was suggested that nitric oxide (NO) may play a role in the release of HGF/SF from the extracellular matrix, implicating NO in MyC activation. Inhibition of nitric oxide synthetase reduces MyC activation in response to trauma *in vivo* (Anderson, 2000).

Identification of factors that stimulate or inhibit MyC proliferation and differentiation has resulted from *in vitro* experiments with cultured MyC. Among the most effective growth factors tested in rat MyC were insulin-like-growth factors (IGF I and II), basic fibroblast growth factor (FGFb) and transforming growth factor β (TGF-β). FGFb stimulated proliferation but depressed differentiation while IGF-I stimulated both proliferation and differentiation (Allen and Rankin, 1990). TGF-β depressed proliferation and inhibited differentiation (Olson *et al.*, 1986). When administered in combination, these factors induce MyC activities in culture that mimic those typical of MyC found *in vivo* in growing muscle. Alterations in the concentration of these growth factors in the muscle environment represent potential mechanisms for regulating MyC activity *in situ* (Allen and Rankin, 1990).

Several growth factors secreted by circulating macrophages and muscle precursor cells were also shown to stimulate MyC proliferation and differentiation (Grounds *et al.*, 1998). Many of these growth factors (such as FGFs) appear to be stored in the basal lamina and extracellular matrix by binding to heparin sulfate proteoglycans, which play an important role in muscle regeneration (Grounds *et al.*, 1998).

Pitkänen *et al.* (2001) reported that growth hormone (GH) and/or IGF-I may also be involved in MyC proliferation. In mammals, MyC are activated by biomechanical shear, stretching or cold exposure, with activation of neuronal nitric oxide synthase as a potential intracellular regulator (Anderson, 2000).

IV.6 Myogenesis

The formation of muscle fibers from muscle stem cells is called myogenesis (see Fig. I-1). Myogenesis is characterized by several myogenic factors synthesized during proliferation and differentiation of muscle MyC. In adult vertebrates, the mechanisms implicated in MyC recruitment from the basal lamina to new fibers remain under debate. When MyC are recruited in mammals and birds, embryonic development is repeated (Hawke and Garry, 2001), including the transcription of the same four myogenic regulatory factors (MRFs), namely MyoD, myogenin, MRF4 and myf5. MRFs are basic helix-loop-helix (bHLH) transcription factors synthesized by the cell during growth and assist proliferation and differentiation, successively (see Fig. I-2). MRFs act by forming a heterodimer with E protein (an additional transcription factor that also contains the bHLH structure), then binding to specific regions of the DNA termed the E-box (CANNTG), which activates the transcription of genes specific to muscle protein synthesis such as myosin light and heavy chains (Wentworth *et al.*, 1991), α- and β-actin (Sartorelli *et al.*, 1990), and creatine kinase (Braun *et al.*, 1990). MRFs gene sequences were reported for several fish species, including carp (Kobiyama *et al.*, 1998), trout (Rescan *et al.*, 1995) and salmon (MyoD: AJ851827; myogenin: DQ294029). The expression pattern of MRFs during embryonic development varies with species, especially between mammals and fish, where myogenin and MyoD appearance is opposite (Watabe, 2001). However, MRFs usually have the same function between species and share a high homology in the DNA binding domain between species (93 to 98% identity) (Kobiyama *et al.*, 1998). However, the entire sequence of carp myogenin showed 69, 55 and 51% sequence identity with those of rainbow trout (Rescan *et al.*, 1995), chicken (Fujisawa-Sehara *et al.*, 1990), and mouse

(Edmondson and Olson, 1989), respectively. The whole MyoD sequence showed a higher homology between species than myogenin with 93, 81 and 71% with zebrafish (Weinberg *et al.*, 1996), rainbow trout (Rescan *et al.*, 1994) and chicken (Lin *et al.*, 1898), respectively. Myf-5 and MyoD are implicated in the determination of myogenic cell fate and the formation of myoblasts during embryogenesis (proliferation), while myogenin and MRF-4 have a role in the activation of muscle differentiation (Feldman and Stockdale, 1991). Myocyte specific enhancers (MEF2) represent another family of proteins that stimulate muscle growth and MRFs transcription and cooperate with MRFs in activating skeletal muscle specific transcription. Another protein named Id (also a HLH factor) does not possess a DNA binding domain, and acts as a negative regulator of MRFs (Johnston, 1999).

Figure I-1: Myogenesis processes. Undiferentiated myogenic satellite cells are activated, proliferate (called myoblasts), then differentiate into myotubes and mature muscle fiber.

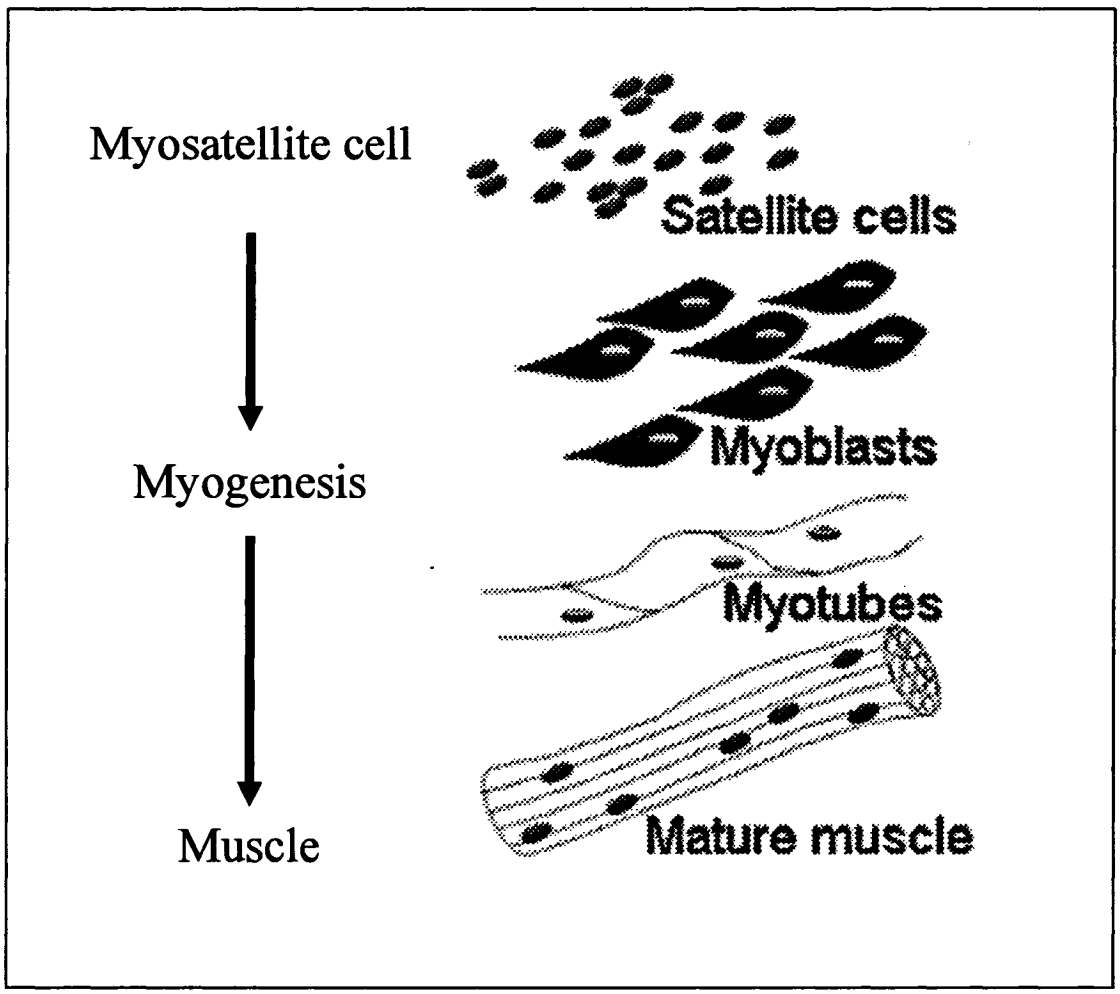
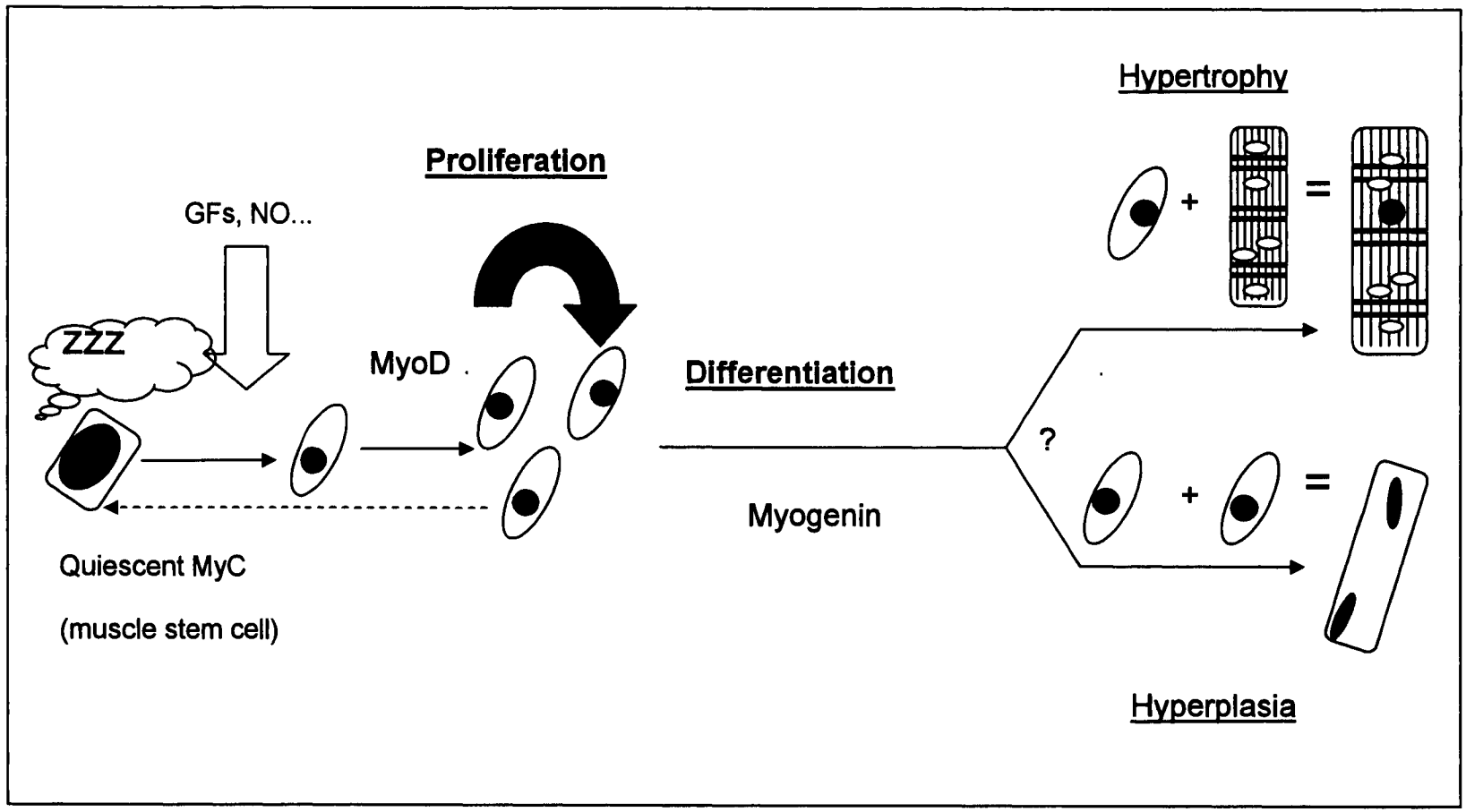


Figure I-2: Myosatellite cell proliferation and differentiation. Quiescent myosatellite cells (MyC) are activated and enter into a proliferation cycle characterized by the expression of MyoD. After successive proliferations, MyC can either return to the quiescent state or enter into the differentiation state characterized by the expression of myogenin. MyC differentiation can lead to the formation of a new muscle fiber (hyperplasia) or the enlargement of an existing muscle fiber (hypertrophy).


Hypertrophy
Proliferation
GFs, NO...
ZZZ
MyoD
Differentiation
Myogenin
?
Quiescent MyC
(muscle stem cell)
Hyperplasia

IV.7 Factors affecting growth

Many factors affect fish growth including external factors such as temperature (cold winter temperature decreases growth rate) or food availability, and internal factors, principally hormones and transcription factors (acting through protein synthesis, modification of metabolism).

IV.7.1 Temperature and photoperiod

Seasonal effects on the morphology and the physiology of muscle tissue are mainly connected with changes in environmental temperature, at least within temperate zone species. The general relationship between temperature and growth is that there is little or no growth below a certain temperature, while above this temperature the growth rate increases proportionally with the increase in temperature up to a maximum. The growth rate decreases as the temperature approaches the lethal limit (Dutta, 1994). Temperate fish in nature experience cyclic temperature changes either because of the seasonal changes in water temperature or because the fish move between different thermal zones.

Temperature is known to have major effects on early muscle development in teleosts, altering the timing of myofibril assembly, and the number and the size of embryonic muscle fibers (Stickland *et al.*, 1988; Vieira and Johnston, 1992; Matschak and Stickland, 1995). A recent study in rainbow trout showed that MRF expression was delayed at low compared to high egg incubation temperatures (Xie *et al.*, 2001). Rearing embryonic and larval stages at different temperatures has different effects on hypertrophic and hyperplasic growth. Higher temperatures result in greater hyperplasia but reduce hypertrophy at least in Atlantic herring (*Clupea harengus*) (Vieira and Johnston, 1992;

Johnston et al., 1995). In other species such as salmon the opposite is found (Stickland *et al.*, 1988; Galloway *et al.*, 1999).

Water temperature can also influence the relative importance of different metabolic fuels in muscle. An increase in the utilization of fatty acids is associated with cold acclimation (Jones and Sidell, 1982) as is also observed in human muscle (Timmons *et al.*, 1985). Cold acclimation also results in increased glycogen content (Johnston and Maitland, 1980) and an increase in the activities of enzymes of the mitochondrial electron transport chain, citric acid cycle and aerobic glucose utilization for both red and white skeletal muscles (Sidell, 1980; Johnston *et al.*, 1985). The data for temperature acclimation suggest a general trend toward a greater proportion of aerobic fibers and a greater power production by these fibers in cold adapted fish (Sänger and Stoiber, 2001).

IV.7.2 Hormones and growth factors

The role of hormones on muscle metabolic pathways and growth was previously reviewed (Moon *et al.*, 1989; Mommsen, 2001), but few experiments have reported the effects of hormones on MyC development.

Hormones, among many actions, affect nutrient metabolism, act on nutrient transporters and act on metabolic enzymes. Many hormones also affect growth and protein uptake in vertebrates, including fish. Fish muscle contains several hormone receptors including receptors for insulin and IGF-I (Mommsen and Moon, 2001). Hormones are implicated in the control of growth in vertebrates and studies document the interactions between the endocrine control of somatic growth and metabolism in teleost fish (Pérez-Sanchez *et al.*, 1995).

It is not possible to study the effect of all hormones, but some appear particularly important for growth and metabolism, since they increase protein synthesis and cell differentiation.

Growth hormone

Growth hormone (GH) is a peptide (20-22 kDa) produced and released by somatotroph cells of the anterior pituitary (Mommsen, 2001). GH has been isolated from many species and is well conserved among vertebrates. Growth hormone synthesis is regulated by glucocorticoids, retinoic acid and cAMP (Yowe and Epping, 1995; Wong *et al.*, 1996; Yang *et al.*, 1997). Mammalian GH increases protein synthesis (Fauconneau *et al.*, 1996), amino acid uptake and incorporation, lipolysis and affects reproduction in trout (Mommsen and Moon, 2001). GH also increases appetite (Markert *et al.*, 1977; Johnsson and Bjornsson, 1994), dominance (Johnsson *et al.*, 1996), nitrogen retention, and amino acid incorporation into muscle (Cheema and Maty, 1978).

GH has functions other than somatic growth in fish, including smoltification and seawater adaptation in salmonids (McCormick, 1996) and the synthesis of hepatic antifreeze proteins in marine teleosts (Idler *et al.*, 1989). GH is released from somatotroph cells in a pulsatile manner, with target tissues responding to the frequency and amplitude of the hormone peaks (Mommsen, 1998). Growth hormone binding protein protects GH from degradation. GH is also regulated by its actual concentration in the organism. GH also stimulates the production of IGF-I in skeletal muscle. Transcripts of growth hormone receptor are present in mammalian muscles.

GH stimulates MyC proliferation in the chicken (Halevy *et al.*, 1996) and administration of GH increases pig skeletal muscle growth and IGF-I mRNA in liver and

skeletal muscle (Grant *et al.*, 1991). In fish, growth efficiency and feed conversion increased in salmon transgenic for a gene construct encoding growth hormone, resulting in body mass gains of up to 3-fold (Devlin *et al.*, 2001). GH can influence muscle protein synthesis directly through muscle GH receptors or local (or hepatic) IGF-I production. GH also has a positive influence of bone and cartilage growth in fish (Takagi and Yamada, 1992). However, GH effects are inconclusive when injected directly into the fish. One study reported an increase in small diameter muscle fibers in rainbow trout (Fauconneau *et al.*, 1997), another an increased body length in salmon (Foster *et al.*, 1991; Fauconneau *et al.*, 1997), and finally, no effect in trout and Atlantic salmon (Fauconneau *et al.*, 1996).

All roles of GH described above make it an excellent candidate for enhancing fish growth in aquaculture. To date several fish species have been made transgenic for GH including salmon, trout and tilapia (Fletcher *et al.*, 2004; Devlin *et al.*, 2001). Insertion of an additional IGF or GH gene into the genome does not necessarily result in increased growth in healthy fish (Chen *et al.*, 1995), although improved growth is reported by several authors (Chen *et al.*, 1995; Devlin *et al.*, 1995). In order to express the GH transgene the construct must be accompanied by an adequate promoter in the host genome (De la Fuente *et al.*, 1999). The conventional approach uses very active promoters to drive the expression of the transgene. A transgene seems to have less chance to be expressed efficiently if it contains non-animal sequences, is devoid of introns, and is integrated in multiple copies. Therefore, all these elements should be included or controlled in the DNA construct (Rocha *et al.*, 2004). However, commercial exploitation of transgenic fish is likely to result in at least some consumer resistance (Aerni, 2004). The transgenic fish generated to date are used only as models to better understand growth processes in vertebrates. The public and

scientific debate behind the use of genetically modified animals will not be addressed in this thesis, but for further information on this subject, see Aerni (2004).

Insulin-like growth factor (IGF-I)

IGF-I is a peptide hormone (7.5-kDa) of the insulin superfamily (Mommsen, 2001) that is thought to mediate many of the growth promoting activities of GH (Duan and Plisetskaya, 1993) including increases in protein synthesis, amino acid uptake, glucose transport, cell proliferation (McFarland et al., 1993) and decreases in protein degradation (Mommsen and Moon, 2001). IGF-I concentrations are influenced by the nutritional status of the fish and the concentration of other hormones. The normal amount of IGF-I in fish plasma is approximately 25 $ng \cdot ml^{-1}$, but only 0.1% of this is present in the biologically active form (Plisetskaya, 1998). In mammals IGF-I induces cell differentiation (Florini and Ewton, 1992). In fish, circulating IGF-I levels are regulated by ration size, dietary protein and energy levels in trout (Pérez-Sanchez et al., 1995), and IGF-I affects mitogenesis to promote hyperplasic growth in fishes (Mommsen, 2001). The binding of IGF to rainbow trout muscle MyC and cell membranes has been reported (Fauconneau and Paboeuf, 2000).

Fibroblast growth factor (FGF)

Members of the fibroblast growth factor family increase muscle MyC proliferation and reduce differentiation (Clegg *et al.*, 1987; Olwin and Rapraeger, 1992). Skeletal muscle MyC express FGFs, and during differentiation *in vitro*, FGFs are down-regulated (Moor *et al.*, 2000). FGF directly represses myogenesis in mammalian muscle cell lines. The binding of the ligand to its receptor induces a signaling pathway that leads to the phosphorylation and inactivation of MRFs (Li and Bernard, 1992). However, FGFs are expressed at

appropriate times and places, and play a role in the regulation of myogenesis during development (Marcelle *et al.*, 1995). FGFs exert their effects through a family of four high-affinity receptors of the tyrosine kinase superfamily (Fantl *et al.*, 1993).

Hepatocyte growth factor (HGF)

HGF is a multifunctional cytokine that has pleiotropic effects on several cells and tissues. It was independently identified as a 'scattered' factor (inducing cells migration), which increased the motility of several cells (Stoker *et al.*, 1987). HGF is presumed to play an important role in activating hepatocytes during liver regeneration and to be involved in the proliferation of renal tubule cells, endothelial and epithelial cell lines (Gal-Levi *et al.*, 1998). The HGF receptor is a protein encoded by the proto-oncogene c-met, a 190 kDa transmembrane tyrosine kinase (Naldini *et al.*, 1991; Bottaro *et al.*, 1991). Jennische *et al.* (1993) was the first to detect HGF mRNA in skeletal muscle. In addition, HGF and c-met were shown to be expressed in developing limb buds by *in-situ* hybridization (Thery *et al.*, 1995; Takayama *et al.*, 1996). HGF was shown to activate quiescent muscle MyC in rats to proliferate and differentiate (Allen *et al.*, 1995; Anastasi *et al.*, 1997).

Adrenergic agonists

In vertebrates including fish, the circulating catecholamine hormones noradrenaline and adrenaline are synthesized and released from chromaffin cells upon preganglionic, autonomic neural stimulation (Reid *et al.*, 1996). A wide variety of external and internal stimuli including stress, can elicit the release of catecholamines in fish (Wendelaar Bonga, 1997; Fabbri *et al.*, 1998). Catecholamines in vertebrates including teleost fish are reported to have numerous physiological effects on carbohydrate, protein and lipid metabolism

(Sheridan, 1994; Moon *et al.*, 1995; Fabbri *et al.*, 1998). Ractopamine (RACT) and clenbuterol (CLEN) are synthetic catecholamine analogues from the group of phenethanolamines (Mustin and Lovell, 1992) used in domestic animal production (Roberts and McGeachie, 1992). These β_2-adrenergic-agonists redirect nutrients from fat deposition to muscle protein synthesis (Beermann, 1993; Vandenberg *et al.*, 1998). In sheep (Aurousseau *et al.*, 1993), pigs (Blanchard *et al.*, 1993) and rats (Maltin *et al.*, 1992), RACT and CLEN increase growth and reduce carcass fat. Lortie *et al.* (2004) reported a significant increase in fractional protein synthesis rates in whole protein, myofibrillar protein and sarcoplasmic soluble protein fractions in white and red muscles of rainbow trout fed 40 ppm RACT or CLEN for 30 days. Clenbuterol increases MyC proliferation in mice (Roberts and McGeachie, 1992) and muscle fiber hypertrophy in Lister rats (Maltin *et al.*, 1992). Ractopamine has also been tested in rainbow trout (Vandenberg and Moccia, 1998) and channel catfish (*Ictalurus punctatus*) (Mustin and Lovell, 1992) with less pronounced effects than in mammalian vertebrates; CLEN has been used on trout (Brambilla *et al.*, 1994). The effect of these β_2-adrenergic-agonists on fish MyC is unknown.

IV.8 Vertebrate metabolism 101

Metabolism is the study of the different fuels, either coming from the diet or from body reserves, used by an animal to sustain daily activities, growth and reproduction. Metabolism, consists of anabolism or the synthesis of molecules needed for animal life, and catabolism, or the degradation of fuels in order to produce energy used to sustain daily activities, including anabolic processes. Metabolism utilizes carbohydrates, lipids and proteins as fuels. Carbohydrates are utilized within the glycolytic pathway, where lactate is

the final product under anaerobic conditions, whereas if oxygen is available, acetyl CoA is produced and enters the mitochondrial citric acid cycle. The citric acid cycle completely oxidizes acetyl CoA into CO_2 and H_2O with the production of energy. Lipids also enter the citric acid cycle through β-oxidation that generates acetyl CoA, or lipids are esterified into triacylglycerides, the main source of lipid storage. Amino acids from the diet can be used directly to form proteins or be oxidized as well by the citric acid cycle (Lehninger *et al.*, 2004).

The liver is a key metabolic organ, and can control the plasma levels of many metabolites. Skeletal muscles, as noted above, use glucose from glycogen or lipids as principal fuels. These metabolic pathways are all controlled by enzymes.

IV.8.1 Use of metabolites as a source of energy by fish

Carbohydrates are key fuels in vertebrate metabolism (Moon and Foster, 1995), yet many fish preferentially use proteins and lipids, and use carbohydrates only poorly as compared with mammals based upon their carnivorous nature (Legate *et al.*, 2001; Moon, 2001). A previous hypothesis related to glucose intolerance in fish (Palmer and Ryman, 1972) proposed a reduced capacity to convert glucose to glucose-6-phosphate due to an absence of glucokinase (GK) activity (Tranulis *et al.*, 1991). Recent studies, however, reported the cloning of a cDNA sequence encoding GK in three fish species, one omnivorous, the common carp, and two carnivorous species, the rainbow trout and gilthead sea bass (Blin *et al.*, 1999). A glucose meal increased the assayable GK transcript levels in the carnivorous trout (Panserat *et al.*, 2000). Glucose transporters, important molecules facilitating the transport of glucose across cell membranes, are also reported in trout. A glucose transporter (onmyGLUT-1) was cloned from rainbow trout and found to be similar

to the mammalian GLUT-1 (Teerijoki *et al.*, 2001) and another glucose transporter, homologous to mammalian GLUT-4, was detected in skeletal muscle, kidney and gill of brown trout (*Salmo trutta*) (Planas *et al.*, 2000). These data suggest that at least some fish have the potential to use dietary carbohydrate.

The study of glucose metabolism in fish and its possible use as a nutrient is becoming increasingly important as its use in fish diets would result in significant environmental and economic savings for the aquaculture industry (Bergot, 1979; Arnesen *et al.*, 1995; Erfanullah and Jafri, 1998; Hemre *et al.*, 2001)

IV.8.2 Enzymes

Metabolic enzyme activities are closely related to food availability (Yang and Somero, 1993) and a high correlation exists between enzyme activities and growth (Pelletier *et al.*, 1993a). I will use enzymes from liver and muscle of cod and trout as an indicator of pathway use and of growth.

Specific enzymes analyzed in this thesis are used to indicate potential changes in the utilization of specific metabolic pathways; i.e. enzymes are used as markers. These enzymes include the glycolytic enzymes lactate dehydrogenase (LDH), pyruvate kinase (PyK) and hexokinase (HK), the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK), the citric acid cycle enzymes malate dehydrogenase (MDH) and citrate synthase (CS), enzymes regulating amino acid metabolism such as glutamate dehydrogenase (GDH), aspartate transaminase (AST), and alanine transaminase (ALT), and glucose-6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (IDH) and malic enzyme (ME) as indicators of NADPH production. These enzymes are used commonly for this purpose (Pelletier *et al.*, 1994; Moon and Foster, 1995; Pelletier *et al.*, 1995).

Enzyme activities studied in fish are reported to be good indicators of muscle accretion. For example, in red muscle, oxidative enzymes such as citrate synthase and hydroxyl-Coenzyme A dehydrogenase increase with muscle growth, while these same activities decrease in white muscle (Pelletier *et al.*, 1994).

IV.9 Implication for Flesh quality in Farmed fish

Aquaculture provides an important alternative to the exploitation of wild fish stocks for human consumption. The eating of fish flesh is recommended by nutritionists for its low concentration of saturated fats and its source of polyunsaturated saturated, especially omega-3 fatty acids (Henderson and Tocher, 1987). The fatty acid composition of the flesh and its nutritional value is largely associated with the composition of the diet of the fish (Bell *et al.*, 1991). It was shown that the amount and composition of fish muscle lipids can influence both the taste and the texture of the fish flesh (Sheehan and Allen, 1999). Increasing the fat content of the diet ultimately increases fish growth rate but could decrease the flesh quality. The consumer will also be concerned by the color (especially in salmon), firmness and moisture of the fillet (Johnston, 1999). These characteristics can vary with the type of processing leading to the product (cooked, salted, smoked, dried...). Muscle fibers are surrounded by collagen fibers (Sänger and Stoiber, 2001), so the amount of connective tissue per muscle volume will be influenced by the diameters of the fibers. This can definitely have an effect on the firmness of the meat after cooking. Any parameter that influences fish growth may also have an effect on the nutritive value and the appeal of the fillet. Even if the goal of my thesis is really upstream of the consumption level, these issues need to be considered.

This study will successively examine the effects of temperature and indirectly photoperiod on Atlantic cod (*Gadus morhua*) and rainbow trout (*Oncorhynchus mykiss*) morphology and metabolism, the effects of the growth hormone transgene on Atlantic salmon (*Salmo salar*) myogenesis and metabolism, and finally the effects of β-agonist feeding on trout growth, metabolism and myogenesis. These results will increase our current knowledge of fish muscle regulation and mechanisms and hopefully provide useful tools for future studies on improving fish muscle accretion for aquaculture.

[1]CHAPTER II: EFFECTS OF SEASONAL TEMPERATURE AND PHOTOPERIOD ON ATLANTIC COD (*GADUS MORHUA*) I. MORPHOMETRIC PARAMETERS AND METABOLITES

Abstract

Age 1+ to age 3+ Atlantic cod (*Gadus morhua*) were held at either a constant (~9 °C) (CST) or at ambient (ABT) ocean temperatures (Newfoundland) from December 2000 to June 2002 under natural photoperiod and *ad libitum* feeding. Body mass and length, liver and muscle glycogen and protein, plasma glucose and lactate were assessed over this period. Both groups increased body mass, length and liver mass, with a significantly greater increase for the CST-group at all sampling dates. Both groups demonstrated a growth phase from August to October 2001, while the ABT-group showed a compensatory growth phase from May to July 2001. Gonad mass significantly increased in males and females from both groups in June 2002. Most tissue metabolites demonstrated seasonal patterns consistent with photoperiod; the effect of temperature was primarily quantitative, implicating that temperature changes food conversion efficiencies as both groups of cod had the same access to food. Under the conditions of this study, Atlantic cod

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[1] Published as Levesque, H.M., Short, C., Moon, T.W., Ballantyne, J.S., Driedzic, W.R. 2005. Effects of seasonal temperature and photoperiod on Atlantic cod (*Gadus morhua*). I. Morphometric parameters and metabolites. Canadian Journal of Fisheries and Aquatic Science 62, 2854-2863. It should be mentioned that lipids in the livers (Table 1 and 2 in Levesque et al., 2005a) were measured by a technician and are not presented in the result section of this Chapter.

growth is controlled primarily by photoperiod, with temperature affecting the amounts of tissue metabolites.

Introduction

Temperature has a marked effect on growth and developmental rates of teleost fishes (Pelletier *et al.*, 1993a; Purchase and Brown, 2001; Dutil and Brander, 2003) and many fish species, especially in the north temperate regions, demonstrate seasonal patterns of growth and condition in response to temperature changes (Guderley and Gawlicka, 1992; Lambert and Dutil, 1997). Atlantic cod, *Gadus morhua,* experience natural temperature changes due to geographic location and season (Brander, 1995), but little is known regarding the influence of seasonal temperature changes on Atlantic cod physiology. The decline in Atlantic cod populations has renewed interest in farming this fast growing and economically important species, and thus the influence of temperature on growth can be of practical interest for aquaculture. Spatial distribution of cod is predominantly controlled by environmental temperature and salinity (Hedger *et al.*, 2004). Cod are, however widely distributed and can be found in waters that range from 2 to 11 °C and even higher (Brander, 1995; Hedger *et al.*, 2004). Separate cod stocks do exist, each with somewhat different optimal preferred thermal optima (Hedger *et al.*, 2004), but temperature is considered a key factor in stock production (Brander, 1995).

Many studies of cold adaptation report morphometric changes including a proportional increase in organ size. For example, Kent *et al.* (1988) reported an increase in hepatosomatic index (HSI) in channel catfish acclimated at 15 compared with 25 °C, and Blier and Guderley (1988) similarly found an increase in HSI in lake whitefish (*Coregonus*

clupeaformis) acclimated to cold temperature (4 °C). Changes in energy reserves may also occur including increases in blood glucose, liver and muscle metabolites (Martínez *et al.*, 1999; Purchase and Brown, 2001; Hochachka and Somero, 2002). Modifications of lipids resulted in the increased incorporation of unsaturated fatty acids into membrane phospholipids to maintain optimal membrane fluidity at lower temperatures (Seddon and Prosser, 1997). Liver lipids are also the first energetic reserves to be metabolized by fish (Black and Love, 1986; Kirsch *et al.*, 1998) and are an important source of essential nutrients (Mishra and Samantaray, 2001). Seasonal changes also affect prey availability and prey diversity which may influence lipid composition of fish liver and muscle (Kirsch *et al.*, 1998) as well as other tissue metabolites. Temperature also affects RNA and DNA levels and protein synthesis, with higher amino acid incorporation at 15 than at 5 °C in juvenile cod (Foster *et al.*, 1992).

Seasonal changes in morphometrics and biochemistry may reflect more than simply temperature because of the co-occurrence of dietary and photoperiod changes. Several studies report that muscle and liver metabolic organization varies seasonally, independent of temperature changes (Kleckner and Sidell, 1985; Seddon and Prosser, 1997). Photoperiod regulation of seasonal changes in physiology is a well-known phenomenon in vertebrates (McCormick *et al.*, 2000). Isolating and identifying the roles of photoperiod versus temperature on various adaptive characteristics of cod can be used to develop improved methods for enhancing growth under aquaculture conditions.

The hypothesis tested in Chapters II and III is that temperature has an effect on fish muscle growth and metabolism. The predictions are that fish exposed to ambient temperature will exhibit low growth rate and metabolic rate when exposed to low temperature and compensatory growth when exposed to rising temperatures. For this reason

we conducted a long-term study to investigate the morphometric and biochemical changes of age 1+ to age 3+ Atlantic cod with high food availability as would occur in an aquaculture situation. Fish were held either at natural ocean temperatures (area of St. John's, Newfoundland) or at constant temperature (~9 °C) over an 18 month period. The constant temperature of ~9 °C was chosen based upon the preferred thermal range of Newfoundland cod stocks (Purchase and Brown, 2001), the relationship between cod growth and temperature (Brander, 1995), and the fact that 9 °C is an intermediate water temperature found in waters used within the cod holding facility during the summer (see Fig. II-1); this temperature is within the lower growth temperature range for cod (Pedersen and Jobling, 1989; Pelletier *et al.*, 1993a). Fish were sampled at regular intervals over this period and a variety of morphometric and biochemical parameters determined. A second part of this study (Levesque *et al.*, 2005b; Chapter III) examines enzyme changes that accompany these growth and tissue metabolite changes. The aim of this study is to determine at which temperature and during which season cod grow the most and if seasons influence metabolite storage in Atlantic cod.

Materials and Methods

Experimental animals and procedures

Atlantic cod (*Gadus morhua)* hatched in March 1999 from stocks caught in Gooseberry Cove, Newfoundland (NAFO region 3L; 48° N, 53° W) were maintained at the Ocean Science Centre, Memorial University of Newfoundland, St. John's, Newfoundland & Labrador. Fish were divided into two groups in October 2000 and kept in seawater tanks

under natural photoperiod. In late November 2000, one group was placed under natural ocean water temperatures while a second group was held at a constant temperature of ~9 °C. Each tank was 5 m in diameter and 1.2 m in height containing approximately 20 000 L of seawater; cod density was approximately 2 g fish per L throughout the sampling period. The temperature and photoperiod changes experienced by both groups are presented in Figure II-1. Both groups were fed twice daily to satiation on a commercial pellet feed (Shur-Gain, Truro, N.S.: 45.0% protein; 22.0% fat; 4.0% fibre; 1.6% calcium; 1.2% phosphorus; 0.7% sodium; 10 000 $IU \cdot kg^{-1}$ vitamin A; 4 000 $IU \cdot kg^{-1}$ vitamin D; 250 $IU \cdot kg^{-1}$ vitamin E). Ten cod from each group were sacrificed by a blow to the head every two to three months. Length and mass were recorded for each fish. Blood was collected from the caudal vessels into a heparinized syringe and centrifuged at 12 000 g for 3 min. The resulting plasma was frozen in liquid nitrogen and stored at -80 °C until used. Liver and gonad weights were recorded and samples of hypaxial white muscle (at the dorsal fin level) and liver were immediately frozen in liquid nitrogen and stored at -80 °C. Condition factor [(body mass (g) / length (cm^3)) x 100], somatic indices for gonad and liver [(organ mass (g) / body mass (g)) x 100] and growth rate (% body $mass \cdot day^{-1}$) [(ln (mass b) – ln (mass a)) / (time b-time a)] were calculated.

Biochemical analyses

Glycogen analyses

Liver and muscle glycogen was determined as glucose equivalents after amyloglucosidase hydrolysis of the sample and measuring the difference in glucose content before and after hydrolysis according to Keppler and Decker (1974). Briefly, frozen pieces

of 100 mg of tissue were homogenized by a 4-s burst from a cell ultrasonic disrupter (Kontes) in 3 vol 6% perchloric acid (PCA), followed by centrifugation at 10 000 *g* for 5 min. After the addition of 0.5 vol 2 mol · L^{-1} $NaHCO_3$, supernatants were digested with 10 vol amyloglucosidase (10 mg ml^{-1}) in acetate buffer (pH 4.8) for 2 h at 37 °C with constant agitation. The reaction was stopped by the addition of 25 µL 70% PCA followed by centrifugation at 7 000 *g* for 10 min. Supernatants were used for glucose analysis (see below).

Plasma lactate and glucose

Plasma lactate and glucose were measured enzymatically using lactate dehydrogenase (LDH) and hexokinase plus glucose-6-phosphate dehydrogenase, respectively, by following the appearance of NADH at 340 nm using a microplate reader (SpectraMax Plus 384; Molecular Devices, Sunnyvale, California USA) and values calculated from standard curves using Soft Max Pro.

Statistics

Results are presented as means ± standard error. Statistical analyses were performed using SPSS (version 12.0) and SYSTAT (version 10). Normality was tested using Shapiro-Wilk; all the data were normaly distributed. One-way analyses of variance (ANOVA) were performed at each time point, with a Bonferoni adjusted critical value (α) of 0.00625. Two separate one-way ANOVA were performed on each treatment. Two ways ANOVA were perfomed on the same data set and gave the same significant differences. When the one-way ANOVA showed significant effects, multiple mean comparisons were made using the Tukey-Kramer comparison. A Pearson correlation was performed on SYSTAT results.

Results

Physical condition of the fish

Atlantic cod at all temperatures continued to eat and remained active throughout the entire experimental period. The initial mass, length and condition factor of the cod at the first sampling date did not differ between groups (Fig. II-2A,B,C). Body mass and length increased for both groups from December 2000 to June 2002, with a significantly ($p<0.01$) greater increase for fish held at the constant temperature (CST) compared with the ambient temperature (ABT) (Fig. II-2A,B). Both the CST and ABT fish exhibited a significant increase in mass between the August 2001 and October 2001 sampling times. The ABT fish exhibited a significant increase in growth between May 2001 and July 2001 that was not observed in the CST fish (Fig. II-2A,B). Condition factor remained unchanged between groups and across the entire period of the study, except for the CST-group in October 2000 where condition factor was significantly elevated (Fig. II-2C).

Liver mass and hepatosomatic index (HSI) followed the same trend in both groups (Fig. II-3A,B). HSI followed an annual cycle that was opposite to that of photoperiod (cf. Fig. II-3B with Fig. II-1). Liver mass was significantly greater in the CST-group from May 2001 to January 2002, while the HSI was not significantly different between groups except in June 2002 where it was higher in the ABT-group (Fig. II-3A,B). Liver mass exhibited a significant increase in both groups between August 2002 and October 2002. Liver mass per body length followed a pattern identical to that of the HSI (data not shown).

The 3+ individuals were reproductively mature in June 2002 as evidenced by the increased gonad somatic index (GSI) for both females and males (Fig. II-4A,B). GSI was different between groups only in June 2002, with GSI for the CST-group 2-fold higher for

females and 1.5-fold higher for males compared with the ABT-group (Fig. II-4A,B). The pattern of variation in gonad weight was not different from that of the GSI (data not shown).

Plasma and liver carbohydrate and liver and muscle protein

Plasma glucose of the CST-group was higher in December 2000 and February 2001 and lower in August 2001, compared with the ABT-group (Fig. II-5A). Plasma lactate followed the same pattern in both groups, with the highest levels in May 2001 (Fig. II-5B). Plasma lactate was higher from December 2000 to May 2001 in the CST-group (Fig. II-5B). Plasma glucose was lowest while plasma lactate was highest in May for both groups, suggesting an enhanced anaerobic metabolism at this time.

Total liver glycogen was the highest in October 2001 and January 2002, and declined significantly in the CST-cod in June (Fig. II-6A). There were no differences between the CST- and ABT-group across the period studied. Muscle glycogen was significantly higher in the CST-group in May 2001 and June 2002 (Fig. II-6B). There was more than a two-fold increase in muscle glycogen between May 2001 and July 2001 in both groups. Liver glycogen was positively correlated with HSI (r^2=0.62; $p<0.001$).

Total liver protein was also higher in October 2001 and January 2002 (Fig. II-7A). Total liver protein was highly correlated with total liver glycogen (r^2=0.73; $p<0.001$), fish weight (r^2=0.78; $p<0.001$) and length (r^2=0.76; $p<0.001$) and HSI (r^2=0.58; $p<0.001$). Muscle protein (Fig. II-7B) was similar between thermal groups (except at the beginning of the experiment), with changes occurring in a cyclic manner. One interesting point is that a sharp, significant decrease in muscle protein between August 28 and October 19 is associated with a period of the most rapid body mass increase (cf. Fig. II-7B with Fig. II-2A).

Discussion

The present study was conducted to gather basic data on the effect of fluctuating environmental temperature relative to constant temperature on Atlantic cod morphology and physiology. These studies were undertaken in young cod fed *ad libitum* as would occur in aquaculture conditions. By understanding changes in body parameters it should be possible to establish the impact of temperature and season on cod metabolism and especially growth.

Body mass and length significantly increased for both groups during the experiment, with a significantly higher growth for the CST group compared with the ABT group. Overall body mass from the beginning to end of the experiment increased 2.8-and 4.5-fold, respectively for the ABT and CST cod, showing that temperature does impact body mass gain over the year. Pedersen and Jobling (1989) previously reported that low temperatures depressed Atlantic cod growth. In our study, food was not limited and body mass and length increased linearly from February 2001 for the CST group and from May 2001 for the ABT group until the end of the experiment. The significantly higher specific growth rate between August and October 2001 for both groups (1.19% body mass per day for CST- and 1.40% body mass per day for ABT-group) must be considered as the period of the year dedicated to accelerated somatic growth for the Atlantic cod. What is interesting is that this rapid growth period is associated with a decrease in muscle protein, supporting the idea that weight gain may be initially a result of water content change that is only later replaced by tissue carbon (Mommsen, 1998). In fact, muscle protein does not return to prior growth levels until January 2002 (Fig. II-7B). At this time, cod are not sexually maturing, so energy can be directed specifically to somatic rather than gonadal

growth. This mass gain also occurs at a time when cod are preparing for winter, when photoperiod and temperature are declining. Photoperiod was identical for both groups in this experiment which may explain why both the CST- and ABT-groups exhibited a higher growth rate prior to winter, implying that mass gain may be affected by photoperiod. The ABT-group also exhibited a higher specific growth rate between May and July 2001 (1.56% body mass per day). This period coincides with the time of the year when ocean temperatures change rapidly (from nearly 0 to 9 °C). This accelerated growth noted for the ABT-group may represent compensatory growth following the low temperature winter period. Compensatory growth is described as exceptionally fast growth that occurs following a slow growth period caused by reduced feeding (Bélanger *et al.*, 2002). It has been shown that Atlantic cod tend to restore their energy reserves after a period of food restriction (Black and Love, 1986; Guderley *et al.*, 2001). In our experiment, the low temperatures experienced by the ABT-cod in winter probably depressed appetite, thus inducing compensatory growth in the spring. Presumably appetite remained constant in the CST-group, while it increased in the ABT-group as temperatures rose so that only the ABT-group exhibited a second period of accelerated growth during the year. Guderley *et al.* (2001) showed that food intake increased in the stickleback as temperatures rose.

Liver mass and HSI showed the same cyclic pattern for both groups, but liver mass was consistently higher in the CST-group compared with the ABT-group, consistent with the higher body mass of CST-group (cf. Fig. II-3 with Fig. II-2A). This pattern of a higher liver mass and HSI in the fall and early winter and lower in spring and summer was also reported by Lambert and Dutil (1997) in their study of the St. Lawrence Atlantic cod. Increased liver mass at cold temperature has been reported in lake whitefish (Blier and Guderley, 1988) and channel catfish (Kent *et al.*, 1988). The current work with Atlantic cod

indicates that metabolic reserves are accumulated in the liver coincident with the maximum growth period, and then these reserves are mobilized as needed during the winter. In our study both groups experienced the same pattern of HSI variation supporting the hypothesis that changes in HSI are driven by photoperiod more than temperature and metabolic demand. Cod liver stores considerable energy during the feeding period (Black and Love, 1986), uses these hepatic reserves during starvation periods, thus reducing the need to mobilize proteins (and thereby enzyme activity) from skeletal muscle (Martínez *et al.*, 1999). Lipid accumulation is typical of growing cod (Black and Love, 1986) and leads to high values of HSI.

GSI increased in June 2002 in both groups but the increase was higher in the CST group than the ABT group. Photoperiod is a more important factor than temperature for sexual maturation, as reported by Johnston *et al.* (2003) in Atlantic salmon. In our study, Atlantic cod initiated sexual maturation in June 2002 as 3+ fish. Gødo and Haug (1999) reported that growth rate is correlated with cod sexual maturity, and rapidly growing individuals will attain maturity at a younger age and at a shorter length than slow-growing and late maturing individuals.

It is well established that the fatty acid composition of a diet influences the lipid composition of tissues such as liver and muscle (Dos Santos *et al.*, 1993; Kirsch *et al.*, 1998). It is also known that temperature affects both digestion and utilization of feed and growth (Shcherbina and Kazalauskene, 1971). Liver is the primary site of lipid deposition and storage in fish. Adult wild cod feed on various prey items including a wide variety of fish species as well as various invertebrates (Lilly, 1991). In the wild, the prey available for cod changes with seasons, whereas in our experiment diet quantity and quality remained the same and abundant all over the entire study period. The differences between groups and

seasons can be attributed to the temperature. Cod fatty acids and lipids are also important metabolites to look at, as they are the precursor of many hormones, in this context both lipid quality and quantity are both important for fish growth and development (Mishra and Samantaray, 2001). Levesque *et al.* (2005a) reported that total lipids and triglyceride (TAG) per g liver measured in these same cod followed the same pattern as liver mass and HSI, suggesting fat accumulation contributes to changes in liver weight. In contrast to mammals where adipose tissue is the main site of lipid storage, many fish species use the liver and skeletal muscle (i.e. red muscle) for lipid storage (Jobling, 1988). Enlarged livers develop in cultured gadoid fish (Nanton *et al.*, 2003), in part due to diet and lifestyle of these species, and this is especially problematic in aquacultured Atlantic cod (Grant *et al.*, 1998). Liver TAG and total lipids had roughly identical values (Levesque *et al.*, 2005a). TAG are the storage form of lipids and a key energy source for locomotion and reproduction in fish. The fatty acid composition of the liver of cod found in our study is close to the composition reported in another cod study by Dos Santos *et al.* (1993) and the composition of juvenile haddock liver (Nanton *et al.*, 2003). However, there were very few differences between the CST- and ABT-groups or the sampling periods in liver lipid composition in those cod (Levesque *et al.*, 2005a), supporting previous reports that diet lipid composition and possibly age are the main factors determining fatty acid composition in Atlantic cod liver (Dos Santos *et al.*, 1993; Shahidi and Dunajski, 1994; Nanton *et al.*, 2003). The increase in the sum of the poylunsaturated fatty acids and omega-6 fatty acids also indicates that these fatty acids accumulate in the body with age, and presumably with reproductive activity as suggested by Henderson and Tocher (1987).

Plasma glucose in the ABT-cod followed an annual cycle comparable with that of liver mass, HSI and total lipids, except that it was shifted to the left, with the highest

plasma glucose values in August 2001. The CST-group showed no trend, although values were significantly higher than the ABT-fish between December 2000 and February 2001. These significantly higher plasma glucose values may indicate the CST cod were stressed at this early period, as plasma glucose levels generally rise with stress in fish (Wendelaar Bonga, 1997). Plasma lactate showed the same trend in both groups, with the highest values noted in May 2001. It is interesting to note that plasma lactate is highest when plasma glucose values are the lowest in both groups in May 2001, when ambient photoperiod and temperature are increasing. These data support those of Mayerle and Butler (1971) that showed blood glucose decreased as blood lactate increased in the American eel (*Anguilla rostrata*) as water temperature rose. Atlantic cod liver glycogen in our study varied in an unpredictable manner, as reported by Seddon and Prosser (1997) in channel catfish. It is important to note, however that in the ABT-group liver glycogen is higher from May 2001 to June 2002, which could be explained by a compensatory elevation of glycogen levels following winter temperatures for this group as suggested by Kleckner and Sidell (1985) in chain pickerel. The higher liver glycogen levels in May and July for the ABT group coincide with the compensatory growth noted in body mass and length for this group. The trend in Atlantic cod muscle glycogen is similar between the two groups with levels in the CST group generally higher than the ABT-group. The annual cycling of muscle glycogen parallels levels of plasma glucose for the ABT-group, with higher values in July and August, and lower values in winter. The lowest value for muscle glycogen is in May as reported by Mayerle and Butler (1971) in the American eel, just as ocean temperature begins to increase.

This long term study of Atlantic cod morphology and metabolism shows similar trends between the ABT and CST groups for most parameters studied, suggesting that

photoperiod, which followed natural ambient light for each group, is the main factor controlling cod physiology as suggested by Johnston *et al.* (2003) for Atlantic salmon. The temperature regime, per se did not have a significant impact except to quantitatively change some of the parameters studied. The natural internal cycle of hormone and metabolite concentrations were maintained at least for the first two years for the Atlantic cod used in our experiment, regardless of the thermal conditions. Significant increases in tissue metabolites coincided with increases in body mass during the May to October period, implicating this as the period of most effective conversion of food to body mass when food is not limiting. Cod exposed to an ABT regime did demonstrate a compensatory growth period as water temperatures rose, as is consistent with depressed feeding at low temperatures. Although this group of cod demonstrated this added growth phase compared with the CST group, the ABT cod did not reach the mass or length of the CST group after two years. Thus temperature appears to impact food utilization as food was provided *ad libitum* to each group in this experiment. Ambient photoperiod therefore may be needed to obtain optimal growth and to maintain the physiological cycles needed to achieve this growth; repeating this experiment by manipulating photoperiod would be needed to further test this hypothesis. The patterns of tissue metabolites observed in this study could also be useful in defining the quantity and quality of feed used across the seasons in an aquaculture facility. Our results demonstrate that although there are quantitative differences in growth rates as a function of temperature, qualitative changes in most tissue metabolites of Atlantic cod follow seasonal changes contrary to photoperiod. Regardless of thermal history, and within the period and the parameters studied, constant temperature does not disturb normal seasonal cycles of the Atlantic cod. Our results also show that photoperiod is more important than food availability in controlling either metabolite storage or appetite (as we

did not evaluate how much was eaten). Cod have a great capacity to acclimate to temperature changes, as only a few differences were observed between groups.

Figure II-1: Temperature and photoperiod profiles of Atlantic cod (*Gadus morhua*) (sampling dates noted by a ▼) held under constant (solid line) or ambient (dotted line) seawater temperatures from November 2000 to June 2002. •, represent photoperiod.

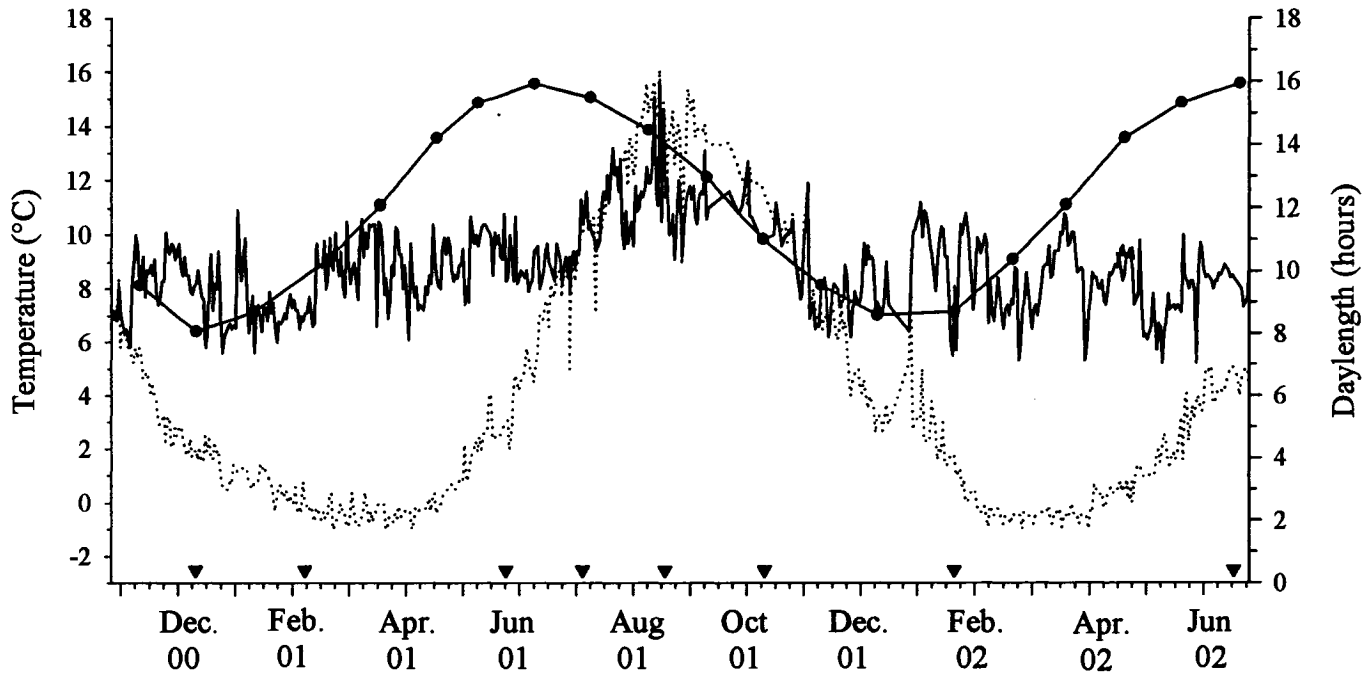


Figure II-2: Body morphometric parameters of Atlantic cod (*Gadus morhua*) throughout the 18-month sampling period (○, represents constant temperature cod; ●, represents ambient temperature cod; sampling dates noted by a ▼). Body mass in g (A); body length in cm (B); condition factor [(body mass (g)·length (cm3)-1) x 100] (C). Each point represents the mean ± standard error (SE) of N = 10. *, indicates values significantly different between groups at the same time point. Letters that are different represent significant differences between sampling month within the same group; upper case letters indicate differences within the constant temperature group and lower case letters indicate differences within the ambient temperature group. Numbers on each line represent the rate of change in specific growth rate (body mass · day^{-1}) between the two time points.

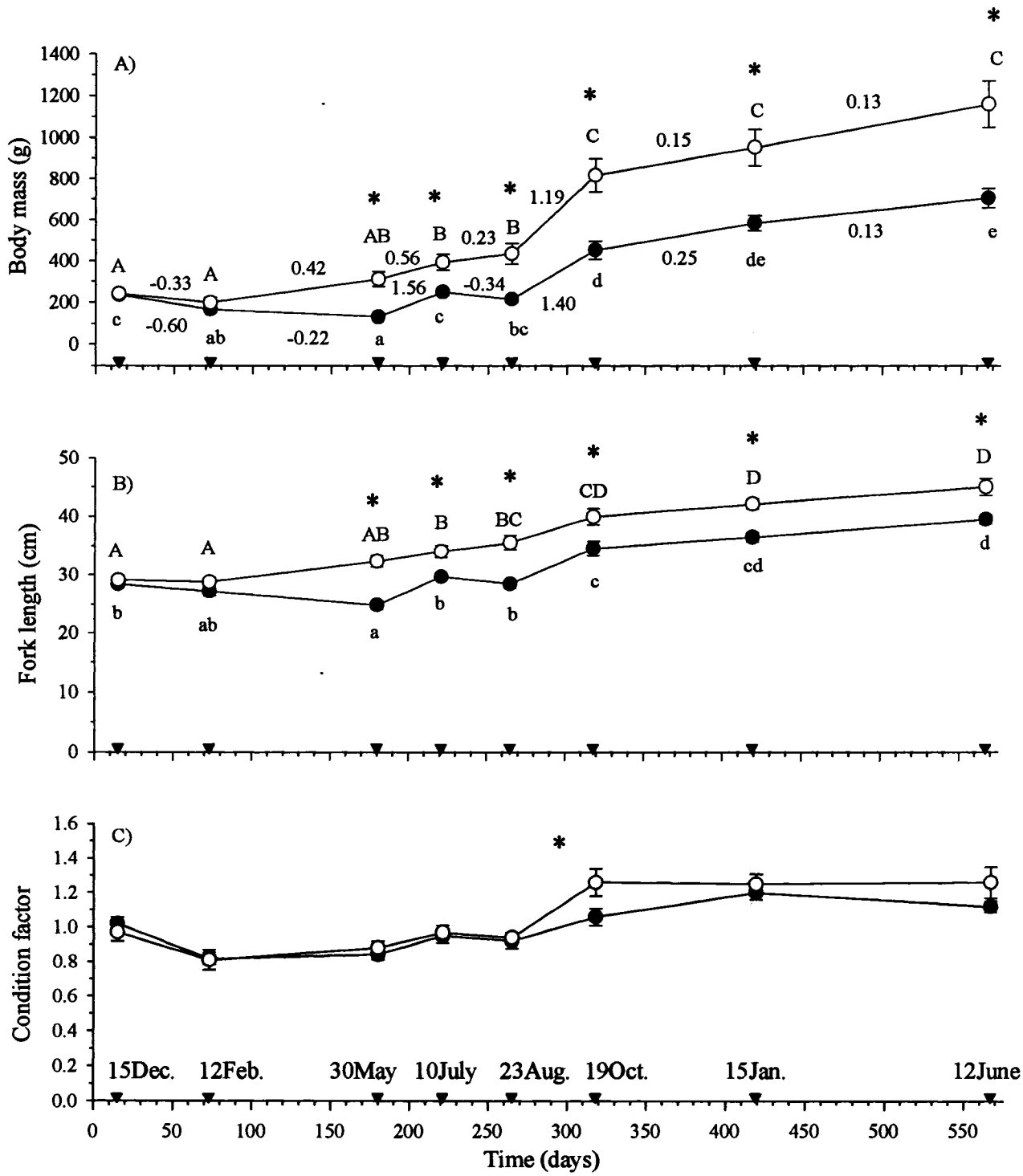

A)
Body mass (g)
1400
1200
1000
800
600
400
200
0
A
-0.33
A
0.42
AB
0.56
B
0.23
B
1.19
C
0.15
C
0.13
C
c
-0.60
ab
-0.22
a
1.56
c
-0.34
bc
1.40
d
0.25
de
0.13
e
B)
Fork length (cm)
50
40
30
20
10
0
A
A
AB
B
BC
CD
D
D
b
ab
a
b
b
c
cd
d
C)
Condition factor
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
15Dec.
12Feb.
30May
10July
23Aug.
19Oct.
15Jan.
12June
0
50
100
150
200
250
300
350
400
450
500
550
Time (days)

Figure II-3: Changes in hepatic parameters of Atlantic cod (*Gadus morhua*) throughout the 18-month sampling period (○, represents constant temperature cod; ●, represents ambient temperature cod; sampling dates noted by a ▼). Liver mass in g (A); hepatosomatic index (HSI) [(liver mass (g)·body mass $(g)^{-1}$) x 100] (B). Each point represents mean ± standard error, N = 10. *, indicates values significantly different between groups at the same time point. Letters that are different represent significant differences between sampling month within the same group; upper case letters indicate differences within the constant temperature group and lower case letters indicate differences within the ambient temperature group.

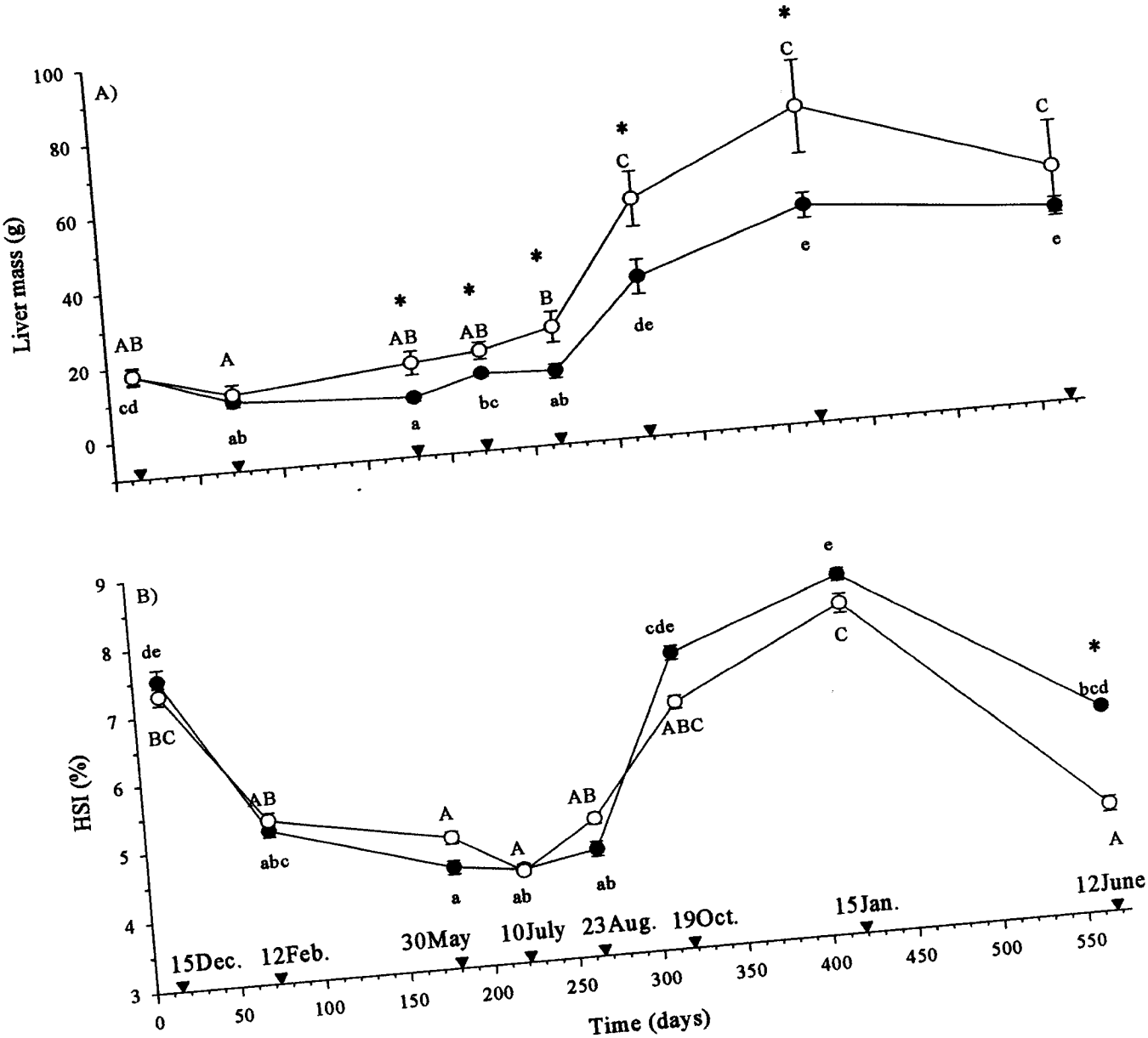

A)
Liver mass (g)
100
80
60
40
20
0
AB
A
AB
AB
B
C
C
C
cd
ab
a
bc
ab
de
e
e
*
B)
HSI (%)
9
8
7
6
5
4
3
de
BC
AB
abc
A
a
A
ab
AB
cde
ABC
e
C
*
bcd
A
15Dec.
12Feb.
30May
10July
23Aug.
19Oct.
15Jan.
12June
0
50
100
150
200
250
300
350
400
450
500
550
Time (days)

Figure II-4: Changes in the gonad somatic index of Atlantic cod (*Gadus morhua*) [GSI = (gonad mass (g)·body mass (g)$^{-1}$) x 100] throughout the 18-month sampling period for females (○, represents constant temperature cod; ●, represents ambient temperature cod; sampling dates noted by a ▼). (A) and males (B). N numbers in June 2002 are 5; N values for all other dates are between 1 and 9 due to random sampling. Each point represents mean ± standard error, when N>2. *, indicates values significantly different between groups at the same time point.

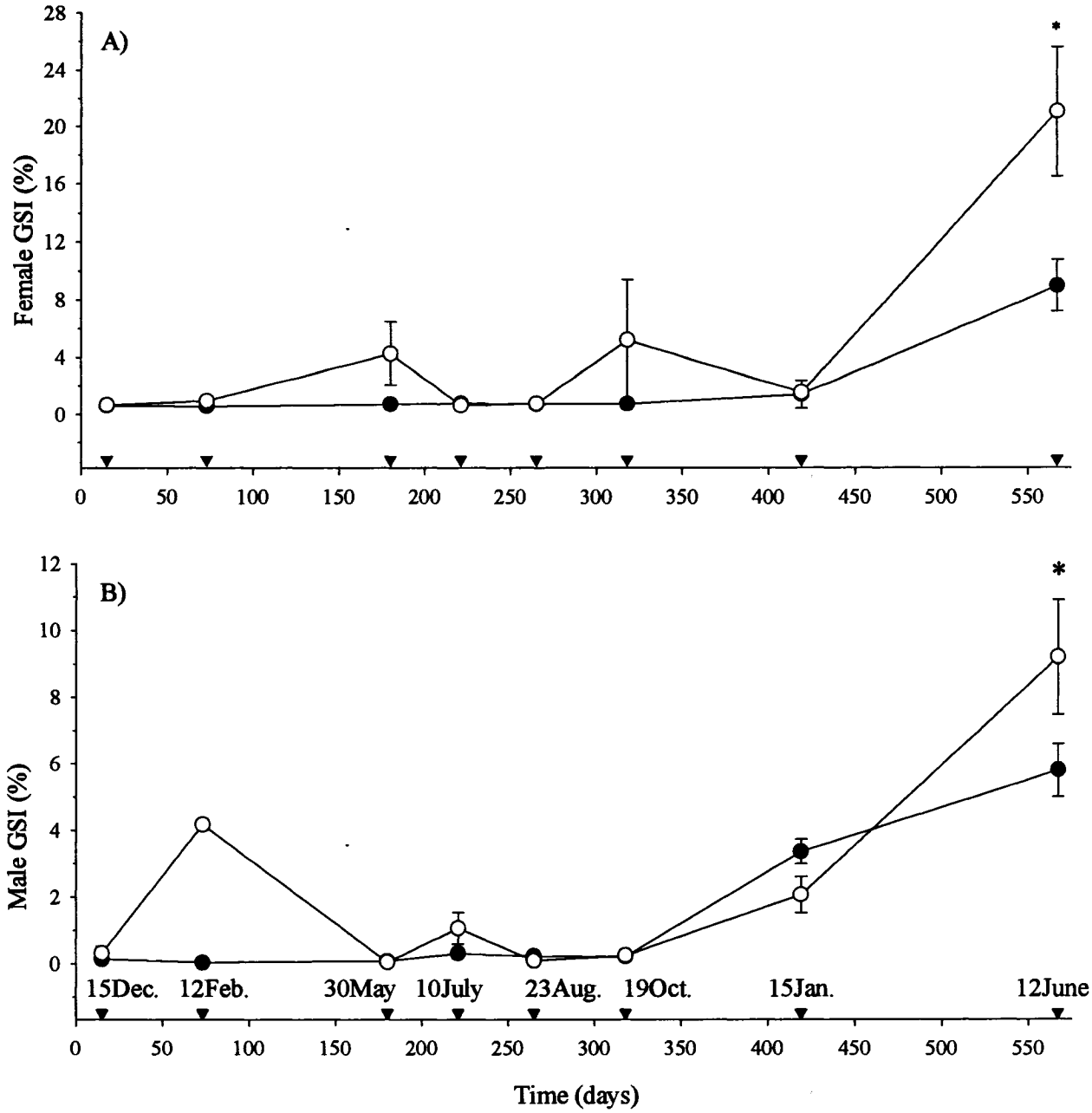

A)
Female GSI (%)
28
24
20
16
12
8
4
0
*
0
50
100
150
200
250
300
350
400
450
500
550
B)
Male GSI (%)
12
10
8
6
4
2
0
*
15Dec.
12Feb.
30May
10July
23Aug.
19Oct.
15Jan.
12June
Time (days)

Figure II-5: Plasma glucose (A) and lactate (B) concentrations in Atlantic cod (*Gadus morhua*) throughout the 18-month sampling period. Each point represents mean ± standard error, N = 10 (○, represents constant temperature cod; ●, represents ambient temperature cod; sampling dates noted by a ▼). *, indicates values significantly different between groups at the same time point. Letters that are different represent significant differences between sampling month within the same group; upper case letters indicate differences within the constant temperature group and lower case letters indicate differences within the ambient temperature group.

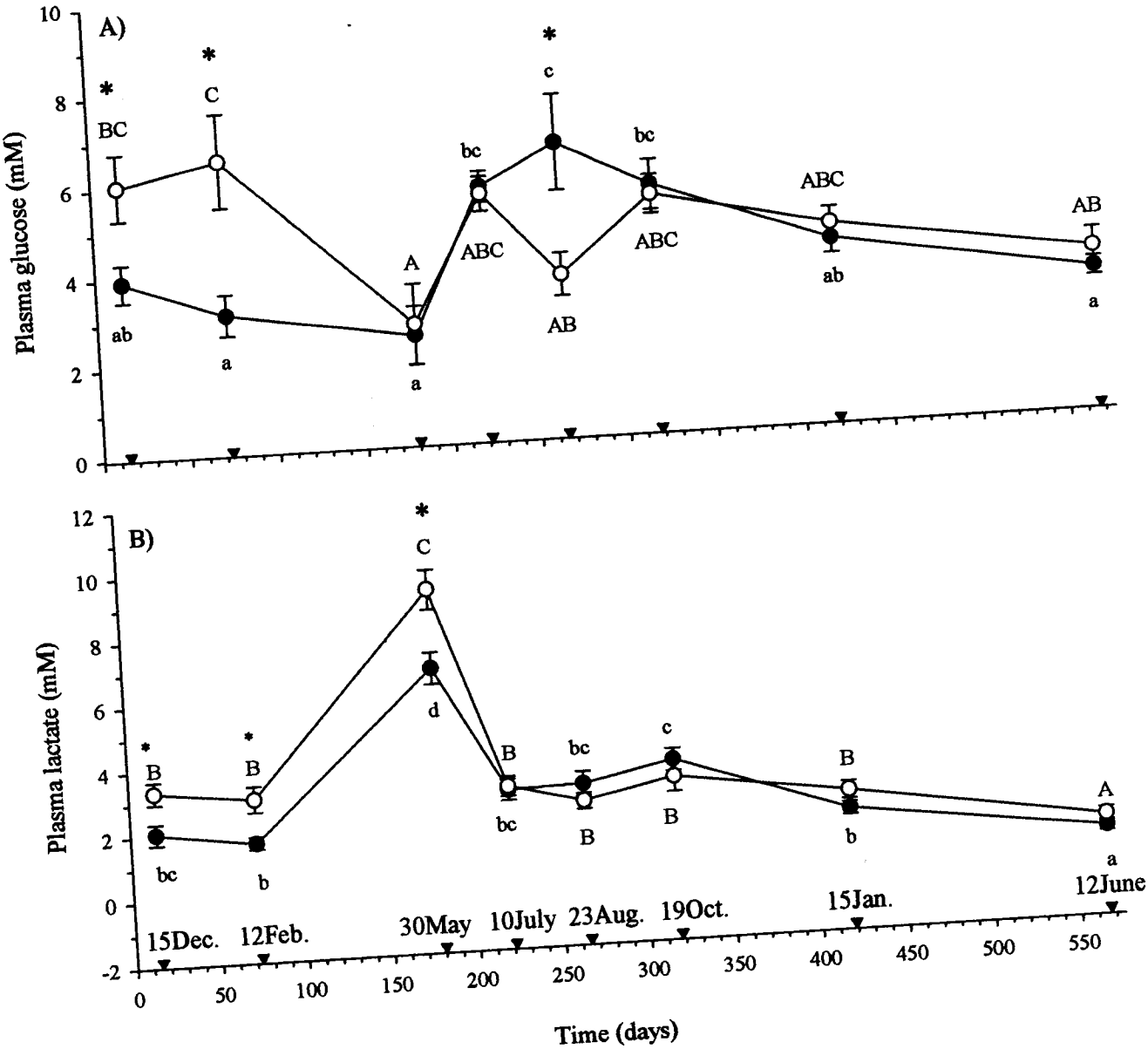

A)
Plasma glucose (mM)
10
8
6
4
2
0
*
*
*
BC
C
c
bc
bc
ABC
AB
A
ABC
ABC
AB
ab
a
a
ab
a
B)
Plasma lactate (mM)
12
10
8
6
4
2
0
-2
*
*
C
d
B
B
B
bc
c
B
A
bc
b
bc
B
B
b
a
15Dec.
12Feb.
30May
10July
23Aug.
19Oct.
15Jan.
12June
0
50
100
150
200
250
300
350
400
450
500
550
Time (days)

Figure II-6: (A) Total liver glycogen and (B) muscle glycogen (mg glucose equivalents·g^{-1}) concentrations in Atlantic cod (*Gadus morhua*) (○, represents constant temperature cod; ●, represents ambient temperature cod; sampling dates noted by a ▼) throughout the 18-month sampling period. Each point represents mean ± standard error, N = 10. *, indicates values significantly different between groups at the same time point. Letters that are different represent significant differences between sampling month within the same group; upper case letters indicate differences within the constant temperature group and lower case letters indicate differences within the ambient temperature group.

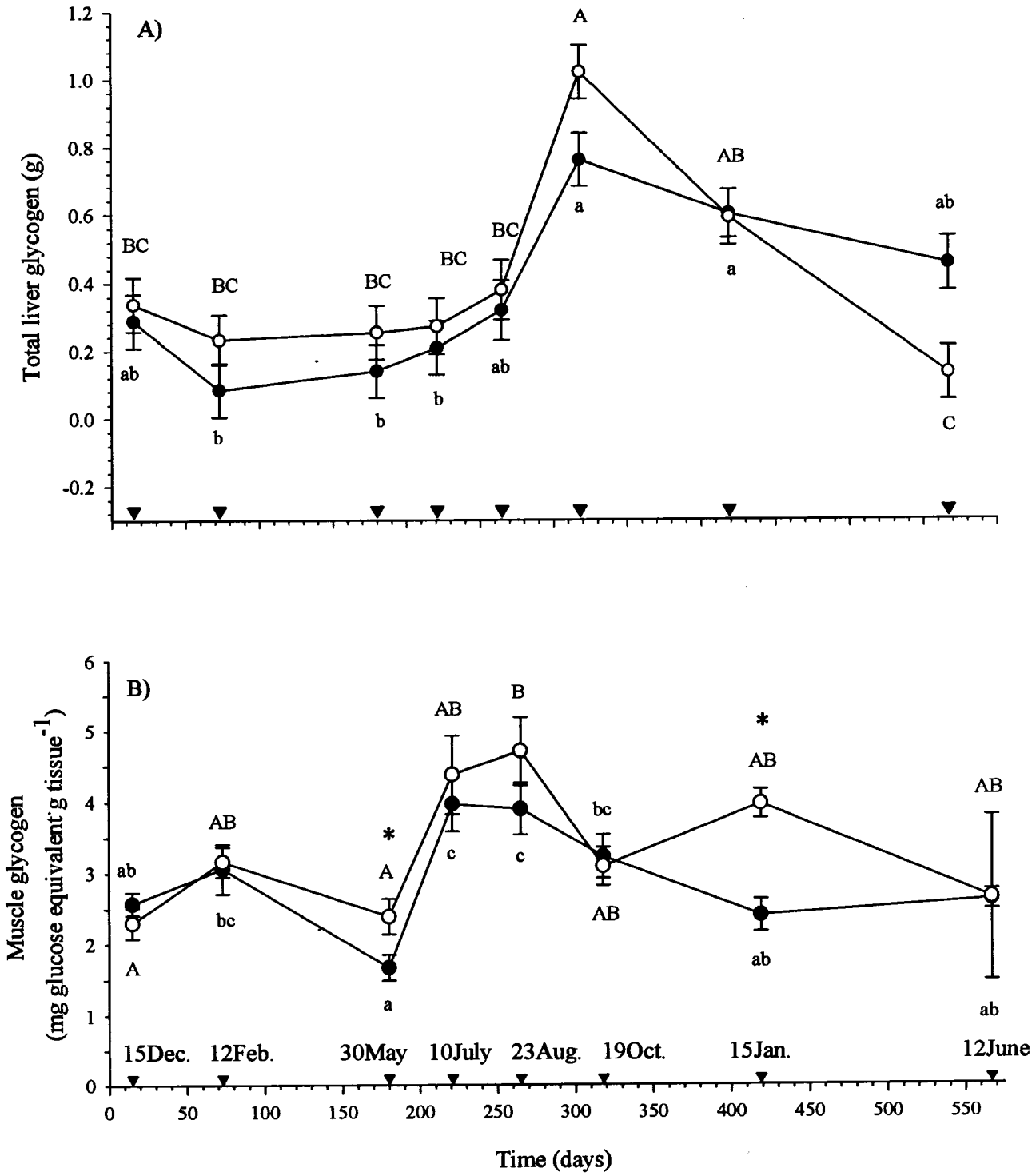
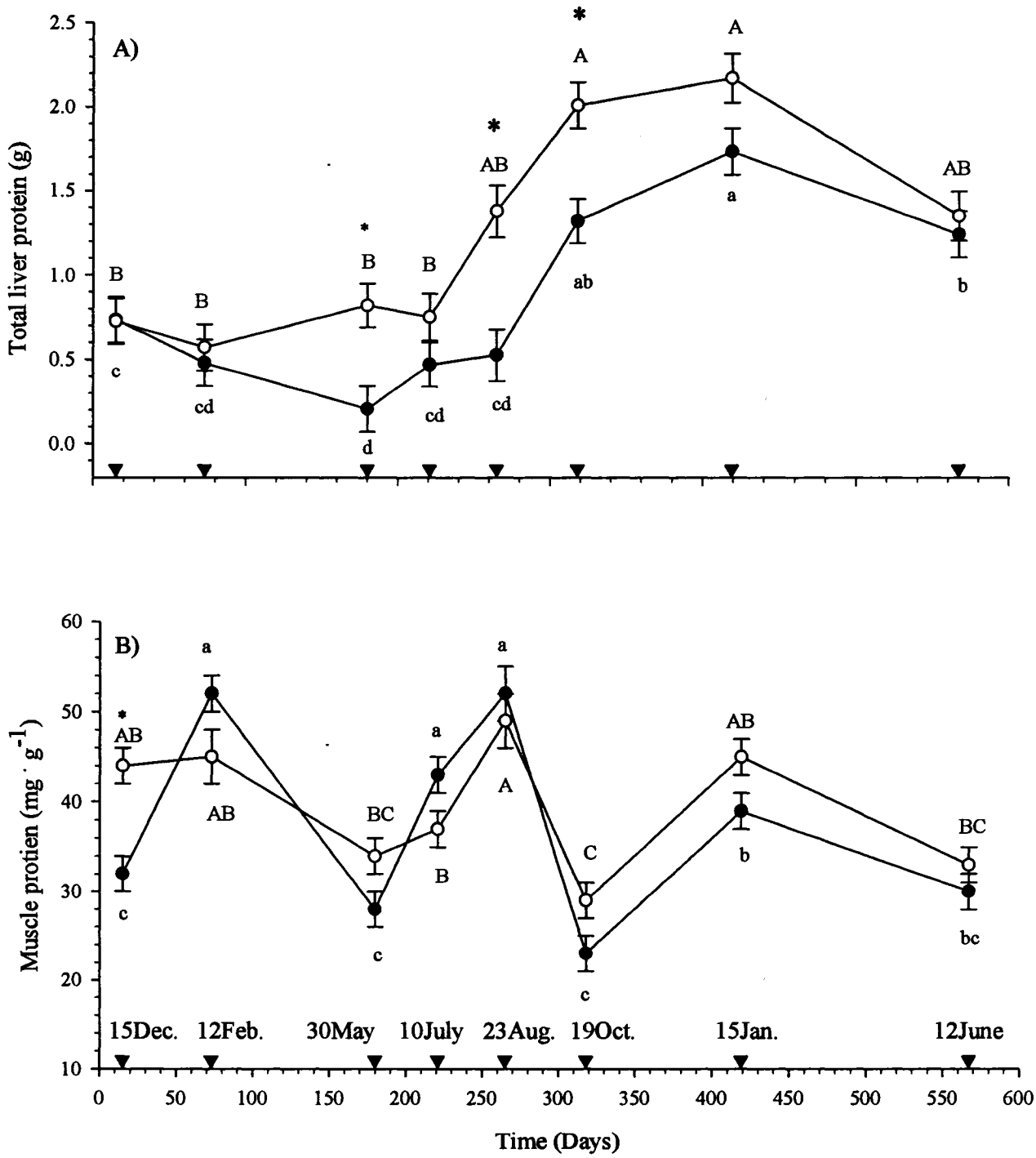

A)
Total liver glycogen (g)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
BC
BC
BC
BC
BC
A
AB
ab
ab
b
b
b
a
a
C
B)
Muscle glycogen
(mg glucose equivalent g tissue -1)
6
5
4
3
2
1
0
AB
B
AB
AB
AB
ab
A
bc
A
c
c
bc
AB
ab
ab
a
*
*
15Dec.
12Feb.
30May
10July
23Aug.
19Oct.
15Jan.
12June
0
50
100
150
200
250
300
350
400
450
500
550
Time (days)

Figure II-7: (A) Total liver protein and (B) muscle protein content ($mg \cdot g^{-1}$) concentrations in Atlantic cod (*Gadus morhua*) (○, represent constant temperature cod; ●, represent ambient temperature cod; sampling dates noted by a ▼) throughout the 18-month sampling period. Each point represents mean ± standard error, N = 10. *, indicates values significantly different between groups at the same time point. Letters that are different represent significant differences between sampling month within the same group; upper case letters indicate differences within the constant temperature group and lower case letters indicate differences within the ambient temperature group.


A)
Total liver protein (g)
2.5
2.0
1.5
1.0
0.5
0.0
B
B
B
B
AB
A
AB
c
cd
d
cd
cd
ab
a
b
*
*
B)
Muscle protien (mg · g-1)
60
50
40
30
20
10
a
a
a
AB
AB
BC
BC
A
C
AB
BC
c
c
B
c
b
bc
15Dec.
12Feb.
30May
10July
23Aug.
19Oct.
15Jan.
12June
0
50
100
150
200
250
300
350
400
450
500
550
600
Time (Days)

[2]CHAPTER III: EFFECTS OF SEASONAL TEMPERATURE AND PHOTOPERIOD ON ATLANTIC COD (*GADUS MORHUA*) II. ENZYMES OF INTERMEDIARY METABOLISM

Abstract

Atlantic cod (*Gadus morhua*) were held at either constant (~9 °C) or ambient ocean temperatures from December 2000 to June 2002 under natural photoperiod. The activities of gluconeogenic, glycolytic, lipid and amino acid-related enzymes were measured in liver and white muscle at eight time points over the experimental period. Principal component analysis reduced the 13 liver enzymes to two Factors and the nine muscle enzymes to three Factors. Factor scores were used to investigate the effects of season, temperature and photoperiod on cod physiology. Liver Factor I (LFI) and muscle Factor III (MFIII) were significantly correlated with mass and length of the fish. Only LFI was significantly correlated with tissue metabolites parameters. Significant differences between thermal groups were only observed for MFIII in June 2002. This study demonstrates that temperature is not a major factor determining the activity of the selected enzymes in age 1+ to age 3+ cod. Photoperiod and internal physiological cycles are more important in determining liver and muscle enzyme activities. Most liver enzymes measured are better

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[2] Published in Levesque, H.M., Bondy, J., Short, C., Ballantyne, J.S., Driedzic, W.R., Moon, T.W. 2006. Effects of seasonal temperature and photoperiod on Atlantic cod (*Gadus morhua*). II. Enzymes of intermediary metabolism. Canadian Journal of Fisheries and Aquatic Science 62, 2864-2873

It should be mentioned that CCO, HOAD and CPTwere measured by J. Bondy.

indicators of fish growth than muscle enzyme and liver protein is the parameter that best correlates with body mass, length and liver mass.

Introduction

Many environmental factors influence the activities of metabolic enzymes in animal tissues including fish tissues. Primary among them are temperature, photoperiod and food availability. Temperature is a known determinant of enzyme activities (Pelletier *et al.*, 1993a) and also affects growth and developmental rates of teleosts (Ayala *et al.*, 2001). Thermal acclimation leads many temperate zone species to adjust tissue metabolic capacity by enzyme changes (Johnston *et al.*, 1985; Guderley *et al.*, 2001). Enzyme activities may also show seasonal variation (Martínez *et al.*, 1999). Liver 3-hydroxyacyl coenzyme A dehydrogenase (HOAD) increased from July to September in the crucian carp (*Carassius carassius*), while lactate dehydrogenase (LDH) and cytochrome C oxidase (CCO) (the enzyme catalyzing the last reaction in the electron transport chain) activities increased in July (Lind, 1992). Muscle and liver metabolic organization can also vary across seasons independently of temperature (Vezina and Guderley, 1991), possibly reflecting an affect of reproductive activities, changing food availability, or photoperiod on enzyme activities. Guderley *et al.* (1996) showed that Atlantic cod (*Gadus morhua*) muscle LDH activities changed in response to feeding ration and to natural seasonal changes in food availability and environmental conditions. Glucose-6-phosphate dehydrogenase (G6PDH) also demonstrated laboratory acclimation in channel catfish (*Ictalurus punctatus*) liver (Kent *et al.*, 1988).

Fish size or growth rate is also an important factor determining metabolic enzyme activities (Somero and Childress, 1980; Martínez *et al.*, 1999). Previous studies reported that muscle glycolytic and oxidative enzyme activities are correlated with fish body mass and length (Pelletier *et al.*, 1993a,b) (Martinez *et al.*, 2000). Atlantic cod intestinal CCO and citrate synthase (CS) activities also correlate with growth rate (Pelletier *et al.*, 1994; Couture *et al.*, 1998; Dutil *et al.*, 1998). White muscle LDH, pyruvate kinase (PK) and CCO are positively correlated with growth in largemouth bass (*Micropterus salmoides*) and Atlantic cod (Goolish and Adelman, 1987; Pelletier *et al.*, 1993a, Martínez *et al.*, 1999, 2000). Aerobic and glycolytic capacities of muscle, liver and intestine followed growth in mass of fish kept in the laboratory (Pelletier *et al.*, 1993a; Houlihan *et al.*, 1995; Pelletier *et al.*, 1995; Martínez *et al.*, 1999). Enzyme activities also change during the life of an organism and the expression of a positive allometric relationship of white muscle glycolytic enzymes are influenced by the physiological status of the fish and position along the length of the fish (Martínez *et al.*, 1999; 2000).

The effects of seasonal temperature changes on Atlantic cod (*Gadus morhua*) intermediary metabolism are poorly understood. The developing Atlantic cod aquaculture industry will require an understanding of this parameter to achieve success. Most previous studies looked at selected enzymes and correlated activities to changes in a limited number of environmental variables. Looking at selected enzymes may obscure changes in metabolic pathways. In Chapter II (see also Levesque *et al.*, 2005a), we demonstrated significant changes in a variety of body parameters and metabolites in Atlantic cod held at natural ocean temperatures and at a constant temperature (~9 °C) under natural photoperiod over an 18 month period. This study estimates activities of glycolytic, gluconeogenic, oxidative, electron transport chain, pentose phosphate pathway and fatty acid enzymes in liver and

white muscle and correlates these with the morphological and metabolic changes noted in this previous study. We predicted that the observed changes in storage fuels and metabolites are correlated with enzyme activities associated with the pathways involved with these metabolites and the growth phases observed in Chapter II. This experiment when coupled with Chapter II (Levesque *et al.*, 2005a) represents a comprehensive analysis of growth and metabolic storage of Atlantic cod as a function of season and temperature variations.

Materials and Methods

Experimental animals and procedure

Atlantic cod (*Gadus morhua*) were held at the Ocean Sciences Centre (St. John's, Newfoundland) aquaculture facilities under a natural photoperiod and divided into two groups in late November. See Levesque *et al.* (2005a; Chapter II) for a detailed description of fish holding and husbandry. One group was held under natural ocean water temperatures and the other group under a constant temperature (~9 °C), representing the lower end of the optimal growth temperature for Atlantic cod (Pelletier *et al.*, 1993a). Ten cod from each group were sacrificed every two to three months by a blow to the head, total mass and length were determined and samples of hypaxial white muscle (at the level of the dorsal fin) and liver were dissected and immediately frozen in liquid nitrogen and stored at -80 °C; see Chapter II for further details.

Enzymes activities

Enzyme activities were measured under saturating substrate conditions according to standard procedures (Moon and Mommsen, 1987). Briefly, tissue pieces (0.1 g) were minced using a razor blade and homogenized (1:5 W/V) in a buffer containing 0.5 M glycerol, 20 mM Na_2HPO_4, 0.5 mM EDTA, 5 mM β-mercaptoethanol, 0.2% bovine serum albumin and a cocktail of protease inhibitors (Sigma Chemical Co., St. Louis, MO; P-8340). Exceptions were made for CS where the homogenizing buffer was 50 mM Tris-HCl (pH 7.4) and for HOAD, CCO and carnitine palmitoyl transferase (CPT) where the homogenization buffer was 0.2% triton X-100 in Tris-HCl buffer (pH 7.4). Homogenization was on ice by grinding the tissue in a 1.5 mL conical tube with a plastic pestle. The homogenate was centrifuged for 10 min at 4 °C and 10 000 *g* (Micro Centaur, Sanyo, VWR Scientific); the resulting supernatant was used immediately for enzyme analysis. Reaction rates were assayed spectrophotometrically at 340 nm following the appearance or disappearance of NAD(P)H for all enzymes except CS and CPT that were monitored at 412 nm to detect the reduction of 5,5' dithiobis-2-nitrobenzoic acid (DTNB), and CCO monitored at 550 nm following the reduction of oxidized cytochrome C. The extinction coefficients used for NAD(P)H, cytochrome C and DTNB were 6.22, 19.1 and 13.61 $mM^{-1} \cdot cm^{-1}$, respectively. Enzyme activities were measured at room temperature (22 °C) using a plate reader (Spectra Max Plus 384; Molecular Devices, Sunnyvale, CA) and SOFTmax pro software to calculate activities, except for the HOAD, CCO and CPT that were measured at 10 °C using a Cary/Varian 50 spectrophotometer. Substrate was added last to initiate the reaction; any activity in the absence of substrate was subtracted from total activity to give substrate specific activities. Activities were corrected either for tissue weight or protein content (bicinchoninic acid (BCA) method, Sigma).

All assays used 50 mM imidazole buffer (pH 7.8) with minor exceptions and conditions were as follows:

Lactate dehydrogenase (LDH; E.C.1.1.1.27): NADH (0.15 mM), pyruvate (5 mM).

Glutamate dehydrogenase (GDH; E.C.1.4.1.3): α-ketoglutarate (0.875 mM), NADH (0.15 mM), ADP (1 mM), EDTA (0.1 mM), NH_4Cl (0.125 mM).

Malate dehydrogenase (MDH; E.C.1.1.1.37): NADH (0.15 mM), oxaloacetate (0.5 mM).

Glucose-6-phosphate dehydrogenase (G6PDH; E.C.1.1.1.49): $MgCl_2$ (10 mM), $NADP^+$ (0.5 mM), glucose 6-phosphate (1 mM).

Malic enzyme (ME; E.C. 1.1.1.40): $MgCl_2$ (10 mM), $NADP^+$ (0.5 mM), malate (1 mM).

Isocitrate dehydrogenase (IDH; E.C.1.1.1.42): $MgCl_2$ (10 mM), $NADP^+$ (0.5 mM), isocitrate (2 mM).

Pyruvate kinase (PK; E.C.2.7.1.40): KCl (25 mM), $MgCl_2$ (10 mM), NADH (0.15 mM), ADP (5 mM), PEP (5 mM), excess PK-free LDH.

Phosphoenolpyruvate carboxykinase (PEPCK; E.C.4.1.1.32): $MnCl_2$ (1.75 mM), 2-deoxyguanosine diphosphate (0.4 mM), $NaHCO_3$ (20 mM), PEP (5 mM), NADH (0.15 mM), excess MDH.

Aspartate aminotransferase (AST; E.C.2.6.1.1.): α-ketoglutarate (13 mM), aspartate (20 mM), NADH (0.15 mM), pyridoxal phosphate (2.5 mM), excess MDH.

Alanine aminotransferase (ALT; E.C.2.6.1.2): α-ketoglutarate (13 mM), alanine (100 mM), NADH (0.15 mM), pyridoxal phosphate (2.5 mM), excess LDH.

Citrate synthase (CS; E.C.4.1.3.7): Tris-HCl (50 mM, pH 8.0), DTNB (0.1 mM), acetyl-CoA (0.3 mM), oxalacetate (0.5 mM).

3-Hydroxyacyl CoA dehydrogenase (HOAD; E.C.1.1.1.35): acetoacetyl CoA (0.1 mM), NADH (0.1 mM).

Cytochrome C oxidase (CCO; E.C.1.9.3.1): cytochrome C (sodium hydrosulfite reduced) (50 μM).

Carnitine palmitoyl transferase (CPT; E.C.2.3.1.21): DTNB (0.2 mM), palmitoyl CoA (0.1 mM), L-carnitine (0.5 mM).

Statistics

Statistical analyses were performed using SYSTAT (version 10) and SPLUS (version 8.0). Normality was tested using Shapiro-Wilk; all data were normaly distributed. Principal component analysis (PCA) was performed to reduce the number of variables (enzymes), by combining those that are correlated (express the same pattern of variations) (Jackson 2003). Liver and muscle enzyme PCA were followed by an oblimin rotation. Males and females were analyzed together except for gonadal-somatic index as no gender differences were identified in the data set. One-way analyses of variance (ANOVA) were performed at each time point and two separate one-way ANOVAs were performed on each treatment, on Factors resulting from the PCA and individual enzymes, with an adjusted critical value (α) of 0.00625, Bonferoni corrected. When the one-way ANOVA showed significant effects, multiple mean comparisons were made using the Tukey-Kramer comparison. Two way ANOVA were perfomed on the same data set and gave the same significant differences. Simple correlations and multiple regressions were performed on enzyme Factors and the morphological and metabolic parameters estimated in Chapter II (see Levesque *et al.*, 2005a); SYSTAT was used to assess their level of significance

Results

Principal component analysis (PCA) was performed as a means of grouping enzymes that showed the same pattern in the same tissue over the experimental period. This analysis is, however sensitive to factors that may vary over this period, and in particular tissue mass and protein content. As noted in Chapter II liver mass, total protein and hepatosomatic index (HSI) varied in a similar pattern, remaining low when cod growth rates were low but increasing and achieving a plateau as growth rates increased from August to October 2002. Muscle protein varied in a less predictable fashion. PCA was performed on enzyme activities expressed as either activity per mg protein or per g tissue mass or per whole organ (liver only). Depending upon the expression of activity, both the number of Factors (enzyme groupings) and in some cases the specific enzymes represented by a particular Factor, changed. It was decided to perform PCA on liver enzyme activities expressed as activity per whole liver (or activity per g x total liver mass) as this estimates the total potential of the liver to catalyze a particular reaction. Muscle enzyme activities could not be reported in this manner, but are reported as activity per g of muscle to reflect the potential of that enzyme in a unit of muscle mass.

Liver enzymes

Total liver enzyme activities are presented in Table III-1. Performing PCA reduced the 13 liver enzymes to two Factors (Table III-2), explaining approximately 85% of the variation of liver enzyme activities. All 13 liver enzymes contributed to each Factor but only enzymes that contributed more than 0.5 loading value to a particular Factor were considered relevant to that Factor. The closer the loading value is to 1 (Table III-2), the

greater that enzyme contributes to that Factor (Jackson, 2003). Enzymes that contribute to the same factor are correlated. As noted on Table III-2, CS, CCO, HOAD and CPT all have loading values greater than 0.9 and are all grouped in Factor I (FI); interestingly these enzymes are exclusively found in the mitochondria. In addition, the amino acid-related enzymes (ALT, AST, GDH), the gluconeogenic enzyme (PEPCK), the glycolytic enzyme (PK), and the NADPH producing enzyme IDH contribute to FI more than to liver FII (Table III-2). As these enzymes contribute the most to FI, this indicates that the activities of these enzymes followed the same trend and are correlated (Fig. III-1A). For each individual sample, PCA estimates a value for each Factor that is a function of the contribution of all enzymes to that Factor which is then plotted as a function of sampling period. According to PCA, the more positive the Factor is the higher are the activities of the collective enzymes making up that Factor (Jackson, 2003). Enzymes contributing to FI had constant activities from December 2000 to August 2001, after which activities continuously increased until the final sampling point (June 2002). Liver FI enzymes were significantly different between sampling times ($p < 0.05$) but not between thermal groups (Fig. III-1A). Liver CCO is included in FI and is presented as an example of the behaviour of each FI enzyme (Fig. III-1B).

Liver FII is explained mainly by the dehydrogenases LDH, MDH and G6PDH, an NADPH-generating enzyme of the pentose-phosphate pathway. As with FI, FII enzyme activities were constant from December 2000 to August 2001, then generally increased from October to December 2001 and decreased to June 2002 (Fig. III-2A). There were no activity differences between the ambient and constant temperature groups. Liver MDH is the enzyme most representative of FII enzymes and is presented here as a specific example of this Factor (Fig. III-2B).

Muscle enzymes

Total activities of nine muscle enzymes are presented on Table III-3. Following PCA, activities of these nine enzymes could be reduced to three Factors accounting for approximately 80% of the variance in activities of these muscle enzymes. Muscle FI (Table III-2) was explained by an oxidative metabolism enzyme (CS), an anaerobic metabolism enzyme (LDH) and a glycolytic enzyme (PK). Muscle FI activities remain constant from December 2000 to July 2001 and slowly increased to January 2002 and decreased to June 2002 (Fig. III-3A). There were no differences between temperature treatments and muscle FI is highly correlated to liver FII (Table III-4). Muscle CS is included in muscle FI enzymes and is shown as an example of these FI enzymes (Fig. III-3B).

Muscle FII includes two amino acid metabolism enzymes (ALT and AST) and two oxidative enzymes (IDH and ME). Muscle FII enzymes remain constant from December 2000 to July 2001 then increased until January 2002 and remained elevated through to June 2002 (Fig. III-4A). Again there were no differences between temperature treatments. The activity of muscle ALT is included in muscle FII and is presented here as an example of FII enzymes (Fig. III-4B).

Muscle FIII includes two oxidative enzymes (GDH and MDH). The activities of FIII enzymes remained stable from December 2000 to July 2001, then decreased and remained stable in the ambient temperature group, whereas they increased in January 2002 and decreased in June 2002 in constant temperature group (Fig. III-5A). A temperature affect is noted for muscle FIII enzymes at the January and June 2002 sampling points, with the constant temperature group showing significantly higher activities than the ambient group in January a trend which was reversed in June 2002. MDH is included as an example of a muscle FIII enzyme (Fig. III-5B).

Simple correlations and multiple regressions

We examined correlations between the Factors (enzyme activity groupings) and the morphologic and metabolic parameters (see Levesque *et al.*, 2005a; Chapter II) obtained for individual cod at the different sampling times and temperature regimes (Table III-4). Significant correlations were obtained between liver FI and muscle FIII and the morphological parameters estimated. Liver FI was also significantly negatively correlated with liver glycogen ($p<0.01$) but positively with total liver protein ($p<0.001$); muscle FI was significantly correlated with total liver glycogen ($p<0.001$) (Table III-4).

The most significant correlate of body mass and length was total liver protein (Table III-5), a variable that alone explains 60 and 57%, respectively of the variability of body mass and length ($p<0.001$). Liver FI and FII, condition factor and HSI were also good indicators of cod body mass and/or length, but their contribution is relatively small compared with liver protein (Table III-5). The optimal multivariate model explains 86% of the variability in body mass and 77% of length. Adding more variables did not increase the coefficients of determination to a level that could justify their inclusion in the model. The optimal multivariate model for liver mass explains 92% of the variability in liver mass. Total liver protein was the most significant correlate of liver mass and alone explained 89% of the liver mass variation ($p<0.001$). Total liver glycogen and liver FI and FII are also significant indicators of liver mass (Table III-5).

Discussion

Previous studies using Atlantic cod report activities of numerous enzymes, and it is clear from these studies that activities vary depending upon season, food availability, body

mass and physiological conditions. In fact, specific muscle enzymes are reported as useful indicators of growth in this species. These studies, however examined enzymes at specific time points rather than using the same group of fish over an extended period of time. Our study sampled cod at eight time points across a 18 month period beginning when the cod were age 1+ (20 months post hatch) and fed *ad libitum*. Growth and metabolite changes across this period better reflected changes in season/photoperiod than temperature, although cod held under a constant (~9 °C) compared with a variable (ambient) thermal regime had higher growth. We predicted that these changes in growth and tissue metabolites reflect changes in tissue enzyme activities. Thirteen liver and nine muscle enzymes were analyzed reflecting a variety of metabolic functions. Principal component analysis (PCA) was used to reduce the number of variables (enzyme activities) and group together those enzymes that varied in a similar manner into the same Factor. Liver Factors defined from the PCA used in this study represented 85% of the variance in enzymes activities. However, depending upon the expression of enzyme activity (i.e. units per mg protein, per g wet tissue weight, or per whole tissue), the number of Factors defined by PCA diferred. This is based upon the fact that liver mass, protein and HSI and muscle protein vary across the sampling period (Levesque *et al.*, 2005a; Chapter II). The issue of how enzyme activities should be expressed in fish growth studies is discussed by Pelletier *et al.* (1995). Although correlations existed between enzyme activities and growth rate when expressed per g wet muscle mass, the strongest correlation existed between growth and enzyme activity per μg DNA. This study expresses liver enzyme activities per mass of liver to reflect the total potential activity of a particular enzyme in the liver. For white muscle, activities per g of

wet muscle mass were used as Pelletier *et al.* (1995) did find a significant correlation between growth rate and this enzyme measure.

The 13 liver enzymes were reduced to two Factors, FI and FII. Liver FI represented 10 of the 13 enzymes assayed (Table III-1), consisting of oxidative, glycolytic and gluconeogenic enzymes. The pattern described for these enzymes (Fig. III-1) reflects that noted for liver mass and total protein (Table III-4) over the sampling period (Levesque *et al.*, 2005a; Chapter II). Unfortunately, PCA did not group the enzymes into definable metabolic pathways that can assist in understanding activities of pathways responsible for the changes we noted in metabolite stores. This one liver Factor (FI) was, however, significantly correlated with cod body mass and length, liver mass and GSI (Table III-4) supporting a key role for the liver in the activities of muscle and gonad. Interestingly, there is a negative correlation with glycogen per g liver (Table III-4). Liver glycogen content increases concurrently with the rise in cod growth rates, then declines even though liver mass remains high (Levesque *et al.*, 2005a; Chapter II). Liver FI enzymes increase even though liver mass remains stable providing further evidence that the liver serves as fuel source for the rest of the fish body. The oxidative enzymes (CS, HOAD, CPT and CCO) are included in liver FI and their loading values are high relative to the other enzymes found in this Factor (Table III-1). The high loading values for these particular enzymes implicates the role of lipid oxidation in providing energy for growth in the cod. The fact that liver FI is correlated with liver total protein and liver mass indicates a strong effect of photoperiod, as liver mass and liver total protein are inversely following photoperiod variations (Levesque *et al.*, 2005a; Chapter II).

Liver FII is represented by three dehydrogenases (G6PDH, LDH and MDH) whose activities are generally high throughout the sampling period, increase concomitantly with

the rapid rise in growth rate, then fall as growth rates decline (Fig. III-2). This pattern is not correlated with any body parameter or tissue metabolite measured (Table III-4). One of these enzymes, LDH has previously been shown as a muscle enzyme to be the strongest correlate of growth rates in cod (Pelletier *et al.*, 1995; Martínez *et al.*, 1999) probably based upon the well established relationship between size and metabolic capacities (Somero and Childress, 1980; Sullivan and Somero, 1983). Our study would suggest that enzymes other than muscle LDH may also be good indicators of growth rates.

The muscle enzymes expressed as units per g white muscle could be reduced to three Factors representing 80% of the variance in activities (Table III-1). Again as with the liver Factors, the enzymes did not group into clearly definable metabolic pathways. Muscle FI and FII enzymes remained relatively constant throughout the 18 month sampling period with two exceptions; muscle FI increased significantly just before the peak growth period then significantly fell (Fig. III-3), FII enzymes also rose before the peak growth period but remained constant as FI enzymes fell (Fig. III-4). The increase in FI enzymes, including LDH supports previous work that demonstrated a strong correlation between LDH, activities and growth rates in cod (Martínez *et al.*, 1999). The period January to June 2002 represents a slow growth period for cod (Levesque *et al.*, 2005a; Chapter II) and a period when the gonadal somatic index (GSI) is significantly increasing. A decrease in muscle FI enzymes (PK, LDH, CS) would reduce oxidation of carbon while high activities of some FII enzymes (in particular ALT, AST) would enhance muscle protein breakdown, ultimately providing additional synthetic carbon for gonadal anabolism. Certainly muscle FI is also correlated with both HSI and total liver glycogen (Table III-4) which may also be important for increases in GSI. Muscle FIII enzymes (GDH, MDH) are negatively correlated with body mass and length (Table III-4), as would be expected by the sharp

decline in activities at a time when cod are actively growing. The precise reason for this pattern is still under investigation.

A major conclusion from this study is that temperature has little impact on the activities of the assayed enzymes. With one exception (muscle FIII, last two sampling points; Fig. III-5), there were no activity differences between holding the cod at a constant (~9 °C) compared with a variable (ambient) temperature regime. Although temperature has a direct impact on enzyme activities (Hazel and Prosser, 1974), it is clear from this study that activities when measured at the same temperature are unaffected by water temperatures suggesting that the activities measured here are thermally compensated (Seddon and Prosser, 1997) to facilitate optimal performance at all temperatures (Guderley *et al.*, 2001). This appears true for both mitochondrial and cytosolic enzymes. This implies that the pattern of enzyme activities shown here is responding to other conditions that are changing over the sample period.

Growth in length necessarily involves the synthesis of structural molecules, while growth in mass can include restoration and accumulation of energy reserves (Couture *et al.*, 1998). Growth in length appears to dominate in young fish, while growth in mass become relatively important as fish reach adulthood (Ricker, 1973). Growth in length is also more permanent than growth in mass, since food deprivation leads to loss of body mass (Couture *et al.*, 1998). In our study, the regressions determined for body mass and length are very similar (Table III-5). Both liver FI and FII, HSI and total liver protein explain the most mass and length variation in the cod studied. Allometries of white muscle glycolytic enzymes are not always found depending on size, reproductive status and season of sampling (Martínez *et al.*, 1999) and allometry of enzyme activities changes during an organism's life. For example, Martínez *et al.* (1999) reported a higher allometric

relationship in Newfoundland cod in fall and early winter. Our data indicate that lipid catabolism is enhanced during the period of enhanced growth that occurred from August 2001 to October 2001 (Levesque *et al.*, 2005a; Chapter II) and when photoperiod is increasing, as previously reported by Jones and Sidell (1982). None of the muscle factors are included in the mass and length multiple regressions, although MFIII is significantly correlated with fish mass and length. This finding is contrary to previous research which reported a positive correlation between the activity of muscle CS (Mathers *et al.*, 1992; Pelletier *et al.*, 1993a) and glycolytic enzymes (Somero and Childress, 1980; Sullivan and Somero, 1983, Pelletier *et al.*, 1993a; 1994; Dutil *et al.*, 1998) with growth rate. In our study, growth in mass and in length were mainly explained by total liver protein content and HSI. HSI plays an important role in fish mass regression but this may be an artifact as organ mass and fish mass are always highly correlated (Pelletier *et al.*, 1994).

In conclusion, the pattern of enzyme activities studied here generally reflect changes in organ mass and growth rates, or the physiological changes occurring within the cod over the sampling period. There are few direct effects of the thermal regime at which the cod were held. As we previously noted (Levesque *et al.*, 2005a; Chapter II), photoperiod is a prime driver of cod physiology, with the temperature regime having a limited quantitative impact on the outcome. Examining enzymes does not assist our understanding of the differences noted in growth rates between the constant and variable temperature groups. The PCA technique is effective in studies of this kind containing multiple variables that may affect distinct processes. Unfortunately this technique did not provide additional information correlating enzymes activities to changes in specific metabolites. What it does suggest, however is that a limited number of patterns of enzyme variation exist and what factor(s) may generate these patterns. This study plus the results in Chapter II permit the

integration of changes in morphometry, metabolites and enzymes activities in young cod throughout the year. This is the first time that so many physiological and morphological factors were studied in cod for 18 month and were integrated together. Given the low temperature encountered during the winter in Atlantic Canadian waters, it is important to understand the interactions among temperature, growth and intermediary metabolism, if growth is to be optimized in cultivated Atlantic cod.

Table III-1: Enzyme activities (mean ± standard error) in the liver of Atlantic cod (*Gadus morhua*) from December 2000 to February 2002. Liver cytochrome C oxidase (CCO) and liver malate dehydrogenase (MDH) activities are shown in Fig. III-1 and 2, respectively and are not presented here. Data are expressed as $\mu mol \cdot min^{-1}$ per whole liver, except for *, which is $mmol.min^{-1}$ per whole liver; n-values between 6 and 10. Enzymes were assessed at 22 °C or 10 °C (see Materials and Methods). CST, Atlantic cod held at constant temperature (~9 °C); ABT, Atlantic cod held at ambient temperature. Phosphoenolpyruvate carboxykinase (PEPCK), alanine aminotransferase (ALT), aspartate aminotransferase (AST), pyruvate kinase (PK), lactate dehydrogenase (LDH), glutamate dehydrogenase (GDH), citrate synthase (CS), isocitrate dehydrogenase (IDH), glucose-6-phosphate dehydrogenase (G6PDH), 3-hydroxyacyl CoA dehydrogenase (HOAD), carnitine palmitoyl transferases (CPT), malic enzyme (ME).

Liver enzymes		Dec. 2000		Feb. 2001		May 2001		July 2001	
		CST	ABT	CST	ABT	CST	ABT	CST	ABT
gluconeogenic	PEPCK	15.44±4.86	15.92±4.85	11.37±4.85	10.59±5.11	8.91±4.85	2.35±4.85	16.66±5.12	8.93±5.12
glycolytic	LDH*	1.47±0.85	1.07±0.85	0.88±0.85	0.62±0.89	1.77±0.85	0.68±0.85	1.36±0.95	1.03±0.89
	PK	100.95±28.98	98.73±28.98	75.49±28.98	72.69±30.55	71.40±28.98	35.67±28.98	84.39±30.55	58.76±28.98
amino acid metabolism	ALT	171.05±53.55	177.82±53.55	77.43±56.45	91.56±53.55	53.82±53.55	24.77±53.55	154.7±56.45	75.12±53.55
	AST	123.64±29.22	152.66±29.22	83.08±29.22	67.87±30.80	49.78±29.22	11.65±29.22	108.55±30.80	67.2429.22
oxidative metabolism	CS	16.61±3.91	16.91±3.91	9.82±4.13	7.79±4.12	8.27±3.91	5.36±3.91	13.64±3.91	9.92±3.91
	GDH	84.38±24.74	98.04±24.74	59.37±24.74	48.19±26.07	81.25±24.74	36.66±24.74	96.21±26.07	58.04±24.73
	IDH	42.91±13.97	27.17±13.96	29.50±13.97	22.89±14.72	38.66±13.97	16±59±13.96	43.3±14.72	28.75±13.97
NADPH producing	G6PDH	46.51±13.20	50.05±13.92	31.19±13.20	25.51±13.91	29.79±14.76	7.01±14.76	47.07±13.91	28.84±13.20
lipid metabolism	CPT	5.59±7.79	8.50±7.79	11.96±8.33	10.41±7.79	17.64±7.79	2.88±7.79	34.58±8.33	26.30±7.79
	HOAD	6.70±5.99	5.99±5.99	15.63±5.99	14.64±5.99	22.63±6.40	5.22±6.40	34.17±6.92	22.73±6.40
Liver enzymes		Aug. 2001		Oct. 2001		Feb. 2002		June 2002	
		CST	ABT	CST	ABT	CST	ABT	CST	ABT
gluconeogenic	PEPCK	15.37±5.43	9.73±5.43	44.87±4.85	27.52±4.85	46.67±5.12	13.14±4.85	52.87±4.86	41.99±4.85
glycolytic	LDH*	0.87±0.95	0.48±0.95	8.51±0.85	4.16±0.85	8.89±0.85	8.66±0.85	7.74±0.85	7.09±0.85
	PK	101.81±32.40	54.39±32.40	197.61±28.98	132.63±28.98	287.03±28.98	219.65±28.98	347.36±28.98	383.99±28.99
amino acid metabolism	ALT	129.21±69.13	71.57±59.87	324.48±53.55	226.28±53.55	618.81±53.5	336.14±53.55	729.81±53.55	488.39±53.55
	AST	109.68±32.67	60.04±32.67	235.07±29.22	169.72±29.22	407.25±29.22	288.85±29.22	353.22±29.22	306.98±29.22
oxidative metabolism	CS	16.24±4.38	8.22±4.37	40.73±3.91	21.42±3.91	30.28±3.91	25.02±3.91	78.37±3.91	73.46±3.91
	GDH	116.42±27.65	47.11±27.66	187.20±24.74	132.86±24.74	240.52±24.74	149.05±24.74	244.07±24.74	169.26±24.74
	IDH	45.30±15.61	25.97±15.61	90.44±13.97	57.08±13.96	123.38±13.97	88.2±13.97	104.85±13.97	95.62±13.97
NADPH producing	G6PDH	55.10±14.76	29.74±14.76	118.14±13.20	68.74±13.20	167.82±13.20	117.41±13.20	98.90±13.20	115.18±13.20
lipid metabolism	CPT	7.79±7.79	10.71±7.79	24.61±8.33	9.15±8.33	45.82±7.79	45.17±7.79	82.02±7.79	54.89±7.79
	HOAD	5.99±5.99	12.02±5.99	40.19±5.99	23.61±5.99	50.26±5.99	68.81±5.99	77.09±5.99	68.80±5.99

Table III-2: Loading values (contribution) of liver enzymes for the two liver Factors (principal components) and of muscle enzymes for the three muscle Factors (principal components). The closer the loading value is to 1 the higher is the contribution of the enzyme to the Factor. See Table III-1 for the complete name of the enzymes.

	Liver		Muscle		
	Factor I	Factor II	Factor I	Factor II	Factor III
PEPCK	0.71618	0.31118			
ALT	0.80225	0.25434	-0.484	0.753	-0.216
AST	0.68205	0.41512	0.345	0.708	-0.027
PK	0.85029	0.19487	0.923	0.061	0.008
LDH	0.48261	0.60778	0.868	0.049	-0.285
GDH	0.6665	0.38809	-0.02	0.079	0.778
MDH	-0.0748	0.92921	-0.126	-0.041	0.75
CS	0.93318	-0.07226	0.934	0.044	0.042
ME			0.12	0.71	0.168
IDH	0.62387	0.46031	0.332	0.604	0.33
G6PDH	0.47181	0.61771			
CCO	0.96026	-0.05287			
HOAD	0.98699	-0.17138			
CPT	0.99019	-0.19484			

Table III-3: Enzyme activities (mean ± standard error) in the muscle of Atlantic cod (*Gadus morhua*) from December 2000 to February 2002. Muscle citrate synthase (CS), alanine aminotransferase (ALT) and malate dehydrogenase (MDH) activities are shown in Fig. III-3, III-4 and III-5, respectively and are not presented here. Data are expressed as $\mu mol \cdot min^{-1} \cdot g^{-1}$ of tissue; n-values between 6 and 10. Enzymes were assessed at 20 °C (see Materials and Methods). CST, Atlantic cod held at constant temperature (~9 °C); ABT, Atlantic cod held at ambient temperature. See Table III-1 for the complete name of the enzymes.

Muscle enzymes		Dec. 2000		Feb. 2001		May 2001		July 2001	
		CST	ABT	CST	ABT	CST	ABT	CST	ABT
glycolytic	PK	14.75±1.77	11.51±1.77	10.46±1.88	10.14±1.68	15.48±1.88	13.57±1.68	10.10±1.68	8.08±1.68
	LDH	209.1±18.9	192.5±18.9	99.2±20.0	88.7±17.9	142.7±17.9	136.0±17.9	137.4±17.9	103.04±17.9
amino acid metabolism	AST	1.13±0.10	1.02±0.10	1.05±0.11	1.18±0.09	0.97±0.09	0.83±0.09	1.02±0.09	0.76±0.09
oxidative metabolism	GDH	0.81±0.11	0.67±0.11	0.90±0.11	0.91±0.10	0.95±0.10	0.79±0.10	0.58±0.10	0.72±0.10
	IDH	4.61±0.31	4.21±0.31	3.09±0.33	3.48±0.29	3.08±0.29	2.99±0.29	2.99±0.29	2.97±0.29
	ME	0.46±0.03	0.39±0.04	0.39±0.04	0.40±0.03	0.46±0.03	0.46±0.03	0.35±0.03	0.34±0.03
Muscle enzymes		Aug. 2001		Oct. 2001		Feb. 2002		June 2002	
		CST	ABT	CST	ABT	CST	ABT	CST	ABT
glycolytic	PK	13.95±1.88	13.61±1.88	22.75±1.68	14.16±1.68	23.11±1.68	23.41±1.68	4.73±1.68	4.46±1.68
	LDH	254.4±20.0	231.9±20.0	378.2±17.9	400.5±17.9	297.9±17.9	278.0±17.9	52.1±17.9	48.9±17.9
amino acid metabolism	AST	1.02±0.11	0.93±0.11	0.95±0.09	0.93±0.09	0.85±0.09	0.75±0.09	1.87±0.09	1.25±0.09
oxidative metabolism	GDH	0.43±0.11	0.59±0.12	0.21±0.10	0.45±0.10	1.08±0.10	0.68±0.10	0.39±0.10	0.67±0.10
	IDH	3.01±0.33	3.16±0.33	3.72±0.29	3.17±0.29	4.56±0.29	4.13±0.29	2.67±0.29	3.43±0.29
	ME	0.42±0.04	0.38±0.04	0.39±0.03	0.41±0.04	0.64±0.03	0.48±0.03	0.49±0.03	0.42±0.03

Table III-4: Correlations between liver and muscle enzyme Factors and morphometric parameters and tissue metabolites in Atlantic cod.*, $p<0.05$ (Bonferroni probabilities); **,$p<0.001$ (Bonferroni probabilities). LF represent liver Factor and MF, muscle Factor.

Variables	LF I	LF II	MF I	MFII	MFIII
Mass	0.70414**	-0.03194	0.02118	0.22721	-0.42894*
Length	0.68407**	-0.03575	0.08846	0.22859	-0.43218*
Liver mass	0.64573**	0.13333	0.2416	0.30186	-0.28833
HSI	0.20542	0.33036	0.35624**	0.11631	0.07378
GSI	0.43315*	-0.25661	-0.30738	-0.00751	-0.28085
CF	0.46837**	0.13158	0.08803	0.1563	-0.30919
Muscle protein ($mg.g^{-1}$)	-0.31382	-0.05371	0.01867	-0.05547	0.25129
Liver Protein ($mg.g^{-1}$)	-0.36568	0.01281	0.03988	-0.10824	-0.04985
Total liver protein	0.58587**	0.18995	0.34454	0.27977	0.41081
Muscle glycogen ($mg.g^{-1}$)	-0.05854	0.00246	0.12935	0.03793	-0.16342
Liver glycogen ($mg.g^{-1}$)	-0.40435	0.12668	0.22877	-0.15916	-0.09645
Total liver glycogen	0.14223	0.37325	0.53031**	0.23143	-0.28020
glucose (mM)	-0.25548	0.16984	0.15556	-0.00695	-0.05169
lactate (mM)	-0.30874	0.12293	0.05191	-0.0902	0.23418
Tiglyceride	0.11784	0.29715	0.42638	0.22422	-0.21371
Total lipids	0.09412	0.32062	0.42578	0.16194	-0.18416
LF1			-0.0763	0.26292	-0.34535
LF2			0.49780**	0.01834	0.01543

Table III-5: Multiple regression models utilizing the variables that best explain the variability of mass, length and liver mass in Atlantic cod. N, number of cod. R^2, regression factor.

Variable	Mass	contribution	probability	Length	contribution	probability	Liver mass	contribution	probability
N	105			105			105		
R^2	0.866			0.77			0.927		
Intercept	71.9		p<0.001	31.54		p<0.001	-12.22	.	p<0.001
LFI	106.67	11.7%	p<0.001	2.56	11.2%	p<0.001	4.09	1.5%	p<0.001
LFII	-56.18	3.0%	p<0.001	-1.12	2.6%	p<0.001	-2.06	1.2%	p<0.001
CF	37968.78	4.0%	p<0.001						p<0.001
HSI	-5413.31	7.0%	p<0.001	-86.93	6.5%	p<0.001			
Total liver glycogen							9.74	1.0%	p<0.001
Total liver Protein	247.38	60.7%	p<0.001	6.68	57.2%	p<0.001	18.11	89.0%	p<0.001

Figure III-1: (A) General liver Factor I pattern; (B) Liver cytochrome C oxidase (CCO) ($\mu mol \cdot min^{-1}$ per whole liver). Each point represents the mean ± standard error; n-values are between 6 and 8 (○, represents constant temperature cod (~ 9 °C); ●, represents ambient temperature cod; sampling dates noted by a ▼). Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) within the constant temperature group (upper case letters) and within the ambient temperature group (lower case letters).

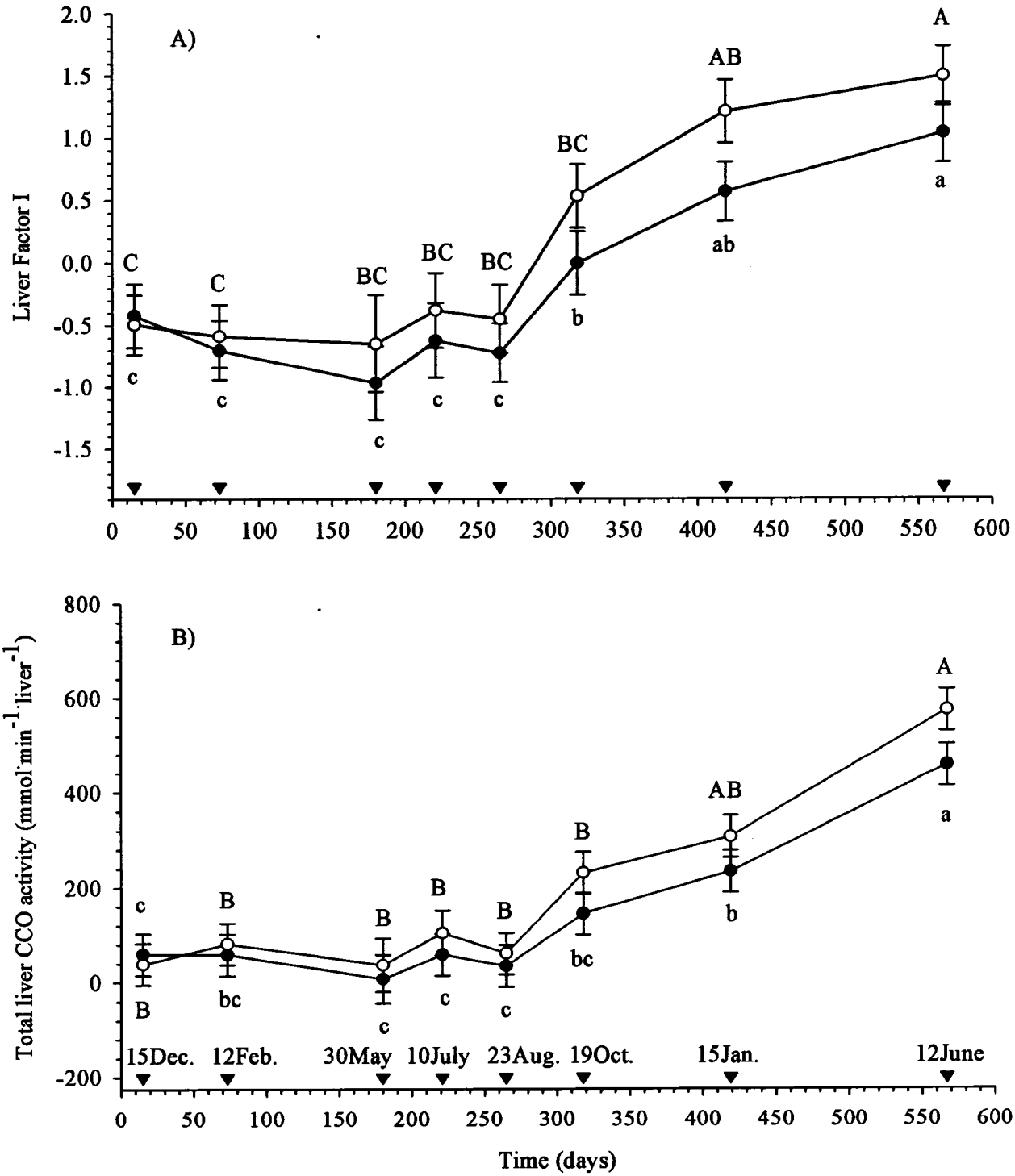
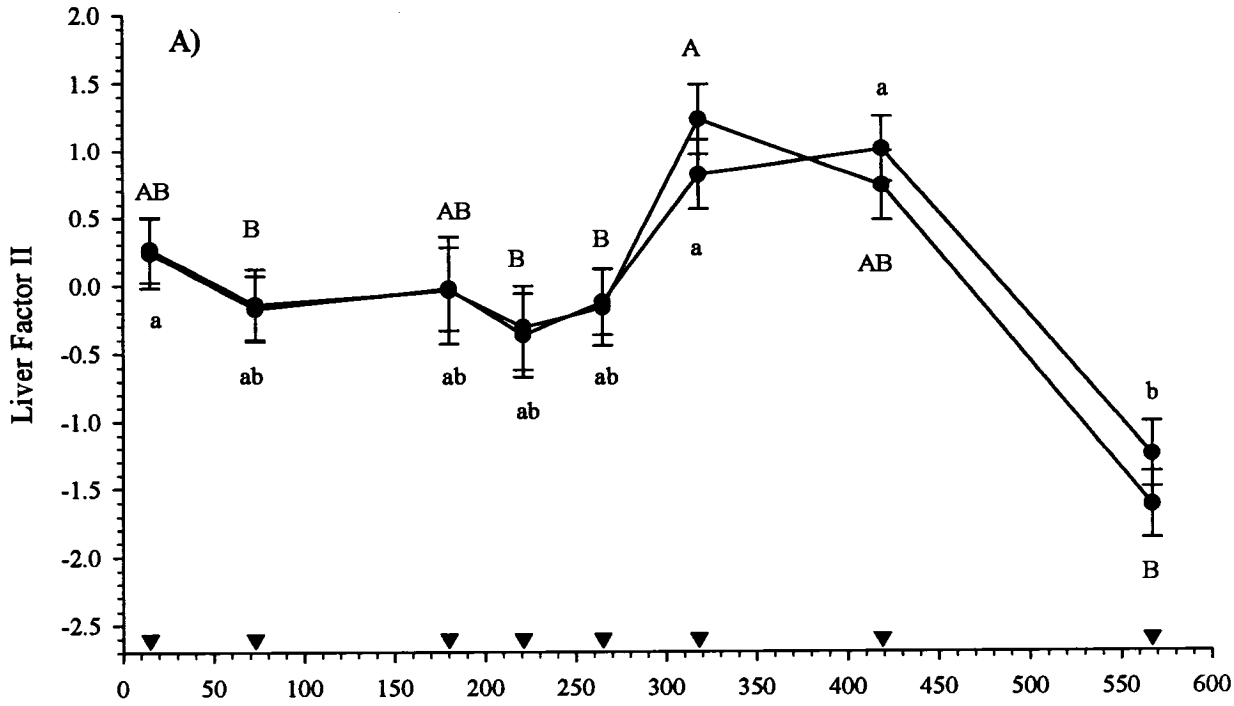

A)
Liver Factor I
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
C
C
BC
BC
BC
BC
AB
A
c
c
c
c
c
b
ab
a
0
50
100
150
200
250
300
350
400
450
500
550
600
B)
Total liver CCO activity (mmol·min-1·liver-1)
800
600
400
200
0
-200
c
B
B
B
B
B
AB
A
B
bc
c
c
c
bc
b
a
15Dec.
12Feb.
30May
10July
23Aug.
19Oct.
15Jan.
12June
Time (days)

Figure III-2: (A) General liver Factor II pattern; (B) Liver malate dehydrogenase (MDH) activities (μmol·min-1 per whole liver). Each point represents the mean ± standard error; n-values are between 6 and 8 (○, represents constant temperature cod (~ 9 °C); ●, represents ambient temperature cod; sampling dates noted by a ▼). Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) within the constant temperature group (upper case letters) and within the ambient temperature group (lower case letters).


A)
Liver Factor II
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
AB
B
AB
B
B
A
a
AB
B
a
ab
ab
ab
ab
b
B
0
50
100
150
200
250
300
350
400
450
500
550
600

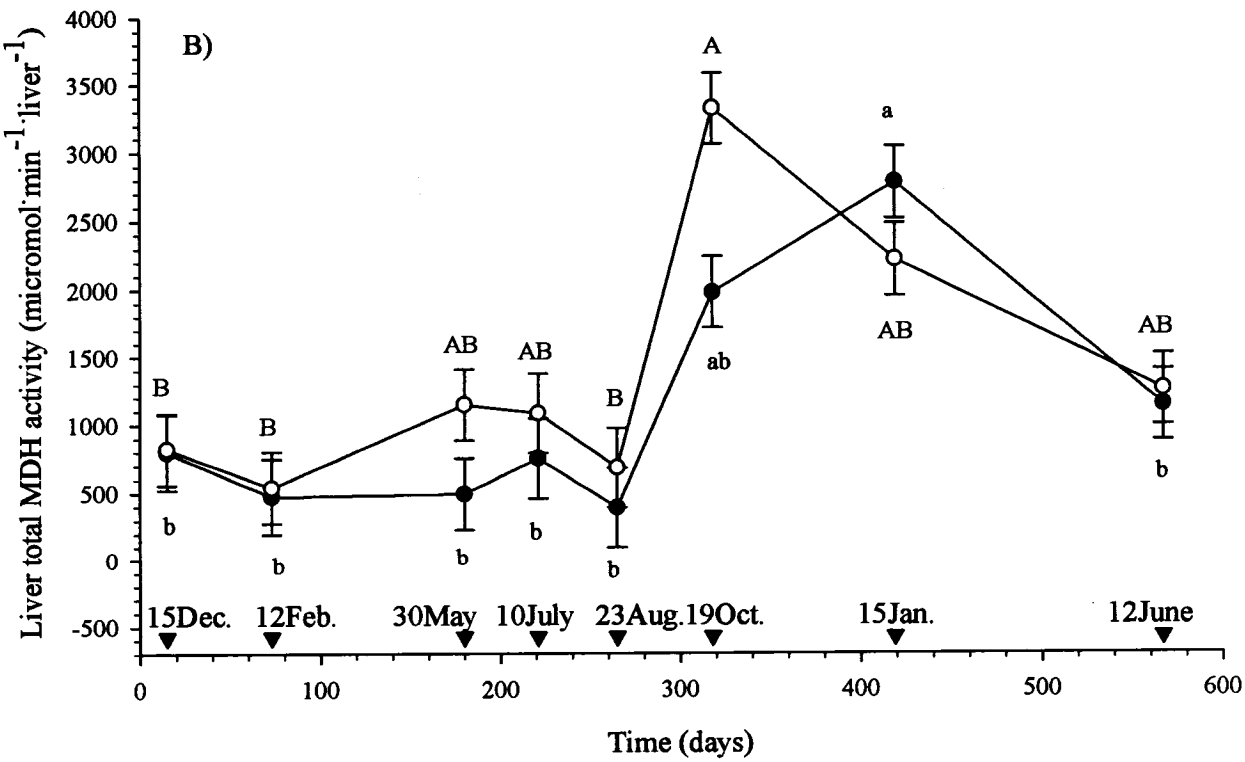

B)
Liver total MDH activity (micromol·min-1·liver-1)
4000
3500
3000
2500
2000
1500
1000
500
0
-500
B
B
AB
AB
B
A
a
AB
AB
b
b
b
b
b
ab
b
15Dec.
12Feb.
30May
10July
23Aug.
19Oct.
15Jan.
12June
0
100
200
300
400
500
600
Time (days)

Figure III-3: (A) General muscle Factor I pattern; (B) Muscle citrate synthase (CS) activities ($\mu mol \cdot min^{-1} \cdot g^{-1}$ tissue). Each point represents the mean ± standard error; n-values are between 6 and 8 (○, represents constant temperature cod (~ 9 °C); ●, represents ambient temperature cod; sampling dates noted by a ▼). Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) within the constant temperature group (upper case letters) and within the ambient temperature group (lower case letters).

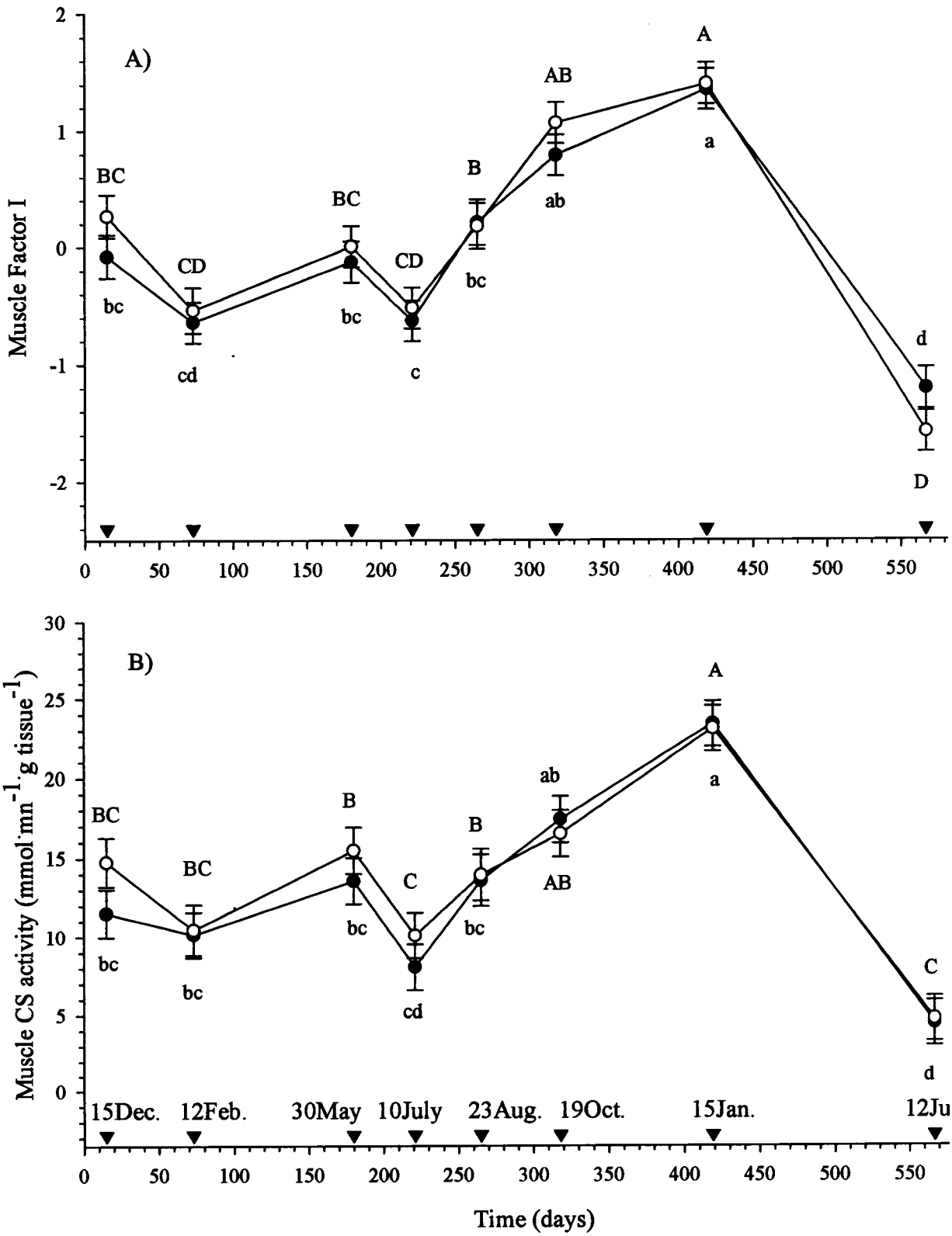

A)
Muscle Factor I
2
1
0
-1
-2
BC
CD
BC
CD
B
AB
A
BC
CD
BC
B
AB
A
bc
cd
bc
c
bc
ab
a
d
D
0
50
100
150
200
250
300
350
400
450
500
550
B)
Muscle CS activity (mmol·mn-1·g tissue-1)
30
25
20
15
10
5
0
BC
BC
B
C
B
ab
A
BC
BC
B
C
B
AB
A
bc
bc
bc
cd
bc
AB
a
C
d
15Dec.
12Feb.
30May
10July
23Aug.
19Oct.
15Jan.
12Ju
0
50
100
150
200
250
300
350
400
450
500
550
Time (days)

Figure III-4: (A) General muscle Factor II pattern; (B) muscle alanine aminotransferase (ALT) activities ($\mu mol \cdot min^{-1} \cdot g^{-1}$ tissue). Each point represents the mean ± standard error; n-values are between 6 and 8 (○, represents constant temperature cod (~ 9 °C); ●, represents ambient temperature cod; sampling dates noted by a ▼). Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) within the constant temperature group (upper case letters) and within the ambient temperature group (lower case letters).

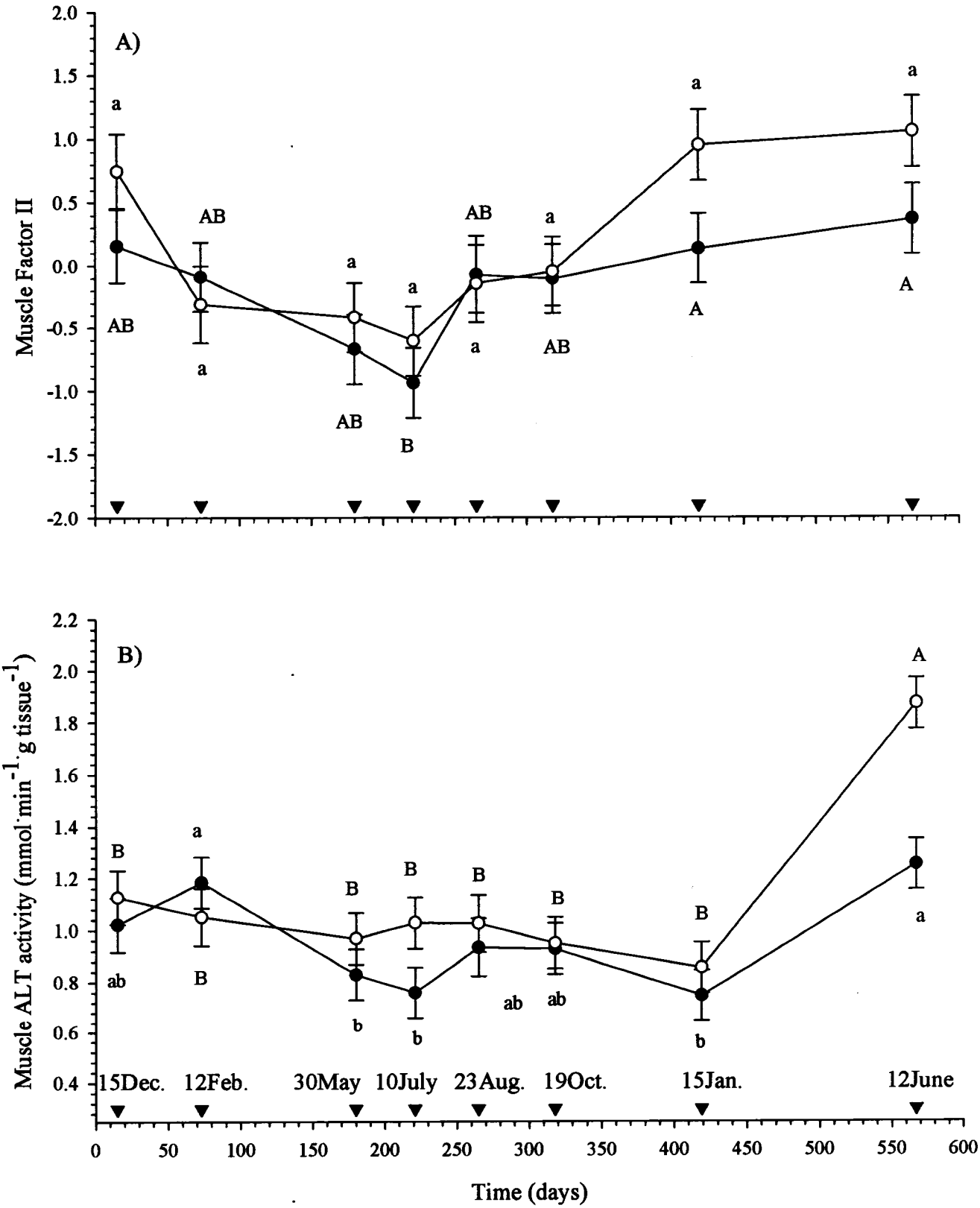
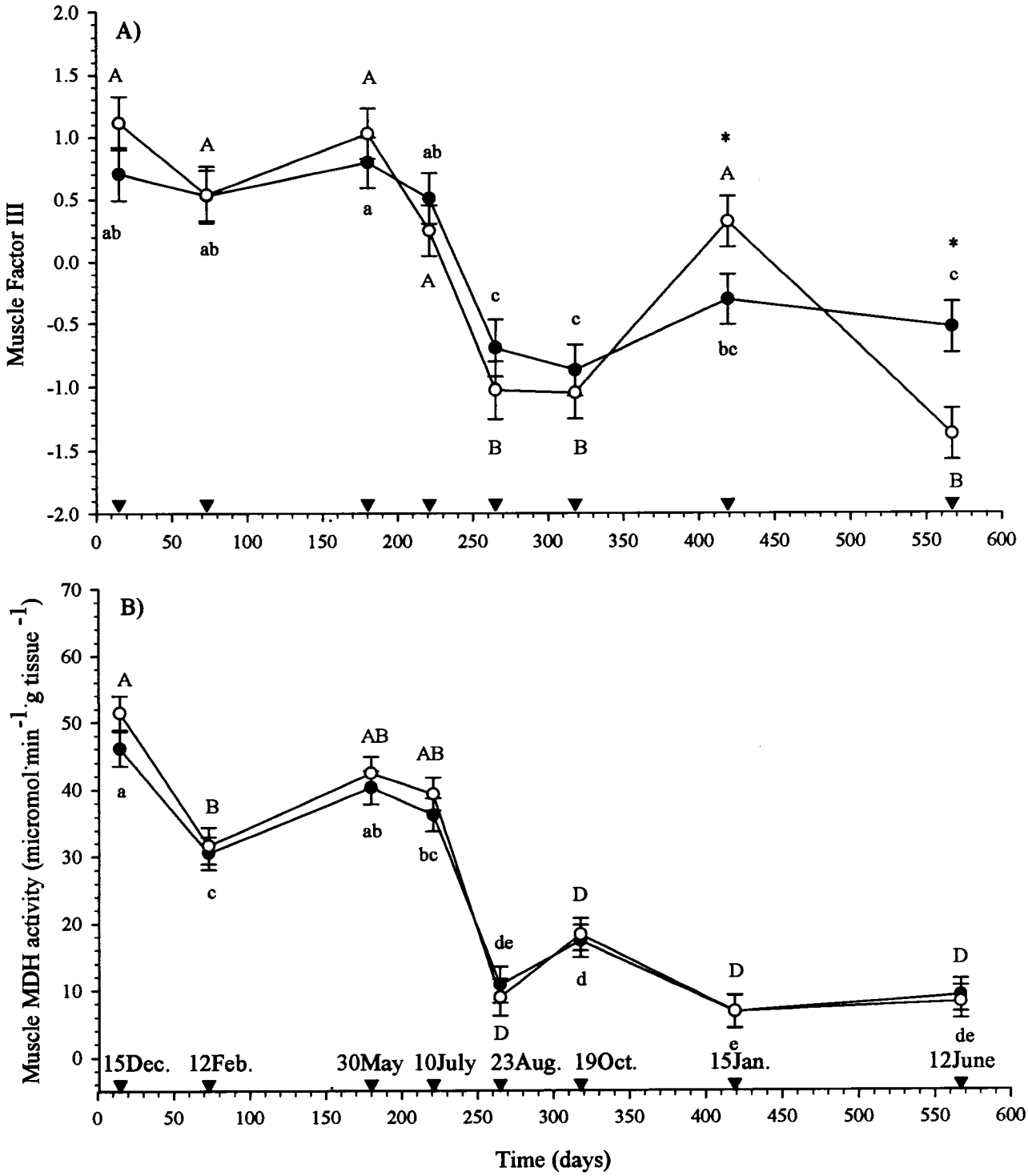

A)
Muscle Factor II
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
a
AB
a
AB
a
a
AB
a
a
AB
a
A
a
A
B)
Muscle ALT activity (mmol·min-1·g tissue-1)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
B
a
B
B
B
B
B
B
B
A
ab
B
b
b
ab
ab
b
a
15Dec.
12Feb.
30May
10July
23Aug.
19Oct.
15Jan.
12June
0
50
100
150
200
250
300
350
400
450
500
550
600
Time (days)

Figure III-5: (A) General muscle Factor III pattern; (B) Muscle malate dehydrogenase (MDH) activities ($\mu mol \cdot min^{-1} \cdot g^{-1}$ tissue). Each point represents the mean ± standard error; n-values are between 6 and 8 (○, represents constant temperature cod (~ 9 °C); ●, represents ambient temperature cod; sampling dates noted by a ▼). *, Differences between ambient and constant temperature groups at a particular sampling time (Tukey-Kramer, $\alpha < 0.05$). Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) within the constant temperature group (upper case letters) and within the ambient temperature group (lower case letters).


A)
Muscle Factor III
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
A
A
A
ab
ab
a
ab
A
c
c
*
A
*
c
ab
bc
B
B
B
0
50
100
150
200
250
300
350
400
450
500
550
600
B)
Muscle MDH activity (micromol·min-1·g tissue-1)
70
60
50
40
30
20
10
0
A
AB
AB
B
a
ab
bc
c
D
de
D
D
d
D
e
de
15Dec.
12Feb.
30May
10July
23Aug.
19Oct.
15Jan.
12June
0
50
100
150
200
250
300
350
400
450
500
550
600
Time (days)

CHAPTER IV: MYOGENESIS AND TISSUE METABOLISM IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) ACCLIMATED TO 5 AND 17 ^{o}C

Abstract

Rainbow trout (*Oncorhynchus mykiss*) were acclimated to 5 and 17 °C. Metabolic enzymes were measured in liver, white and red muscles, and the expression of myogenin and MyoD mRNA were measured in the two skeletal muscle types. Thermal acclimation significantly modified metabolic and myogenic organisation of white and red muscles. LDH was decreased in liver, white and red muscles and amino acid metabolism was decreased while oxidative metabolism was increased in both muscles of 5 compared with 17 °C acclimated trout. There was no difference in the proliferation of myosatellite cells extracted from white muscle of 5 or 17 °C exposed fish, except at day 4 when proliferation was higher in cells from the 17 °C acclimated fish. Relative myogenin and MyoD I expressions were decreased in white muscle while myogenin decreased and MyoD I increased in red muscle at low temperature. When compared to the previous experiments with cod (*Gadus morhua*), this study reveals that acclimation to a constant temperature and photoperiod have different effects on fish metabolic enzymes than acclimatization to natural temperature and/or photoperiod. However, this study shows that myogenic factors are affected by water temperature but that metabolic enzymes and myogenic factors are regulated differently in skeletal muscle tissue, even though both are influenced by temperature.

Introduction

In Chapter II and III, I demonstrated that growth rates of Atlantic cod (*Gadus morhua*) held at ambient water temperatures (-1 to 16 °C) were significantly depressed compared with cod held at a constant ~9 °C temperature (see also Levesque *et al.*, 2005a,b). This same thermal regime had minor effects on tissue metabolic enzymes. Myogenic factors are known to be key to somatic growth but the cod experiment did not allow for the estimation of whether myogenic factor expression was implicated in the different growth responses. This experiment exposed rainbow trout (*Oncorhynchus mykiss*) to two temperatures within their normal thermal range in order to determine the role of myogenic factors in temperature-mediated changes in somatic growth.

Temperature is a crucial environmental factor setting the limits for life and influencing the activity of organisms including growth, reproduction, feeding, assimilation, transformation, excretion and reproduction (Brander, 1995). Temperature has also a direct effect on muscle fiber recruitment patterns, hormone levels and metabolism (Johnston and Wokoma, 1986).

Seasonal effects on the morphology and physiology of muscle tissue are mainly connected with changes in environmental temperature. Low temperatures generally depress metabolic rates, however many poikilotherms, including fish, have the ability to adjust the rates of biological functions through acclimatory changes resulting in the compensation of enzyme activities and metabolic rates in cold exposed animals (Crockett and Sidell, 1990; Sokolova and Portner, 2001; Kawall *et al.*, 2002; Torres and Somero, 2005). Previous studies in fish exposed to low temperature report an increase in mitochondrial densities (Egginton and Sidell, 1989; Battersby and Moyes, 1998; Johnston *et al.*, 1998,) and in

organ size including the liver (Kent *et al.*, 1988; Seddon and Prosser, 1997) and heart (Kent *et al.*, 1988). An increase in mitochondrial enzyme activities in rainbow trout (Battersby and Moyes, 1998), cod (Lannig *et al.*, 2003) and flounder (*Platichthys flesus*), and an increase in flounder aerobic glycolysis and fatty acid oxidation (Johnston and Wokoma, 1986; Guderley and Gawlicka, 1992) was reported in skeletal muscle in cold acclimated compared with warm acclimated fish. An increase in liver enzyme activities in channel catfish exposed to low temperature was also reported (Kent *et al.*, 1988).

Muscle growth is indeterminate in many fish species and myogenic factors (MRFs) are responsible for determination, proliferation and differentiation of muscle precursor cells or myosatellite cells (MyC) into muscle fiber. Muscle growth can happen by an increase in fiber number (hyperplasia) or an increase in the size of existing fibers through the addition of new MyC nuclei into existing fibers (hypertrophy) (Watabe, 2001; Chapter I). An increase in the number of muscle fibers is interesting from an aquaculture point of view, as white muscle fibers have the ability to reach a maximum diameter of 250 μm at least in Atlantic salmon (Johnston, 2001), so the more numerous the muscle fibers are the larger the fish can grow (Stoiber *et al.*, 2002). MRFs are transcription factors containing a basic helix-loop-helix motif (bHLH) (see Chapter I). They include Myf-5, MyoD, myogenin and MRF4 (Rudnicki and Jaenisch, 1995). The MRFs have been implicated not only in the differentiation of muscle precursor cells into muscle cells in many vertebrates but also in the determination of muscle phenotype. According to Hughes *et al.* (1993), MyoD accumulates in fast muscle fibers while myogenin accumulates in slow muscle fibers in mammals. Skeletal muscle is composed primarily of two muscle types, red or slow fibers and white or fast fibers. White skeletal muscle constitutes about half of the total fish mass and 65 to 75% of white muscle protein synthesis is retained as growth (Houlihan *et al.*,

1988). Temperature is known to have major effects on early muscle development in teleosts, altering the timing of myofibril assembly with respect to the somite stage (Atlantic herring, Johnston *et al.*, 1995), as well as the number and size of embryonic muscle fibers in numerous species (*Salmo salar*, Stickland *et al.*, 1988; *Clupea harengus*, Vieira and Johnston, 1992; *Pleuronectes platessa*, Brooks and Johnston, 1993; *Oncorhynchus mykiss*, Matschak *et al.*, 1998; *Gadus morhua*, Galloway *et al.*, 1999). It was shown that MRFs expression was delayed and prolonged at low compared with high egg incubation temperatures in rainbow trout (Xie *et al.*, 2001) and that lower temperatures increase salmonid fiber numbers and their diameter (Johnston and Mclay, 1997) and produce larger alevins (term used to describe yolk-bearing larvae that transform into juveniles, as in salmonids); these differences, however disappear after first feeding. One day old cod larvae hatch at a shorter length at 1 °C and had more but smaller diameter white muscle fibers than those hatched at 5 and 8 °C (Galloway *et al.*, 1999). Again, these differences between temperature groups disappear in 5 day old larvae. A 5 °C difference in acclimation temperature is enough to significantly alter the proportion and distribution of muscle fibers in turbot (*Scophthalmus maximus*, Calvo and Johnston, 1992). In many fish species, the acclimatory change to low temperature seems to involve a switch in myosin gene expression resulting in the appearance of different myosin isoforms (Gerlach *et al.*, 1990; Goldspink *et al.*, 1992; Hirayama and Watabe, 1997).

Most studies on the effects of temperature on fish myogenic factors are undertaken in embryos or larvae and in many cases differences disappear with age after hatching, but for aquaculture it is important to increase the number of fibers in fish muscle during growth. However, few studies report the effects of temperature acclimation on juvenile fish muscle growth. Kobiyama *et al.* (2000) reported that acclimation of adult carp (*Cyprinus*

carpio L.) from 20 to 30 °C significantly decreased myogenin expression in white muscle but did not affect MyoD expression levels.

Several hormones and in particular growth hormone (GH) regulate development and somatic and gonadal growth in fish. Temperature does influence the concentration of plasma GH (Marchant and Peter, 1986; Gabillard *et al.*, 2003) and so GH could mediate the temperature effects noted on fish growth. In another study, I demonstrate an increased proliferation of Atlantic salmon white muscle MyC exposed *in vitro* to GH (see Chapter V). In this study, rainbow trout MyC extracted from untreated fish are exposed to specific growth factors (IGF-I, FGFb and HGF) and β_2-adrenergic agonists (β_2-AA) (clenbuterol and ractopamine). IGF-I expression in white muscle is related to GH and IGF-I does increase myogenesis in mammals (Florini *et al.*, 1996) and MyC proliferation in rainbow trout (Castillo *et al.*, 2004). It is also demonstrated in Chapter VI that proliferation of MyC extracted from β-AA exposed trout increases. FGFb and HGF are shown to increase MyC proliferation in mammals (Sheehan and Allen, 1999) and FGFb increases MyC differentiation in channel catfish at least by visual counting of myotubes (Cyrino and Mulvaney, 1999).

The hypothesis of Chapter IV is that temperature has a direct effect on muscle myogenesis and metabolism. The predictions are that myogenic factors and satellite cells proliferation will decrease in rainbow trout exposed to low temperature and metabolic enzymes will increase in low temperature due to compensation. The aim of this study is to investigate the effect of temperature on the relative expression of muscle MRFs (myogenin and MyoD) and MyC proliferation, and to relate these to differences in metabolic enzymes in juvenile rainbow trout. In addition I investigate the *in vitro* effects of some growth factors and β-AA on these processes.

Material and Methods

Experimental animals and procedures

Juvenile rainbow trout were obtained from Linwood Acres Trout Farm (Campbellcroft, ON). Fish were received at the University of Ottawa Aquatic Care Facility and placed in small 110-115 L fibreglass stock tanks supplied with flowing, aerated, and dechloraminated City of Ottawa tap water at a temperature of 13 ± 1°C. Fish were divided into two groups of 100 fish per tank. Two weeks following their arrival, water temperatures were slowly changed to the desired temperature at a rate of 1 °C per day. A 12L:12D photoperiod was used and a temperature of either 5 (low temperature group) or 17 (high temperature group) °C was maintained for a 16 week period. These two temperatures were choosen as they are within the normal range of temperature encountered by rainbow trout in their natural anvironment and they are the widest range we could obtainable in our aquatic facilities. Throughout this period, fish were hand-fed every day 2% body weight of a commercial diet (Purina Trout Chow) containing 41% crude protein (minimum), 11% fat (minimum), 3.5% crude fibre (maximum), 1% calcium (actual), 0.85% phosphorus (actual), 0.45% sodium (actual), 6800 $IU \cdot kg^{-1}$ vitamin A (minimum), 2100 $IU \cdot kg^{-1}$ vitamin D (minimum), 80 $IU \cdot kg^{-1}$ vitamin E (minimum), and 200 $IU \cdot kg^{-1}$ vitamin C (minimum). Fish weights were assessed throughout the feeding period from which specific growth rates (SGR) were calculated based upon the formula: $[(\ln W_T - \ln W_t) \cdot (T - t)^{-1}] \times 100$, where W_T and W_t are fish masses at time T (end time) and time t (initial). At the end of the exposure period, 10 fish per group were sacrificed to collect samples of blood, liver, white and red muscles for metabolic enzymes. Small pieces of white and red muscle tissues were

collected for RNA extraction. The remaining muscle was used for myosatellite cell (MyC) extraction to characterize *in vitro* proliferation of cells extracted from low and high temperature acclimated fish. mRNA was also extracted from cultured MyC. MyC extracted from untreated trout were used to assess the effects of growth factors (50 $\mu g \cdot ml^{-1}$ and 100 $\mu g \cdot ml^{-1}$ of FGF and IGF-I and 5 and 10 $ng \cdot ml^{-1}$ of HGF) and β_2-adrenergic agonists (1 and 10 ppm of clenbuterol and ractopamine) on proliferation and differentiation capacities using fluorescent and molecular methods (see below).

Myosatellite cell isolation and culture

Myosatellite cells (MyC) were isolated from trout white muscle using the procedure of Fauconneau and Paboeuf (2000) with some modifications. Briefly, trout were removed from the tank, killed by a blow to the head and rinsed with cold water to remove mucus. Trout were then disinfected by washing with a bleach solution (1/2000) followed by PBS and 70% ethanol. The skin was removed and white muscle was filleted from both sides of the fish inside a laminar flow hood and collected in basal medium consisting of DMEM (Dubbelco's Modified Eagle Medium, Sigma D7777) containing $NaHCO_3$ (9 mM), HEPES (20 mM), Posm 300 $mOsm \cdot Kg^{-1}$, pH 7.4, supplemented with 15% horse serum (Sigma H1270), an antibiotic cocktail (final concentration: penicillin 100 $U \cdot ml^{-1}$, streptomycin 100 $\mu g \cdot ml^{-1}$, fongizone 0.25 $\mu g \cdot ml^{-1}$, Sigma A5955) and gentamycine 75 $\mu g \cdot ml^{-1}$ (Sigma, G1397). White muscle pieces were then incubated for 1 h at 4 °C in basal medium with 15% horse serum and 4-fold more antibiotic cocktail in order to ensure the sterility of the tissue. After incubation, tissue pieces were chopped into small cubes and transferred into basal medium supplemented with the initial antibiotic concentration. The tissue was then subjected successively to chemical (0.2% collagenase, Gibco 1700-017 and 0.1% trypsin,

Sigma T4799 in DMEM) and mechanical (1.4 mm X 100 mm needle) digestion. Cells were harvested by centrifugation (300 x g), resuspended in basal medium with 10% fetal calf serum (FCS, Gibco 26140-079) and the antibiotic cocktail and plated on poly-L lysine (Sigma, P5899) and laminin (Sigma, L2020) coated glass cover slips at 3 x 10^5 cells per cm^2. After 40 min, the media was changed to remove non-myosatellite cells and tissue debris. Myosatellite cells were cultured at 18 °C in DMEM supplemented with 10% fetal bovine serum and the cocktail of antibiotics.

Proliferation was assessed by bromodeoxyuridine (BrdU) incorporation as reported in Valente *et al.* (2002). BrdU was added to the cell culture media at a concentration of 10 $\mu mol \cdot L^{-1}$ and added to cultured MyC extracted from the low and high temperature acclimated trout groups and to those cells extracted from untreated trout and exposed to growth factors and β_2-AA. Myosatellite cells were fixed in 50 mM glycine in 70% ethanol 24 h after the BrdU was added. Incorporation of BrdU into MyC was measured using an immunofluorescence kit (Roche diagnostics, QC, Canada). Total nuclei were stained using Hoechst 33258 dye solution (355 nm/450 nm). Total nuclei and nuclei that incorporated BrdU were counted using the Image J 1.33 program (National Institute of Health, Rockville, Maryland) from computer-generated pictures taken using a fluorescent microscope (Zeiss, Germany, Axiophot). Duplicate slides were made for each time point and experiments were repeated 3 times (n = 3) with a different pool of trout white muscle. Three random fields in each slide were counted and each field contained at least 100 cells.

Myosatellite cells extracted from untreated fish were exposed to growth factors [IGF-I (GroPep, Adelaide, Australia), HGF and FGFb (R&D System, Minneapolis, MN)] or β_2-AA (clenbuterol, Sigma, St. Louis, MO and ractopamine, Eli-Lilly, Indianapolis,

Indiana). MyC were extracted from a pool of white muscle and cultured on poly-L lysine and laminin coated glass slides (see above). Cells were cultured in 5% Charcoal/Dextran treated fetal bovine serum (Hyclone, South Logan, UT) instead of 10% fetal calf serum. Growth factors or β-AA were added each day to the culture beginning the day after the extraction and for the next 4 days. At day 1 or 3, 10 $\mu mol \cdot L^{-1}$ BrdU was added to the well and the glass slide from the well was removed 24 h later and fixed in 50 mM glycine in 70% ethanol. Incorporation of BrdU was measured as previously described (see above).

Myogenesis

For RT-PCR studies, RNA was extracted from frozen samples of red and white muscles using 1 ml TRIzol per 65 to 85 mg of tissue and from white muscle MYC cultured in 75 cm^2 flasks (1 $ml \cdot cm^{-1}$) according to the manufacturer's instructions (Invitrogen, Life Technologies, Burlington, ON, Canada). Myogenesis was assessed by the presence of MyoD I, MyoD II and myogenin mRNA in the total RNA isolated from the muscle tissue and MyC from the temperature experiment and the *in vitro* exposure experiment. mRNA was treated with Deoxyribonuclease I (Invitrogen, 18068-015) and transformed into cDNA (Invitrogen, MMLV reverse transcriptase 28025-013) and amplified using Taq polymerase (Invitrogen, 1803842) according to the manufacturer's instructions. Specific primers were developed using the software primer 3 (Whitehead Institute, Cambridge, MA) and MyoD I and MyoD II sequences available in Rescan and Gauvry (1996) and the GenBank sequences for myogenin, PAX7, 18S and β-actin (accession numbers Z46912, AJ618975, AF243428, AJ438158, respectively). See Table IV-1 for the primer sequences used. MyoD I, myogenin, 18S and β-actin were amplified for 27, 27, 13, 22 cycles, respectively for

white muscle tissue mRNA. MyoD I, MyoD II, myogenin, 18S and β-actin were amplified for 26, 27, 26, 13, 20 cycles for red muscle tissue mRNA. MyoD I, MyoD II, myogenin, PAX 7, 18S and β-actin were amplified for 24, 24, 22, 28, 12, 17 cycles for red muscle tissue mRNA. The PCR conditions were 94 °C for 45 s, 59 °C for 30 s and 72 °C for 45 s, except for myogenin where the annealing temperature was 62 °C rather than 59 °C. The amplification products were run on a 1.5% agarose gels containing ethidium bromide. Products amplified with each set of primers were cloned using a TOPO cloning Kit and One Shot TOPO II vector (Invitrogen) to ensure that the right product was amplified. Plasmid DNA containing the insert of interest was purified using QIAprep Spin Miniprep Kit Protocol (Qiagen) and sequencing was undertaken by the Core DNA Facility Center (Ottawa, ON). Optimal conditions for $MgCl_2$ and primers concentration were determined for each set of primers and tissues. A cycle gradient was established for each set of primer for white and red muscle RNA in order to establish the optimal number of cycle necessary to quantify bands of interest using a pool of RNA from the high and low temperature groups. The bands obtained were quantified using the Quantity one computer program (Bio Rad, Hercules, CA) and data are expressed using relative band intensities of the gene of interest against β-actin. β-Actin was used as a control in this study, but it is important to note that the results were the same when 18S was used instead of β-actin as the comparator transcript.

Enzyme activities

Ten rainbow trout from each of the high and low temperature acclimation groups were dissected for tissue enzyme analysis. Tissues were immediately frozen on dry ice and

kept at -80 °C until analyses were performed. Activities of pyruvate kinase (PK), phosphoenol pyruvate carboxykinase (PEPCK), alanine and aspartate aminotransferase (ALT, AST), malate dehydrogenase (MDH), glutamate dehydrogenase (GDH), lactate dehydrogenase (LDH), isocitrate dehydrogenase (IDH), malic enzyme (ME) and glucose-6-phosphate dehydrogenase (G6PDH) were measured in liver and skeletal muscle when possible as decribed in previously (see Chapter III).

Plasma metabolites

Plasma lactate and glucose were measured enzymatically using method described previously (see Chapter II).

Statistics

Statistical analyses were performed using SYSTAT (version 10). Normality was tested using Shapiro-Wilk; all data were normaly distributed. One-way analysis of variance (ANOVA) was performed on each parameter measured. When the one-way ANOVA showed significant effects, multiple mean comparisons were made using the Tukey-Kramer comparison. Simple correlations were performed on enzymes and fish mass; SYSTAT was used to assess their level of significance.

Results

After four months of thermal exposure, body mass, length and hepatosomatic index (HSI) were higher in the 17 (high temperature, HT) compared with the 5 (low temperature, LT) °C acclimated group (Table IV-2). Body mass increased in both groups over the

experiment, but both groups gained equivalent mass until day 70 when the HT mass gain became significantly ($p<0.001$) higher than that in the LT group (Fig. IV-1); this difference was further enhanced at 110 days. Specific growth rates (SGR) were not the same throughout the acclimation period being higher until 45 days, then more-or-less maintaining this rate for the HT group, but slowing significantly in the LT group (see Fig. IV-1). There were no significant differences in condition factor (CF) or liver mass (Table IV-2). Plasma lactate and total protein content were significantly lower ($p < 0.01$) in 5 °C group (LT) (Table IV-2).

The liver glycolytic enzyme LDH, the amino acid enzyme ALT, and IDH were all significantly increased while GDH decreased in the HT compared with the LT group (Table IV-3). In red muscle LDH was increased but the two amino acid metabolism enzymes, ALT and AST and two oxidative enzymes from the citric acid cycle MDH and ME were decreased in the HT group compared with the LT group (Table IV-4). White muscle LDH was increased and AST, MDH, IDH and ME were decreased in the HT group compared with the LT group (Table IV-5).

MyC proliferation capacity assessed by BrdU incorporation after 16 weeks was not different between acclimation groups over 8 days of culture (Fig. IV-2); however at 4 days after plating MyC extracted from the HT group did show a significantly higher proliferation compared with those from the LT group.

There was a tendency for relative MyoD I, MyoD II and myogenin mRNA expression in MyC extracted from LT-acclimated trout white muscle (Fig IV-3) to be lower than in MyC extracted from HT-acclimated trout. This expression pattern was retained in whole white muscle, as both relative MyoD I and myogenin expression was significantly higher in HT- compared with the LT-acclimated groups (Fig. IV-4). The red muscle

expression pattern was somewhat different. MyoD I relative expression were significantly lower ($p < 0.01$) while myogenin expression were significantly higher ($p<0.01$) in the LT compared with the HT group (Fig. IV-5). However there was no significant difference in MyoD II relative expression between the groups.

Myosatellite cells extracted from untreated trout white muscle were exposed to growth factors (HGF, IGF-I and FGFb) and the two β_2-AA clenbuterol and ractopamine for 2 or 4 days after plating. There was a significant increase in BrdU incorporation after 4 days with 50 $ng \cdot ml^{-1}$ IGF-I and after 2 and 4 days with 100 $ng \cdot ml^{-1}$ IGF-I (Fig. IV-6A). After 2 days there was an increase in BrdU incorporation with 50 $ng \cdot ml^{-1}$ FGFb and after 4 days with 50 and 100 $ng \cdot ml^{-1}$ FGFb (Fig. IV-6B). However, 5 and 10 $ng \cdot ml^{-1}$ HGF had no effect and 50 $ng \cdot ml^{-1}$ was lethal to the cultured trout MyC (data not shown). There was no effect on BrdU incorporation into MyC exposed to 1 and 10 ppm of the β_2-AA clenbuterol or ractopamine. There was no statistical difference in myogenin, MyoD I and II and PAX7 relative expression in MyC mRNA after 4 days of exposure to 100 $ng \cdot ml^{-1}$ IGF-I or FGFb or to 10 ppm clenbuterol and ractopamine (data not shown).

Discussion

This study demonstrates that rainbow trout acclimated to a low water temperature had a significantly lower growth rate, agreeing with previous studies on channel catfish exposed to 10 °C for 28 days (Weber and Bosworth, 2005) and coho salmon (*Oncorhynchus kisutch*) exposed to 2.5 °C for 2 month (Larsen *et al.*, 2001). Trout used in this study were provided with the same amount of food per body mass regardless of acclimation temperature; however the trout from the low temperature group did eat less, as

also reported in the study of Weber and Bosworth (2005). Unfortunately the amount of food eaten by each group was not recorded, but the data suggest that up to 45 days of exposure both groups were feeding well; after this period the LT group significantly reduced its SGR and were not growing at 110 days (Fig. IV-1). The HT group remained in a positive growth rate throughout the entire period of acclimation. No significant differences occurred in liver mass between groups in contrast to Kent *et al.* (1988) who reported an increase in liver weight with a decrease in acclimation temperature from 25 to 15 °C in channel catfish (*Ictalurus punctatus*). However hepatosomatic index (HSI) did significantly increase in the LT-acclimated trout supporting results of Gabillard *et al.* (2003) in rainbow trout, Blier and Guderley (1988) in lake whitefish (*Coregonus clupeaformis*), Lannig *et al.* (2003) in cod and Seddon and Prosser (1997) and Weber and Bosworth (2005) in channel catfish. An increase in HSI is generally considered to be associated with an accumulation of lipids (Hochachka and Somero, 1972; Hilton, 1982) and preservation of glycogen storage (Capilla *et al.*, 2002) within the liver. Kent *et al.* (1988) reported that the higher liver mass in cold acclimated fish is due to tissue hypertrophy and an increase in metabolite storage.

Plasma glucose content was unchanged although both plasma lactate and total protein decreased with cold acclimation in agreement with Mayerle and Butler (1971) who reported a lower blood lactate and total protein content in 5 compared with 15 °C American eel (*Anguilla rostrata*) indicating a decrease in anaerobic glycolysis and an increase in protein catabolism, respectively. In this study the decrease in plasma lactate level was associated with a decrease in LDH activities in both liver and skeletal muscle suggesting a reduced glycolytic and gluconeogenic potential of this cold acclimated group.

Given the effect of temperature on physiology and metabolism, many fish species compensate for temperature changes by thermal acclimation, a process whereby functional properties of tissues change to adjust to high or low temperatures (Hazel and Prosser, 1974; Guderley and Blier, 1988; Guderley and Gawlicka, 1992). Low temperature acclimation in this study led to significantly decreased LDH activities, indicative of a decrease in anaerobic glycolysis in liver and white and red muscle tissues as reported by Kleckner and Sidell (1985) in liver of acclimated chain pickerel (*Esox niger*). However, Johnston and Wokoma (1986) in flounder and Guderley and Gawlicka (1992) in rainbow trout did not find any differences in LDH activities in either muscle type between cold and warm acclimation. It was shown by Somero and Childress (1980) and Childress and Somero (1990) that the activities of glycolytic enzymes (LDH and PK) in white muscle increase with body size in active pelagic fish. This increase in glycolytic capacity in larger individuals is thought to maintain a constant capacity for burst swimming (Pelletier *et al.*, 1993a). Moreover, Guderley and Gawlicka (1992) reported that LDH activities are correlated with growth rate in trout. Given that the low temperature acclimated trout in my study were both smaller and had a lower growth rate, reduced LDH activities would be predicted.

Activities of the amino acid transaminases generally increased in both muscle types in the LT group, supporting an enhanced protein catabolism in white and red muscle of cold-acclimated rainbow trout. This decrease is indicative of a fasting condition where peripheral amino acids are used to maintain hepatic glycogen and blood glucose levels (Mommsen and Moon, 2001). The higher HSI in the LT-acclimated trout support at least the maintenance of hepatic glycogen levels coming primarily from peripheral amino acids, not lactate.

Cold acclimation enhances the aerobic capacity of muscle by increasing the activities of mitochondrial enzymes and mitochondrial volume density (Battersby and Moyes, 1998) and the activities of aerobic enzymes (Johnston and Maitland, 1980; Sidell *et al.*, 1983; Hubley *et al.*, 1997; Battersby and Moyes, 1998). The increased activities assist in the maintenance of a constant oxidative capacity across a range of temperature (Johnston and Maitland, 1980; Egginton and Sidell, 1989) and also compensate for a reduced diffusion coefficients of cytoplasmic metabolites (Johnston, 1982; Tyler and Sidell, 1984; Sidell *et al.*, 1988; Egginton and Sidell, 1989). Changes in mitochondrial density also help to maintain a constant diffusive flux of oxygen between mitochondria and capillaries (Hubley *et al.*, 1997). In LT-acclimated trout, most citric acid cycle enzyme (mitochondrial enzymes) activities increased in both skeletal muscle types. Battersby and Moyes (1998) reported higher mitochondrial (citrate synthase, CS and cytochrome c oxidase, CCO) enzymes activities in red and white muscles of rainbow trout after a 2 month acclimation to 4 compared with 18 °C fish. Kleckner and Sidell (1985) also reported an increase in enzymes activities from aerobic metabolism in tissues of cold-acclimated chain pickerel.

Glucose-6-phosphate dehydrogenase (G6PDH) is the rate-limiting enzyme of the pentose phosphate pathway and controls the flow of metabolic intermediates to other pathways (Seddon and Prosser, 1997). G6PDH was previously shown to demonstrate a positive compensation in channel catfish (Kent *et al.*, 1988) when expressed on a protein or DNA basis; however no significant differences were found between G6PDH activities in rainbow trout acclimated to 5 and 17 °C.

In addition to acting on metabolic enzymes, temperature also affects the expression of myogenic factors (MRFs) that are related to fish muscle growth. Many studies reported the effects of temperature on embryos and larval MRFs expression levels (see Chapter I,

Introduction). Few studies report the effects of temperature on juvenile rainbow trout muscle proliferation and differentiation. Fauconneau and Paboeuf (2000) and Valente *et al.* (2002) reported that the proliferation rate of satellite cells is strongly dependent on the fish state *in vivo*. In other studies (see Chapters V, VI), I demonstrated that β_2-AA feeding of rainbow trout and the presence of the GH-transgene in Atlantic salmon significantly affect the proliferation capacity of myosatellite cells (MyC) once extracted and cultured *in vitro*. However, in the present study there is no significant difference in BrdU incorporation (proliferation capacity) into MyC extracted from 5 and 17 °C rainbow trout, with the exception of day 4 after extraction where MyC from the 17 °C fish remained in an exponential phase of BrdU incorporation, whereas MyC extracted from 5 °C had already reached a plateau (Fig. IV-2). Further studies are needed to conclude wether this difference is an artefact. More cells were plated than used by Fauconneau and Paboeuf (2000) who also used rainbow trout, based upon previous optimization experiments with Atlantic salmon and rainbow trout (see Chapters V, VI). However, in this particular experiment the white muscle MyC proliferated faster than previously observed, which may be due to the specific group of fish used in this study. It is possible that the high number of cells plated and high proliferation and differentiation rates of these cells masked any differences in proliferation capacity between the two groups. It should be noted, however that the differences in BrdU incorporation at day 4 after plating was accompanied by differences in the relative expression of MyoD (determination and proliferation of myoblasts) and of myogenin (differentiation into myotubes) implicating a possible mechanism to account for this higher proliferation rate at day 4.

Two non-allelic MyoD genes that result from the known genome duplication in salmonids were previously demonstrated in rainbow trout (Rescan and Gauvry, 1996b).

MyoD I and MyoD II mRNA are both present in slow fibers and only MyoD I is expressed in fast fibers of adult rainbow trout (Delalande and Rescan, 1999). MyoD I, MyoD II and myogenin were lower in MyC extracted from LT-acclimated trout. However, the lower expression levels of these MRFs were not significant due most likely to the small sample size. Matschak and Stickland (1995) reported that increasing the culture temperature from 5 to 11 °C led to an increase in differentiation rates 20 days after extraction in Atlantic salmon.

In this experiment, the relative expression of both MyoD I and myogenin was lower in white muscle from 5 compared with 17 °C acclimated trout. Relative expression of MyoD I was lower and myogenin higher in red muscle of the low compared with the high temperature-acclimated fish. This result indicates that myoblast determination and proliferation were decreased in both white and red muscles in the low compared with the high temperature trout. There was no significant difference in red muscle MyoD II expression. However, the specific function of MyoD II in red muscle is not known (Delalande and Rescan, 1999). MyC differentiation was decreased in white muscle but increased in red muscle after cold compared with high temperature-acclimated trout. This result agrees with red muscle generally responding to cold acclimation by an increase in size (Ayala *et al.*, 2001; Sänger and Stoiber, 2001) and with the fact that white and red muscles each develop in a different manner (Stoiber *et al.*, 2002). On the other hand, Kobiyama *et al.* (2000) reported a significant decrease in myogenin levels in carp 14 and 30 days after the temperature was raised from 20 to 30 °C; 30 °C is a temperature above the normal range encountered by carp and could have led to abnormal or deleterious effects on the muscle cells.

This study demonstrates that myogenin and MyoD I are affected by acclimation temperature in juvenile trout and are at least in part mediators of muscle growth in trout. The effects of temperature on MRFs could be explained by the fact that a direct relationship between temperature and myostatin mRNA expression exists (Weber and Bosworth, 2005) at least in channel catfish. Myostatin is a negative regulator of fish muscle growth that inhibits myoblast proliferation (Thomas *et al.*, 2000) and differentiation (Langley *et al.*, 2002).

This study also demonstrated an increase in proliferative capacity of trout MyC when exposed to the growth factors FGF (FGFb) and IGF-I (Fig. IV-6). However, there was no difference in the relative mRNA expression of MyoD and myogenin in these cells, which may come from the low sample size. An increase in MyC proliferation *in vitro* was previously reported for IGF-I in rainbow trout (Castillo *et al.*, 2004) and for IGF-I and FGFb in channel catfish (Mulvaney and Cyrino, 1995). This latter study also reported that FGFb induces MyC differentiation, which was not found in our study; however it was also reported that FGFb is an inhibitor of myoblast differentiation in avian (Marcelle *et al.*, 1995) and murine (Scata *et al.*, 1999) MyC. IGF-I is also believed to promote muscle differentiation in mammals (Allen and Boxhorn, 1989) but this was not demonstrated in our results. Larsen *et al.* (2001) reported a high correlation between IGF-I content and growth in coho salmon exposed to high and low temperatures. No differences were found in BrdU incorporation, MyoDs or myogenin relative expression levels in MyC exposed to HGF, clenbuterol or ractopamine. HGF was shown to increase MyC proliferation and differentiation in mammals (Allen *et al.*, 1995; Gal-Levi *et al.*, 1998; Miller *et al.*, 2000). The fact that no differences were found in proliferation and differentiation of MyC after HGF exposure may be due to the concentration used to stimulate the cells that was based

on concentrations used in mammalian studies which could be too high for fish MyC. The mode of action of ractopamine and clenbuterol in MyC activation is not known and only two concentrations of these compounds were tested.

This study demonstrated that cold acclimation induced compensatory changes in fish muscle enzyme activities, muscle growth and MRF relative expression at least in white muscle. The results are quite different from the cod study (see Chapters II and III) as no significant changes occurred in enzyme levels in cod exposed to ambient and constant temperatures. However this difference may result from the experimental design rather than a real species diffference. In this study, rainbow trout were exposed to the same temperature for a four month period, under constant photoperiod leading to acclimation and the expression of patterns observed in similar fish experiments in the literature. In contrast, one group of Atlantic cod was exposed to water temperatures that continuously fluctuated and both groups were exposed to a natural photoperiod. Constant temperature differs greatly from natural situations, where the animals are subjected to sometimes extremely variable environments within a single season (Larsen *et al.*, 2001; Stoiber *et al.*, 2002). In this experiment, MRFs and metabolic enzymes were both affected by water temperature as reported by Stoiber *et al.* (2002), but in a different manner. Metabolic enzymes respond by increasing amino acid metabolism and oxidative enzymes and by decreasing anaerobic glycolysis. However, we can conclude that the changes in MRFs and metabolic enzymes measured in this study are independent because metabolic enzyme activities changed in a similar way while the relative expression of MRFs differed between the muscle types.

Table IV-1: Sequences of the primers used and length of the products amplified.

Gene amplified	Gene bank sequence	Forward primer	Reverse primer	Product size (bases)
MyoD I	Rescan and Gauvry, 1996	5'CAACTGCTCAGACGGAATGA3'	5'GCTATGGAACCATCCTCCG3'	249
MyoD II	Rescan and Gauvry, 1996	5'TGAAGAGATGCACGTCGAAC3'	5'TCTTGCGGACGTGCACGTTG3'	230
myogenin	Z46912	5'TGAAGAGATGCACGTCGAAC3'	5'TCTTGCGGACGTGCACGTTG3'	232
β-actin	AJ438158	5'CGCCGCACTGGTTGTTGACA3'	5'GCGGTGCCCATCTCCTGCT3'	674
18S	AF243428	5'GGAGGTTCGAAGACGATCAG3'	5'CTCAATCTCGTGTGGCTGAA3'	438
PAX7	AJ618975	5'TCTGTCGGTACCAGGAGACC3'	5'ACAGTGCTTCTGTGCCACAC3'	184

Table IV-2: Body mass (g), length (cm), liver weight (g), condition factor (CF), hepatic somatic index (HSI) and plasma glucose ($mg \cdot ml^{-1}$), lactate (nM) and total protein ($mg \cdot ml^{-1}$) (mean ± SE) of rainbow trout acclimated for 16 weeks to low (5 °C) and high (17 °C) ambient temperatures. CF and HSI were calculated as $[(\text{body mass}) \cdot (\text{body length})^{-3}] \times 100$ and $[(\text{liver mass}) \cdot (\text{total weight})^{-1}] \times 100$, respectively. **, indicates significant differences between groups ($\alpha = 0.01$), *, indicates significant differences between groups ($\alpha = 0.05$).

	Low temperature	High temperature
Initial weight (g)	3.69 ± 0.14	3.80 ± 0.11
Initial length (cm)	6.75 ± 0.08	6.83 ± 0.07
Initial CF	1.19 ± 0.02	1.19 ± 0.02
Final weigh (g)	12.14 ± 0.77 **	35.89 ± 6.24
Final length (cm)	9.80 ± 0.19 **	13.30 ± 0.88
Liver weigh (g)	0.23 ± 0.03	0.41 ± 0.09
Final CF	1.27 ± 0.02	1.45 ± 0.09
HSI	1.92 ± 0.24 **	1.11 ± 0.07
Plasma glucose ($mg \cdot ml^{-1}$)	0.97 ± 0.12	1.12 ± 0.11
Plasma lactate (nM)	1.59 ± 0.28 *	4.57 ± 1.05
Plasma proteins ($mg \cdot ml^{-1}$)	25.29 ± 1.97 *	31.73 ± 1.92

Table IV-3: Enzyme activities (mean ± standard error) in the liver of rainbow trout (*Oncorhynchus mykiss*) acclimated for 16 weeks to low (5 °C) and high (17 °C) temperatures. All data are expressed as $\mu mol \cdot min^{-1} \cdot g\ tissue^{-1}$, $8 \leq n \geq 10$. Enzymes were assessed at 20 °C. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamate dehydrogenase (GDH), glucose-6-phosphate dehydrogenase (G6PDH), isocitrate dehydrogenase (IDH), lactate dehydrogenase (LDH), malic enzyme (ME), malate dehydrogenase (MDH), phosphoenol pyruvat carboxykinase (PEPCK), pyruvate kinase (PK). Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) between groups.

Liver		Low temperature	High temperature
	Protein	67.83 ± 2.11	75.07 ± 2.07
Glycolysis	PK	12.56 ± 0.73	13.35 ± 0.81
	LDH	100.11 ± 3.21 b	142.24 ± 5.92 a
gluconeogenesis	PEPCK	5.42 ± 0.16	5.38 ± 0.25
amino acid metabolism	ALT	15.21 ± 0.57 b	21.41 ± 2.12 a
	AST	21.85 ± 0.83	23.44 ± 1.09
oxidative enzymes	GDH	12.27 ± 0.83 a	9.10 ± 1.12 b
	MDH	93 59 ± 2.88	94.67 ± 10.67
	IDH	5.63 ± 0.25 b	7.96 ± 0.38 a
	ME	7.89 ± 1.63	1.63 ± 0.22
penthose phosphate pathway	G6PDH	7.62 ± 0.62	1.44 ± 0.19

Table IV-4: Enzyme activities (mean ± standard error) in the red muscle of rainbow trout (*Oncorhynchus mykiss*) acclimated to low (5 °C) and high (17 °C) temperatures. All data are expressed as $\mu mol \cdot min^{-1} \cdot g\ tissue^{-1}$, $8 \leq n \geq 10$. Enzymes were assessed at 20 °C. Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) between groups. See Table IV-3 for complete enzyme names.

RM		Low temperature	High temperature
	Protein	29.23 ± 0.61	27.52 ± 1.12
Glycolysis	PK	39.37 ± 2.56	47.79 ± 4.26
	LDH	169.62 ± 11.07 b	209.74 ± 12.30 a
amino acid metabolism	ALT	1.51 ± 0.07 a	1.27 ± 0.06 b
	AST	49.01 ± 2.59 a	34.52 ± 1.62 b
oxidative enzymes	GDH	5.79 ± 0.34	5.63 ± 0.22
	MDH	139.81 ± 5.42 a	100.76 ± 7.47 b
	IDH	18.79 ± 0.86	16.52 ± 0.87
	ME	1.69 ± 0.07 a	1.44 ± 0.07 b

Table IV-5: Enzyme activities (mean ± standard error) in the white muscle of rainbow trout (*Oncorhynchus mykiss*) acclimated to a low (5 °C) and a high (17 °C) temperature. All data are expressed as $\mu mol \cdot min^{-1} \cdot g\ tissue^{-1}$, $8 \leq n \geq 10$. Enzymes were assessed at 20 °C. Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) between groups. See Table IV-3 for complete enzyme names.

WM		Low temperature	High temperature
	Protein	29.45 ± 0.81	28.27 ± 1.14
Glycolysis	PK	23.44 ± 2.18	33.52 ± 5.22
	LDH	121.52 ± 10.15 b	200.80 ± 22.68 a
amino acid metabolism	ALT	0.81 ± 0.05	0.91 ± 0.09
	AST	14.32 ± 0.75 a	10.61 ± 0.63 b
oxidative enzymes	GDH	0.73 ± 0.06	0.79 ± 0.07
	MDH	45.01 ± 1.39 a	38.29 ± 2.67 b
	IDH	4.36 ± 0.22 a	2.77 ± 0.21 b
	ME	0.55 ± 0.03 a	0.43 ± 0.02 b

Figure IV-1: Body mass gain in rainbow trout over the acclimation period. ●, Body mass of trout exposed to 17 °C; ○, body mass of trout exposed to 5 °C. 20 <n > 25 *, indicates significant differences in mass between groups (Tukey-Kramer, $\alpha < 0.05$). Numbers on each line represent the specific growth rate between the two time points.

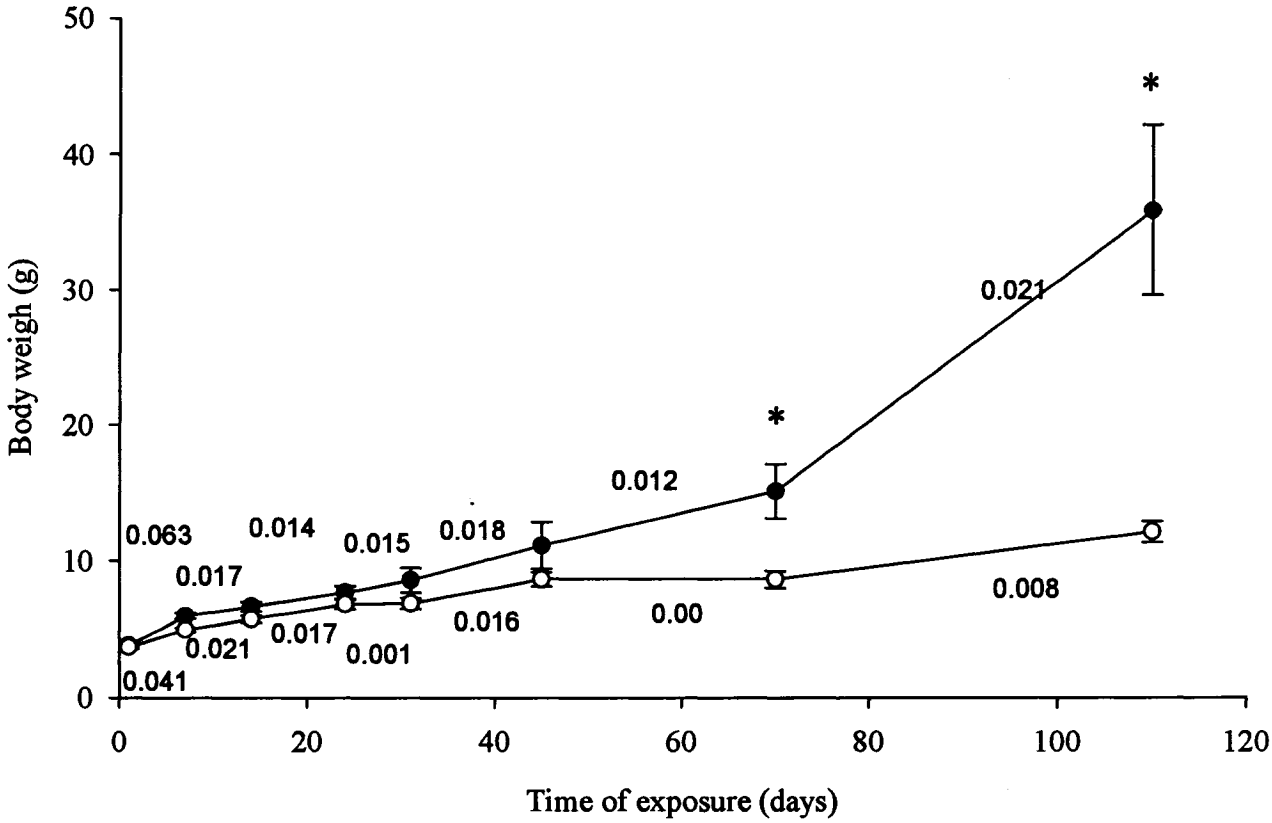


Figure IV-2: Rainbow trout white muscle myosatellite cell proliferation *in vitro*. •, Satellite cells extracted from 17 °C acclimated trout; ○, satellite cells extracted from 5 °C acclimated trout. N = 3 (mean ± standard error); *, indicates significant differences in cell proliferation between groups (Tukey-Kramer, $\alpha < 0.05$).

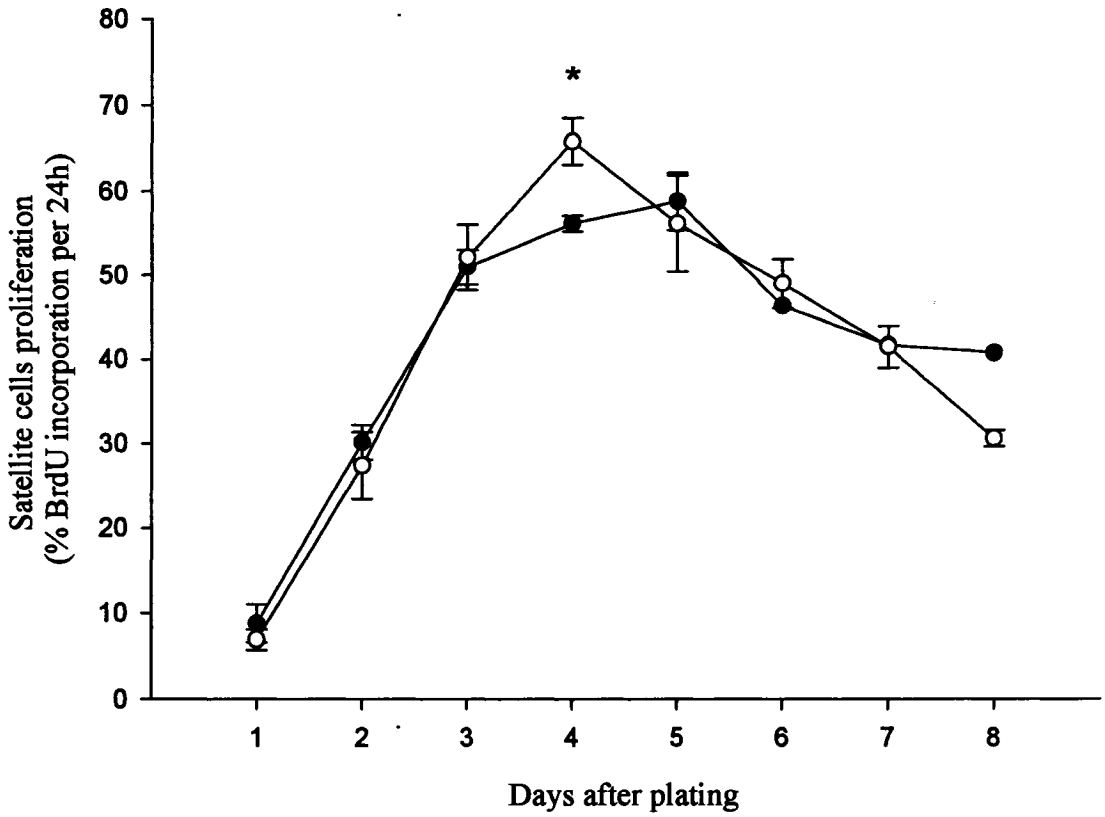


Figure IV-3: Relative mRNA expression of white muscle myosatellite cell A) MyoD I, B) MyoD II and C) myogenin in rainbow trout (*Oncorhynchus mykiss*) acclimated to 5 °C (LT) and 17 °C (HT) temperatures. N = 2 (mean ± standard error). Relative band intensities (gene of interest compared with β-actin) were determined from the densities of the resulting gel bands.

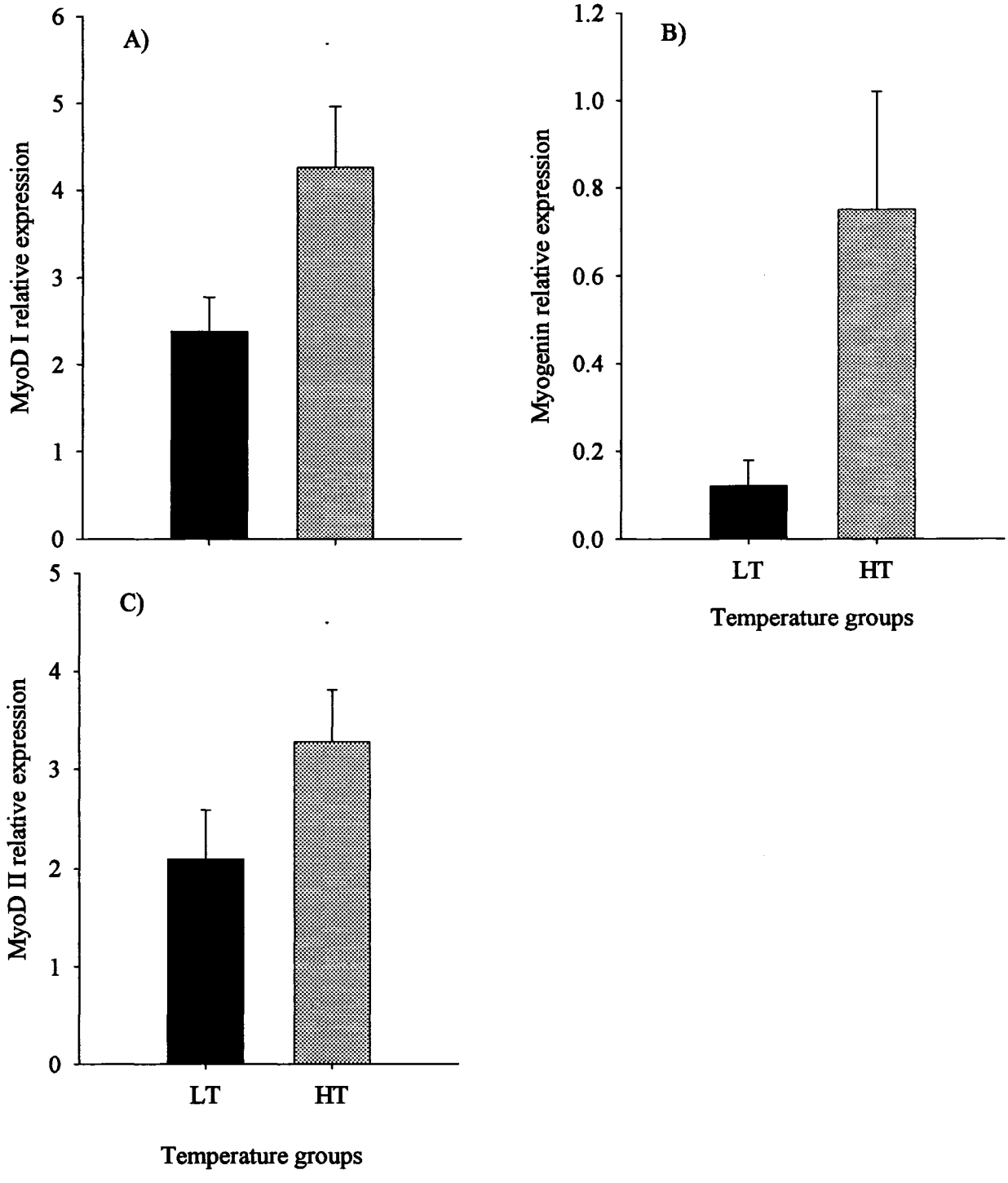

A)
MyoD I relative expression
6
5
4
3
2
1
0
B)
Myogenin relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
LT
HT
Temperature groups
C)
MyoD II relative expression
5
4
3
2
1
0
LT
HT
Temperature groups

Figure IV-4: Relative expression of rainbow trout white muscle A) myogenin and B) MyoD I acclimated to 5 °C (LT) and 17 °C (HT) temperatures. N=10 (mean ± standard error); *, indicated significant differences between groups. Relative band intensities (gene of interest compared with β-actin) were determined from the densities of the resulting gel bands.

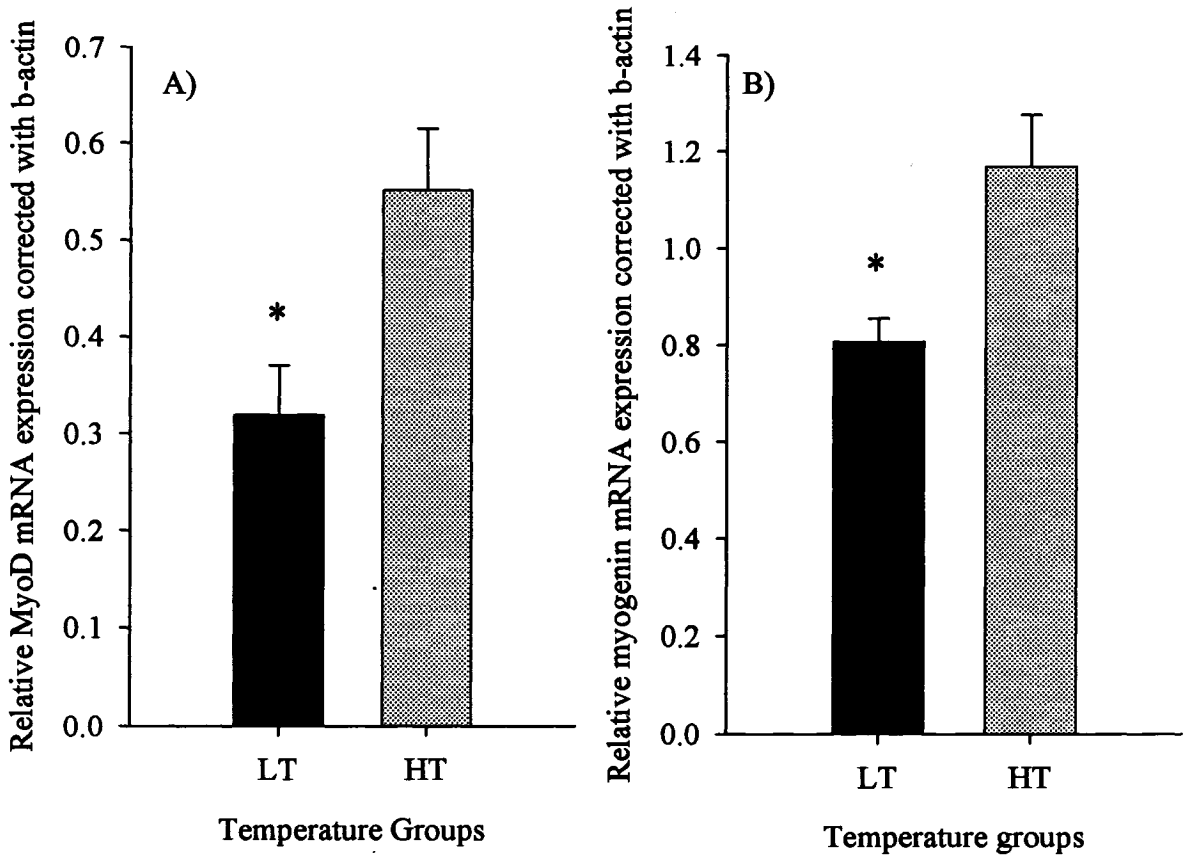


Figure IV-5: Relative expression of rainbow trout red muscle A) myogenin, B) MyoD I and C) MyoD II acclimated to 5 °C (LT) and 17 °C (HT) temperatures. N=10 (mean ± standard error); *, indicated significant differences between groups. Relative band intensities (gene of interest compared with β-actin) were determined from the densities of the resulting gel bands.

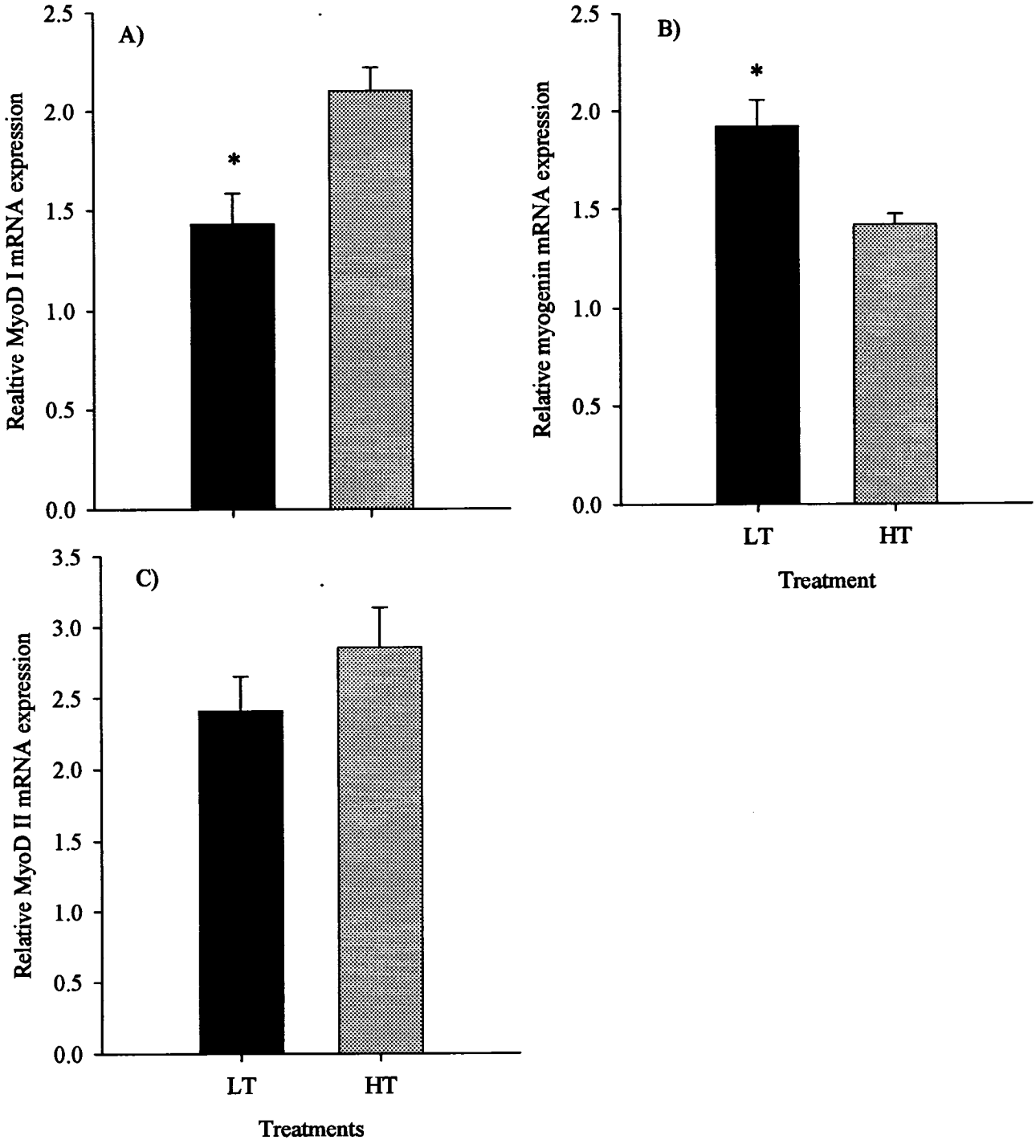
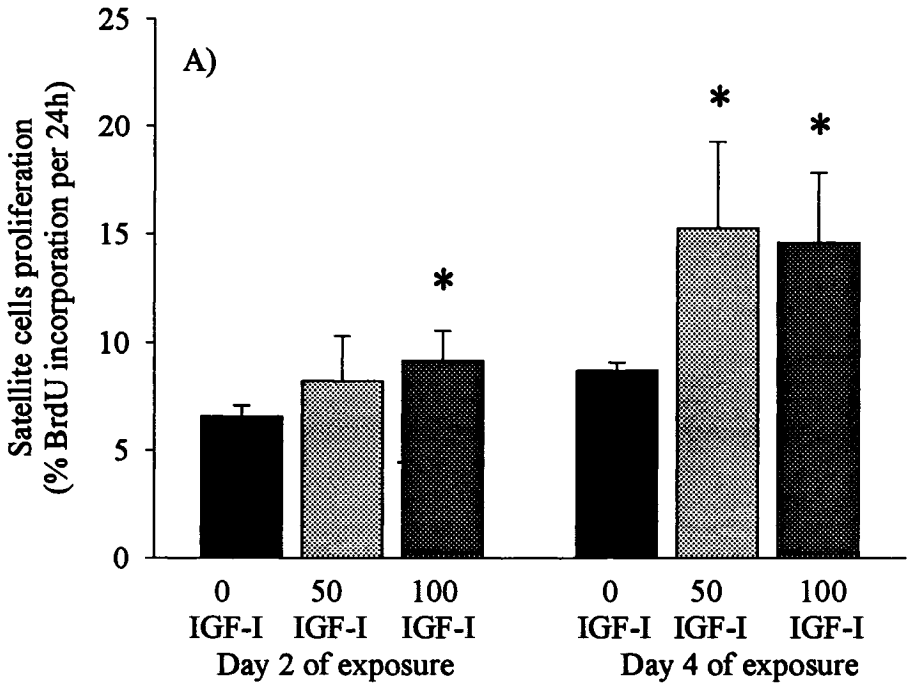
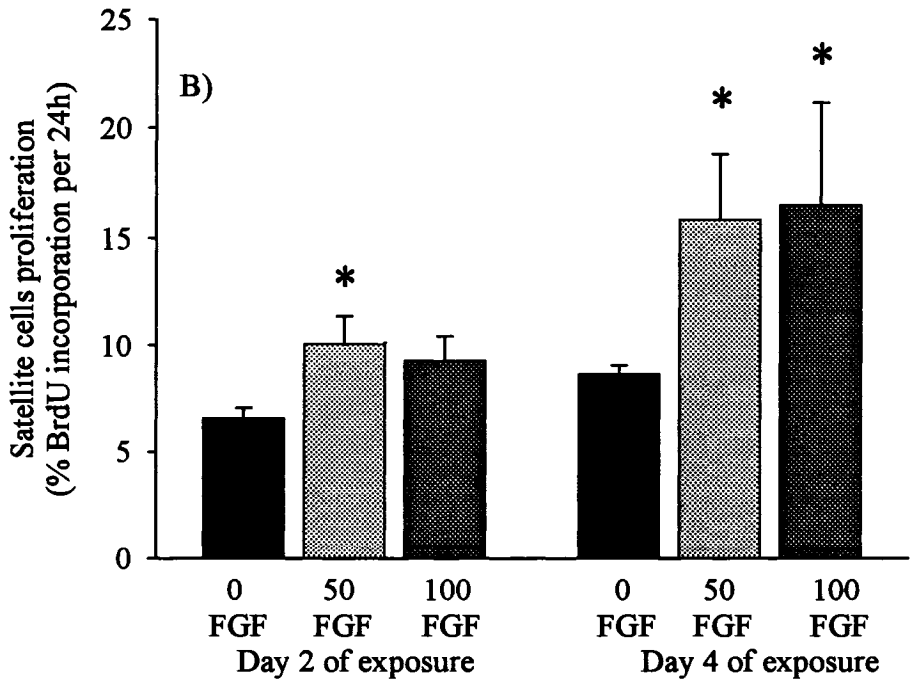

A)
Realtive MyoD I mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
*
B)
Relative myogenin mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
*
LT
HT
Treatment
C)
Relative MyoD II mRNA expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
LT
HT
Treatments

Figure IV-6: White muscle myosatellite cell proliferation *in vitro* using bromodeoxyuridine (BrdU) incorporation into myosatellite cells extracted from untreated rainbow trout and cultured in the presence of A) 50 $\mu g \cdot ml^{-1}$ (gray bars) and 100 $\mu g \cdot ml^{-1}$ (dark gray bars) IGF-I and B) 50 $\mu g \cdot ml^{-1}$ (gray bars) and 100 $\mu g \cdot ml^{-1}$ (dark gray bars) FGFb; black bars in both A and B represent BrdU incorporation into control cells. Cells were exposed to growth factors for 2 (J2) and 4 days (J4); *, indicates significant differences from the control (Tukey-Kramer, $\alpha < 0.05$).


A)
Satellite cells proliferation
(% BrdU incorporation per 24h)
25
20
15
10
5
0
*
*
*
0 IGF-I
50 IGF-I
100 IGF-I
Day 2 of exposure
0 IGF-I
50 IGF-I
100 IGF-I
Day 4 of exposure


B)
Satellite cells proliferation
(% BrdU incorporation per 24h)
25
20
15
10
5
0
*
*
*
0 FGF
50 FGF
100 FGF
Day 2 of exposure
0 FGF
50 FGF
100 FGF
Day 4 of exposure

CHAPTER V: MYOGENESIS AND MUSCLE METABOLISM IN ATLANTIC SALMON (*SALMO SALAR*) MADE TRANSGENIC FOR GROWTH HORMONE

Abstract

Tissues from Atlantic salmon (*Salmo salar*) made transgenic for growth hormone (GH) and from non-transgenic salmon were collected for enzyme estimates and mRNA analyses. White muscle myosatellite cells were isolated for proliferation assays using bromodeoxyuridine (BrdU) incorporation. Myosatellite cell proliferation was higher in cells from GH-transgenic salmon compared with cells from non-transgenic salmon at 4 months and from non-transgenic salmon of the same mass. MyoD I and myogenin mRNA contents were higher in myosatellite cells isolated from non-transgenic fish after 6 days of plating. In white muscle tissue, MyoD I mRNA was higher in transgenic salmon at 4 months indicating a higher proliferation capacity in this tissue *in situ*. White muscle tissue myogenin mRNA content varied with fish size and was lower in transgenic fish at 4 months, indicating a lower differentiation capacity. In red muscle tissue, MyoD II mRNA was lower and myogenin was higher in GH-transgenic fish at 7 months compared with non-transgenic salmon at 7 months. However, red muscle myogenic factor expression was not different between transgenic and non-transgenic fish of the same size. Enzymes activities in white muscle and intestine were correlated with fish body mass. This study shows that myogenic factors and myosatellite cell proliferation are affected by the ectopic GH-transgene, whereas some metabolic parameters are more affected by fish size indicating that, at some point GH-transgenic animal are physiologically closer to older non-transgenic

salmon. Moreover, proliferation and differentiation in white and red muscles happens in successive waves, implicating differences in energy allocation between white and red muscle.

Introduction

Fish growth is generally indeterminate in contrast to mammals and birds, and most fish grow throughout their life by the formation of new muscle cells (hyperplasia) and/or the increase in size of existing cells (hypertrophy) (Fauconneau and Paboeuf, 2001; Chapter I). The cells responsible for muscle growth are myosatellite cells (MyC) which are specific muscle stem cells. Myosatellite cells are small spindle-shaped cells having a heterochromatic nucleus and a small number of organelles (Koumans *et al.*, 1990a) and are located between the sarcolemma and the basal lamina of differentiated muscle fibers (Valente *et al.*, 2002). These cells supply additional nuclei in hypertrophic growth and are the source of new muscle fibers for hyperplasic growth (Allen *et al.*, 1979; Veggetti *et al.*, 1990; Akster *et al.*, 1993; Johnston *et al.*, 1995). In fish, the ratio between the contribution of hyperplasic and hypertrophic growth decreases with age and length/size (Weatherley *et al.*, 1980; Stickland, 1983; Koumans and Akster, 1995), as hypertrophic growth persists long after hyperplasic growth has ceased (Weatherley *et al.*, 1988; Koumans *et al.*, 1993; Rowlerson *et al.*, 1997). The mechanisms involved in regulating hypertrophic vs. hyperplasic growth remain unresolved.

Myosatellite cells proliferate and differentiate to produce myotubes (multicellular muscle cells); this process is called myogenesis. Proliferation and differentiation are regulated by transcription factors called myogenic regulatory factors (MRF) (Hawke and

Garry, 2001). MyoD and myogenin are two basic helix-loop-helix (bHLH) transcription factors that play essential roles in muscle cell determination, proliferation and differentiation (Feldman and Stockdale, 1991) during development and growth of vertebrates including fish (Xie *et al.*, 2001). These two factors together with Myf5 and MRF4 make up the MRF family (Watabe, 2001). In addition to transcription factors, hormones including growth hormone (GH) and insulin-like growth factor-I (IGF-I) are implicated in vertebrate muscle growth (Mommsen and Moon, 2001). Circulating levels of these hormones are affected by ration size, and the composition and energy status of the fish (Mommsen and Moon, 2001).

In vertebrates, most effects of GH are mediated by IGF-I (Pierce *et al.*, 2005). IGF-I induces growth and differentiation, and regulates reproduction and metabolism (Castillo *et al.*, 2004). Fauconneau *et al.* (1996) reported an increase in muscle protein synthesis in WM of rainbow trout juveniles and Atlantic salmon smolts with mammalian GH administration and Roberts *et al.* (2004) recently hypothesized that GH over-expression in transgenic fish was increasing growth rate through a decrease in myostatin expression (a negative regulator of muscle growth). Atlantic salmon made transgenic for GH represent a good model to analyse growth processes as these fish grow much faster and are able to reach market size one year earlier than their non-transgenic siblings (Fletcher *et al.*, 2004; Deitch *et al.*, 2006), although the precise mechanisms involved in this rapid growth have yet to be resolved (Devlin *et al.*, 1994; Fletcher *et al.*, 2004).

Energy metabolism is different between fish red and white muscles. WM fibers rely more on anaerobic glycolysis and use glycogen as the principal fuel (Johnston *et al.*, 2001). WM is used principally at high and burst swimming speeds, whereas RM is used for continuous locomotion and its high mitochondrial and capillary densities allow this fiber

type to oxidize carbohydrates and lipids as fuels and possibly specific amino acids (Moyes *et al.*, 1989; Johnston, 2001).

The hypothesis of Chapter VI is that GH affects Atlantic salmon by acting on myogenesis and muscle metabolic enzyme activities. The predictions are that Atlantic salmon made transgenic for GH will exibit increased levels of myogenic factors, increased proliferation of myosatellite cells and a higher metabolic rate than non-transgenic salmon. The goal of this study is to compare proliferation and differentiation in WM myosatellite cells, and in white and red muscle tissues from GH-transgenic and non-transgenic Atlantic salmon. In addition I want to establish whether the faster growth rate of the transgenic salmon is mediated by higher metabolic enzyme activities or if the GH-transgenic salmon have similar enzyme activities than older, but similar sized, non-transgenic salmon. This study also provides the analysis of fish myogenesis and intermediary metabolism in a stable strain of Atlantic salmon made transgenic for GH.

Material and Methods

Experimental animals and procedures

The transgenic Atlantic salmon (*Salmo salar*) strain (termed EO-1α) used in this study is hemizygous for a single functional ectopic copy of a growth hormone (GH) transgene (Fletcher *et al.*, 2004; Deitch *et al.*, 2006). This strain was created in 1989 by injecting fertilized eggs with a chimeric GH gene construct (opAFP-GHc2) consisting of a chinook salmon (*Oncorhynchus tshawytscha*) GH cDNA regulated by an ocean pout (*Macrozoarces americanus*) antifreeze protein gene promoter (Du *et al.*, 1992).

The Atlantic salmon selected for this study were from the 5th generation of this lineage. Salmon were held at the Ocean Sciences Centre, Memorial University of Newfoundland (OCS, St. John's, Newfoundland) aquaculture facilities in sea water at 13 °C under natural photoperiod. Transgenic salmon were obtained by fertilizing a pool of eggs from seven wild non-transgenic females with sperm obtained from a hemizygous GH-transgenic male. Eggs were incubated at 6-7 °C in a re-circulating incubation system at pH 6.8-7. Fry were transferred to rearing tanks on March 3, 2003; the pH was maintained at 6.8-7 and the temperature was held at 13.0-13.8 °C under natural photoperiod. For further explanation see Dietch *et al.* (2006). Non-transgenic salmon were obtained by crossing the same pool of eggs from non-transgenic females with sperm from a single non-transgenic male. Fin clips were removed from all fish at the termination of the experiment, frozen in liquid nitrogen and stored in a -80°C freezer until analysis by PCR for the presence or absence of the GH transgene according to the method outlined in Deitch *et al.* (2006) (Fig. V-1A).

The experimental plan was to examine the various aspects of muscle growth in pre-smolt salmon, a time when these fish are exhibiting rapid growth. However during this period, the transgenic fish can grow to be 6 to 10-times larger than the non-transgenics. This difference in growth rate makes it impossible to compare size matched fish at the same time. To overcome this difficulty, the transgenics and non-transgenics were first sampled at 4 months when the non-transgenics had sufficient muscle mass for the required sample size. A second sampling occurred at 7 months of age when the non-transgenics had grown to be the approximate size that the transgenics were at 4 months. This experimental design allowed comparisons between age-matched and size-matched transgenic and non-

transgenic fish. All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care for the use of animals in teaching and research.

Myosatellite cell isolation and culture

Myosatellite cells (MyC) were isolated from white muscle (WM) tissue using the procedures of Fauconneau and Paboeuf (2000) as recorded in Chapter IV. GH-transgene expression was verified in MyC mRNA using standard PCR techniques as reported in Deitch *et al.* (2006). In addition to comparing proliferation rates of MyC isolated from transgenic and non-transgenic salmon, an experiment was also carried out to examine the effects of salmon growth hormone (GH) *in vitro* at concentrations of 10 and 50 $ng \cdot ml^{-1}$ (GroPep GHBU020 lot:IJH-GHB1) on cells isolated and cultured from non-transgenic salmon. Cells were cultured in a serum replacement medium (Sigma, S0638) in 24 well plates at an initial density of 3×10^5 cells per cm^2. Proliferation was assessed using BrdU as described above in Chapter IV.

Myogenesis

Myogenesis was assessed by quantifying the mRNA levels of the myogenic regulatory factors (MRF) MyoD I, MyoD II and myogenin in cultured MyC *in vitro* and muscle tissue *in situ* with the use of semi-quantitative RT-PCR as described in Chapter IV. The primer sets used are given in Table V-1. MyoD I, myogenin and β-actin were amplified for 26, 26, and 23 cycles, respectively for WM tissue RNA. MyoD I, MyoD II, myogenin and β-actin were amplified for 25, 28, 24, 21 cycles respectively for RM tissue RNA. MyoD I, MyoD II, myogenin, β-actin and the ectopic GH transgene were amplified

for 27, 26, 20, 18 and 35 cycles, respectively for WM MyC RNA. The PCR conditions used were 94 °C for 45 s, 59 °C for 30 s and 72 °C for 45 s, except for myogenin where the annealing temperature was 62 °C rather than 59 °C. The PCR conditions to amplify the ectopic GH transgene in WM MyC were 94 °C for 45 s, 59 °C for 1 min and 72 °C for 1 min.

The PCR amplification products were run on a 1.5% agarose gel containing ethidium bromide. The gel was scanned under UV light and the density of the bands quantified using the Quantity one computer program. Data are expressed as a ratio of the band density for the gene of interest to the band density for β-actin from the same tissue. β-Actin was used as the control gene as it remains the main housekeeping gene used to normalize mRNA levels in most studies (Selvey *et al.*, 2001). Moreover β-actin is the gene used in most muscle studies, thus facilitating the comparison of our work with previous studies. It is important to note, however that β-actin and 18S mRNA provided similar relative expression patterns and the ratio between β-actin and 18S did not change between treatments.

Enzymes activities

Twelve Atlantic salmon per group at 4 months and ten per group at 7 months of age were dissected and tissues removed for enzyme analyses. WM and RM were always taken from the same side of the fish at each sampling. Two samples were taken from each fish; one was kept for enzyme assays and the other for RNA isolation (as above). Tissues (muscles, liver and intestine) were immediately frozen on dry ice and held at -80 °C until analyses were performed. Enzymes activities were measured under substrate saturating conditions as described in Chapter III.

Statistics

Statistical analyses were performed using SYSTAT (version 10). Normality was tested using Shapiro-Wilk; all data were normaly distributed. One-way analysis of variance (ANOVA) was performed on each parameter measured. When the one-way ANOVA showed significant effects, multiple mean comparisons were made using the Tukey-Kramer comparison. Simple correlations were performed between enzymes and fish mass using SYSTAT to assess levels of significance.

Results

Growth hormone (GH) transgenic and non-transgenic salmon were sampled at 4 and 7 months of age. At both sampling times, the mass of the GH-transgenic salmon was about 6-times larger than the non-transgenic salmon (Table V-2). For each sampling of transgenic fish a non-transgenic of the same age was also harvested to act as an age control. At 7 months of age, non-transgenic salmon had approximately the same mass, length and condition factor (Table V-2) as the GH-transgenic salmon at 4 months. This sampling allowed for a mass control, as reported in Pitkänen *et al.* (2001). The WM MyC yield did not differ between transgenic and non-transgenic salmon at either sampling times, but significantly more MyC were extracted from salmon WM at 4 than at 7 months of age (Table V-2).

A sample of the fin clip from all salmon used in this study was processed through a PCR assay to detect the ectopic GH gene (Fig. V-1A). All GH-transgenic salmon used carried the ectopic GH copy This group showed three bands; two for the endogenous GH gene and one for the transgene, as shown in lanes 2 and 3 (Gabillard *et al.*, 2003). The

transgenic GH copy is shorter as it was made from a cDNA fragment and does not contain an intron. All non-transgenic salmon were found not to possess the transgene (only two endogenous bands as shown in lanes 4 and 5). It is important to note that the amount of DNA used in the assay was not quantified, which explains the differences in the expression of the endogenous GH between samples. Moreover, the presence of the GH-transgene mRNA expression was confirmed in satellite cells extracted from transgenic salmon (Fig. V-1B). In this case endogenous GH was not detected as the primers amplified the promoter region specific to the GH ectopic copy.

Tissue enzymes

Metabolic enzymes were estimated in RM, WM and liver in the transgenic and non-transgenic salmon. Only citrate synthase (CS) was measured in the intestine. Most enzyme activities in WM were negatively correlated with fish mass (Table V-3) except for pyruvate kinase (PK) activity which was positively correlated (Fig. V-2). Aspartate aminotransferase (AST) and malate dehydrogenase (MDH) were not significantly correlated with body mass. CS measured in the intestine was positively correlated with fish body mass (Table V-3).

Most RM enzyme activities were higher in the non-transgenic compared with transgenic salmon at the same mass and at the same age (Table V-4). Red muscle quantity was not adequate to sample in the non-transgenic salmon at 4 months of age.

No differences were found in liver enzyme activities between transgenic salmon and age or mass of the non-transgenic group for phosphoenolpyruvate carboxykinase (PEPCK), alanine aminotransferase (ALT), AST, glutamate dehydrogenase (GDH), lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G6PDH) (Table V-5).

Liver CS was higher in transgenic compared with non-transgenic salmon at both sampling times (Fig. V-3).

White muscle myosatellite cells and myogenesis

Myosatellite cells extracted from transgenic and non-transgenic salmon showed proliferation capacity. A significantly higher proliferation rate was found for MyC extracted from transgenic salmon at 4 months compared with non-transgenic salmon of the same age and of the same mass (Fig. V-4). The proliferation rate of MyC from transgenic salmon at 7 months was not possible to measure due to high cell mortalities, coinciding with the fact that these fish were in precocious reproduction. Proliferation of satellite cells from non-transgenic salmon was significantly enhanced by GH (10 and 50 $ng \cdot ml^{-1}$ media) after a 24 h incubation (Fig. V-5). This experiment was done only with cells from 7 month non-transgenic fish.

MyoD I, MyoD II and myogenin mRNA expression were measured in MyC from transgenic and non-transgenic salmon at 4 months following 6 days of plating. Relative MyoD I and myogenin mRNA expressions were lower in cells extracted from transgenic salmon compared with non-transgenic salmon satellite cells (Fig. V-6A,B). There were no differences in relative MyoD II expression between transgenic and non-transgenic salmon (Fig. V-6B). Unfortunately RNA extraction failed in satellite cells harvested from 7 month old fish.

Tissue myogenesis

MyoD I expression at 4 month was significantly higher ($p < 0.01$) in WM from transgenic compared with non-transgenic salmon of the same age and the same size (Fig. V-7). No differences exist between transgenic and non-transgenic salmon at 7 months. It

was not possible to amplify MyoD II from the WM tissue samples. Relative myogenin mRNA expression was the opposite of MyoD I expression in WM with lower expression in transgenic compared with non-transgenic salmon of the same age (4 months) and to non-transgenic salmon of the same mass (Fig. V-8). No difference was noted between transgenic and non-transgenic salmon at 7 months for myogenin expression.

As noted above, RM could not be obtained from 4 month old non-transgenic salmon due to the small size of the fish. There were no differences between groups for RM MyoD I expression (Fig. V-9A). MyoD I expression was lower in transgenic salmon at 7 months compared with non-transgenic salmon of the same age, but not different at the same mass (Fig. V-9B). Myogenin mRNA expression follows an opposite pattern with respect to MyoD II expression in RM, with higher expression in transgenic salmon at 7 months compared with non-transgenic salmon at the same age (Fig. V-10), but not at the same mass.

Discussion

GH-transgenic salmon

Fish are an interesting model for muscle growth studies as RM and WM are clearly distinct tissues and together represent more than 50% of the total body mass of the animal. They are also the major tissues in terms of protein content (Houlihan *et al.*, 1988). In fish, an increase in body mass is due primarily to growth of WM fibers. WM accretion shows the best relationship to growth and is highly sensitive to whole body growth changes (Houlihan *et al.*, 1988). Thus small changes in white muscle myosatellite cell proliferation and

differentiation, and muscle tissue metabolism may have significant effects on fish growth. In this study, RM was also examined as it does not grow to the same extent as WM and is more stable than WM to whole body growth changes (Houlihan *et al.*, 1988). Also RM and WM are quite different in their expression of MyoD (Rescan and Gauvry, 1996a), physiological use (Johnston *et al.*, 2001), and metabolic enzyme activities (Moyes *et al.*, 1989). An effect of ectopic expression of GH might be expected at different levels in fish RM and WM. The transgenic salmon strain used in this study is a good model to look at muscle growth processes as GH-transgenic salmon clearly grow faster than their non-transgenic siblings (Fletcher *et al.*, 2004), but the precise mechanisms involved remain unresolved. Here myogenic factors and metabolic enzymes were measured to assess their impact in the growth of GH-transgenic salmon.

Metabolism

Metabolic enzyme activities can be affected by several parameters including food availability and composition (Yang and Somero, 1993). Moreover, a high correlation exists between enzymes activities and fish growth (Pelletier *et al.*, 1993a). It was shown in previous studies that the growth-promoting action of GH in tissues is accompanied by various metabolic changes in these tissues (Pitkänen *et al.*, 2001). This study examined whether the higher growth rate of transgenic fish was mediated by an increase in metabolic enzyme activities in tissues.

Most WM enzymes were negatively correlated with fish body mass, regardless of the presence or absence of the GH-transgene. It was shown previously that LDH and CS activities in WM and intestine correlate positively with fish growth rate when expressed in units per mg of protein (Pelletier *et al.*, 1993a; Couture *et al.*, 1998; Dutil *et al.*, 1998) and

that WM CS correlates negatively with fish growth rate when expressed in units per g wet tissue (Couture *et al.*, 1998). Previous studies reported a higher activity of WM CS (an indicator of mitochondrial activity) when expressed in units per g of tissue in ~60 g Coho salmon (*O. kisutch*) made transgenic for GH (Blier *et al.*, 2002) and GH-transgenic tilapia (McKenzie *et al.*, 2003) compared with their non-transgenic siblings of the same size. Deitch *et al.* (2006) also reported a higher activity of myocardial CS that was associated with a higher physical activity; however Deitch *et al.* (2006) did not find any differences in WM CS activity levels between transgenic and non-transgenic salmon. In contrast, I found that CS activity decreased in transgenic salmon WM compared to non-transgenic WM. This may result from the lower weight of the salmon studied in my experiment (15 g). It is important to note that metabolic enzyme activities in WM from GH-transgenic salmon are similar to the activities of older non-transgenic salmon of the same mass.

Activities of the enzymes measured in the RM were all higher in the non-transgenic salmon group compared with the GH-transgenic groups of salmon with a higher mass. This finding is in agreement with Kiessling *et al.* (1991) who reported that metabolic enzymes are strongly affected by age in rainbow trout and are generally lower in fish with a higher mass. Moreover, RM enzymes measured in non-transgenic salmon are also higher than in GH-transgenic salmon of the same age, suggesting a strong effect of the ectopic GH on lowering metabolic activities in this tissue. However, other studies will be needed to understand this last result. Within the liver, only PK, MDH, IDH and CS enzyme activities showed significant differences among groups, suggesting that most enzymes measured in the liver are affected by factors other than growth or GH. MDH, IDH and CS are oxidative enzymes, and IDH also provides NADPH for lipid synthesis. CS is an important enzyme of the citric acid cycle and is higher in transgenic compared with non-transgenic salmon at

both sampling times, suggesting that oxidative metabolism is increased in the liver in GH-transgenic salmon at 4 and 7 months. Previous studies reported higher oxygen consumption in GH-transgenic tilapia (McKenzie *et al.*, 2003) and in exercised GH-transgenic salmon (Stevens *et al.*, 1998) compared with non-transgenic fish of the same size. At 7 months IDH activity is significantly lower in GH-transgenic salmon compared with non-transgenic salmon suggesting a lower lipid synthesis in the liver. This result agrees with previous findings showing that GH treatment inhibited lipogenesis and a reduced fat content was reported in the carcass of GH-treated (Sorensen *et al.*, 1996) and GH-transgenic (Pursel *et al.*, 1990) farm animals. Fauconneau *et al.* (1997) reported a decrease in size of adipose cells after GH treatment in rainbow trout. It was also shown that GH improves feed conversion in both GH-treated (Campbell *et al.*, 1989) and GH-transgenic (Pursel *et al.*, 1990) domestic pigs and in GH transgenic tilapia (De la Fuente *et al.*, 1999). The extra GH in these transgenic salmon could also increase appetite and food conversion and ultimately act on fish metabolism to reduce fat accumulation (Fletcher *et al.*, 2004).

Most of the GH affects in vertebrates are thought to be mediated by IGF-I which is synthesised primarily in the liver. Reports in the literature of IGF-I effects on fish metabolism are rare. Castellio *et al.* (2004e) reported an increase in glucose uptake due to IGF-I stimulation on trout satellite cell.

My study demonstrates that ectopic GH effects fish enzymes. However, the conclusion that higher growth in GH-transgenic salmon is mediated by increases in metabolic enzyme activities is not warranted at this time. White muscle shows a correlation between fish weight and enzymes activities, but it is a negative correlation.

Muscle myosatellite cell proliferation and differentiation

Interest in muscle MyC has increased over the past few decades especially for mammals, thanks to their property of proliferation and differentiation and their role in renewing injured muscle fibers. Little is currently known about these cells in fish. Most fish grow throughout their life, maintaining a pool of quiescent satellite cell over this period (Valente *et al.*, 2002). MyC have been extracted and cultured from WM of various fish species, including salmon (Matschak and Stickland, 1995), but their proliferation was primarily demonstrated in cells isolated from rainbow trout (Greenlee *et al.*, 1995a,b; Rescan *et al.*, 1995; Fauconneau and Paboeuf, 2000). The differences in the yield of MyC between fish of 4 and 7 months agree with previous studies that reported numbers of MyC decrease with age of the fish (Alfei *et al.*, 1994; Greenlee *et al.*, 1995b; Koumans and Akster, 1995). This effect was found in different species including rainbow trout (Fauconneau and Paboeuf, 2001) and common carp (*Cyprinus carpio*) (Koumans *et al.*, 1990b). It is interesting that transgenic and non-transgenic salmon have similar numbers of WM MyC at the same age, indicating that the faster growth rates of the GH-transgenic salmon is not due to higher numbers of MyC in the pool available for muscle proliferation. One might predict that the transgenic salmon would have lower MyC numbers due to their larger size, but size seems to be less important than age in controlling MyC numbers at least in my study. Also, GH and possibly IGF-I that mediates many of the actions of GH, appear not to influence the number of MyC in salmon WM. Instead, the higher growth rate of transgenic salmon is, at least in part, due to a higher proliferative capacity of the WM MyC. This is the first time that a direct effect of GH is shown on teleost muscle MyC. GH is not effective in mammals, as it has no influence on MyC proliferation *in vitro* in ovine (Harper *et al.*, 1987) or rat (Allen and Rankin, 1990), indicating that the effects of GH on

mammalian MyC are mediated by other hormones or growth factors. However, GH did induce proliferation of avian MyC (Halevy *et al.*, 1996; Hodik *et al.*, 1997). Also, Weatherley and Gill (1982) and Fauconneau *et al.* (1997) showed that administration of GH *in vivo* increased WM hyperplasia in juvenile rainbow trout. The effect of IGF-I on MyC proliferation is well documented in vertebrates (Allen and Rankin, 1990; Duclos *et al.*, 1991; Florini *et al.*, 1991). Other studies from our group also revealed an increase in the *in vitro* proliferation of rainbow trout MyC with IGF-I stimulation (see Chapter IV). My results predict that both GH and IGF-I would affect fish MyC proliferation *in vitro*.

Muscle MyC in culture begin to differentiate after a few days into small myotubes. Quantitation of MyC differentiation is rarely demonstrated in fish. Extracted fish MyC express MyoD and myogenin and repeat embryonic myogenesis (Rescan *et al.*, 1994, 1995). The lower expression of MyoD I and myogenin in WM MyC extracted from the transgenic compared with the non-transgenic salmon can be explained by the fact that after 6 days MyC from the transgenic salmon have already reached the maximum differentiation allowed by the size of the plate and can not proliferate further and start to degenerate. It would be interesting in a future study to quantify the amount of MyoD and myogenin in MyC from transgenic and non-transgenic salmon at different times after plating by a method that requires fewer cells to observe any differences in the length of the different myogenic steps in the two salmon groups.

Tissue myogenesis

In my study, tissue myogenesis was examined directly as it represents what is actually happening in the fish at the moment of sampling, in contrast to cultured MyC that indicate the proliferation and differentiation *capacities* of these cells held in similar media.

Two distinct non-allelic MyoD genes, MyoD I and MyoD II are found in salmon. The two MyoDs share a similar sequence (Watabe, 2001) but my study demonstrates that their pattern of expression differs between RM and WM. Other fish species are reported to express two MyoDs including gilthead seabream (*Sparus aurata*) (Tan and Du, 2002) and rainbow trout (*O. mykiss*) (Delalande and Rescan, 1999), possibly explained by the ancient tetraploidization of the fish genome (Rescan and Gauvry, 1996; Delalande and Rescan, 1999). The differences in MyoD I and II expression in the two muscle tissues support the hypothesis of Rescan and Gauvry (1996) that these two homologues evolved separately. WM expresses only MyoD I, whereas both forms are present in RM. MyoD II may be more important in RM proliferation as there are significant differences among groups, whereas no differences exist in RM MyoD I between groups. These differences between MyoD expression in slow and fast muscles were also found in seabream (Tan and Du, 2002) and rainbow trout (Delalande and Rescan, 1999). Also the expression of myogenin was greater in RM as shown by the lower number of cycles necessary for amplification as reported by Rescan *et al.* (1995) in trout. Recent studies in zebrafish (Devoto *et al.*, 1996) and rainbow trout (Rescan *et al.*, 2001) revealed that slow and fast muscle fibers originate from two distinct cell populations. Other differences were reported between WM and RM in myostatin expression, a negative regulator of muscle growth (Roberts *et al.*, 2004). Expression of MyoD I in WM revealed that proliferation is increased in WM of GH-transgenic salmon compared with both age (at 4 months) and size controls. There is no effect of the transgene at 7 months on MyoD I expression. In RM the only difference is at 7 months and is opposite to that seen in WM.

Myogenin is required for myogenic differentiation (Rudnicki and Jaenisch, 1995). My results show that myogenin is expressed at higher relative amounts when relative

MyoD expression is low. Quantitative data on adult myogenesis in fish are scarce but our study suggests that proliferation and differentiation phases are happening at different times during the adult life of the fish.

Conclusions

In this study I tested the effects of a higher GH expression in transgenic Atlantic salmon on metabolism and myogenesis, to see if the properties of MyC or enzyme activities were involved in the higher growth rate found in transgenic fish. This study clearly shows that the process leading to a higher growth rate in transgenic salmon is more complex than simply higher metabolic rates or higher expression of MRFs in the muscles. However, enzymatic activities in WM were correlated with fish mass indicating that when considering metabolism, GH-transgenic salmon are physiologically more similar to their older non-transgenic conspecific. Moreover, the higher proliferation capacity of MyC extracted from GH-transgenic salmon compared with non-transgenic salmon could at least be part of the explanation for the higher growth rate in GH-transgenic salmon.

My study also demonstrated that proliferation and differentiation do not happen at the same time, and might respond to successive waves in both RM and WM showing that energy is not allocated in both muscles for proliferation and differentiation at the same time. Further studies need to be done to confirm this hypothesis. I also show for the first time that GH directly stimulates satellite cell proliferation in Atlantic salmon.

Table V-1: Sequences of the forward and reverse primers used in the study and length of the product obtained for each gene amplified.

Gene amplified	Gene bank sequence	Forward primer	Reverse primer	Product (bases)
MyoD I	AJ557148	5'GTGCTTGGATAAAAACGTGCGC3'	5'CAAAGAAATGCATGTCGC3'	195
MyoD II	AJ557149	5'GAGCCAGGATTACACTCGTTAC3'	5'CGAAGAAATGCATGTCGC3'	141
myogenin	AJ534875	5'GTGGAGATCCTGAGGAGTGC3'	5'GGTCCTCGTTGCTGTAGCTC3'	232
β-actin	AF012125	5'CGCCGCACTGGTTGTTGACA3'	5'GCGGTGCCCATCTCCTGCT3'	674
18S		5'GGAGGTTCGAAGACGATCAG3'	5'CTCAATCTCGTGTGGCTGAA3'	438
GH ectopic copy	Fletcher et al., 2004	5'GTCAGAAGTCTCAGCTACAGC3'	5'ACAGAAGTCCAGCAGGAATAT3'	135

Table V-2: Mass (g), length (cm), condition factor (CF), myosatellite cell yield and growth rate of GH-transgenic and non-transgenic salmon used in the experiment. Myosatellite cell yield is expressed as 10^6 cells per g tissue. Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) between non-transgenic and transgenic salmon. Specific growth rate = $(\ln W_b - \ln W_a) \cdot T^{-1}$, where W is the mean of the weight of the fish and T is the number of month separating the two weights.

	fish age	Mass	Length	CF	Satellite cell yield	Growth rate g per month
Non-transgenic salmon	4 month	2.83 ± 0.75 c	6.57 ± 0.49 c	0.98 ± 0.07 b	2.95 ± 0.24 a	
	7 month	16.15 ± 2.18 b	11.13 ± 0.58 b	1.17 ± 0.11 a	1.92 ± 0.10 b	0.58
Transgenic salmon	4 month	14.33 ± 3.32 b	11.75 ± 0.81 b	0.87 ± 0.10 c	2.71 ± 0.21 a	
	7 month	93.71 ± 21.03 a	21.38 ± 1.80 a	0.94 ± 0.06 b	2.17 ± 0.11 b	0.62

Table V-3: Enzyme activities (mean ± standard error) in the white muscle and intestine of GH-transgenic and non-transgenic Atlantic salmon (*Salmo salar*). All data are expressed as $\mu mol \cdot min^{-1}$ mg $protein^{-1}$, except for * which is in $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$; $N \geq 8$. Enzymes were assessed at 20 °C. ALT, Alanine aminotransferase; AST, aspartate aminotransferase; GDH, glutamate dehydrogenase ; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; IDH, isocitrate dehydrogenase; and, CS, citrate synthase. Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) between non-transgenic and transgenic salmon.

		Non-transgenic 4 month	Transgenic 4 month	Non-transgenic 7 month	Transgenic 7 month	Mass correlation	Probability
White muscle	Protein	30.63 ± 1.88 c	44.71 ± 1.88 b	62.85 ± 2.17 a	61.01 ± 2.17 a		
	AST	0.45 ± 0.03 ab	0.35 ± 0.03 b	0.56 ± 0.04 a	0.55 ± 0.04 a		
	ALT*	38.05 ± 3.35 a	38.26 ± 3.35 a	36.78 ± 3.38 a	20.95 ± 3.87 b	-0.548	p<0.006
	GDH*	35.05 ± 2.62 a	34.97 ± 2.62 a	21.70 ± 3.03 b	15.42 ± 3.03 b	-0.579	p<0.002
	LDH	8.15 ± 0.88 c	10.89 ± 0.88 b	19.48 ± 1.02 a	0.64 ± 1.02 d	-0.615	p<0.001
	MDH	2.36 ± 0.16 a	1.31 ± 0.16 b	1.73 ± 0.19 ab	2.16 ± 0.19 a		
	IDH	0.19 ± 0.01 a	0.14 ± 0.01 ab	0.11 ± 0.01 b	0.09 ± 0.01 b	-0.525	p<0.012
	CS	0.67 ± 0.04 a	0.64 ± 0.04 a	0.61 ± 0.04 a	0.38 ± 0.04 b	-0.695	p<0.001
Intestine	Protein	13.67 ± 0.85	15.62 ± 0.61	16.11 ± 0.70	15 27 ± 0.66		
	CS	0.44 ± 0.03 b	0.47 ± 0.02 b	0.50 ± 0.03 ab	0.57 ± 0.03 a	0.49	p<0.002

Table V-4: Enzyme activities (mean ± standard error) in the red muscle of GH-transgenic and non-transgenic Atlantic salmon (*Salmo salar*). All data are expressed as $\mu mol \cdot min^{-1}$ mg $protein^{-1}$, except for * which is in $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$; $N \geq 8$. Enzymes were assessed at 20 °C. See Table V-3 for the list of abbreviations of the enzymes. n.a.: red muscle could not be sampled from control salmon at 4 months due to the small size of the fish. Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) between non-transgenic and transgenic salmon.

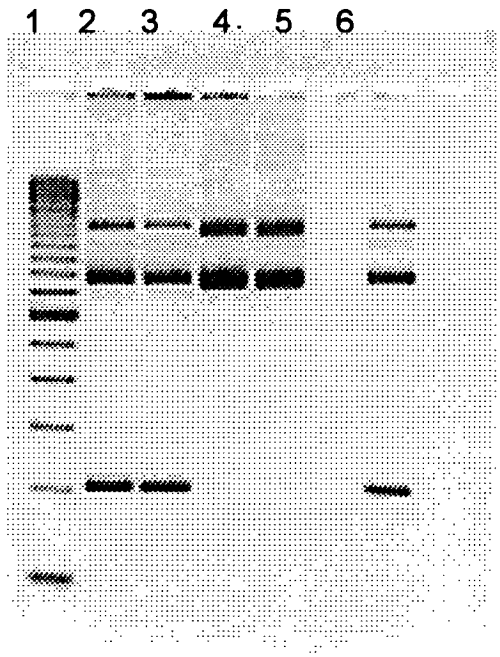
	Non-transgenic 4 month	Transgenic 4 month	Non-transgenic 7 month	Transgenic 7 month
Protein	n.a	10.44 ± 0.38 ab	11.73 ± 0.45 a	9.92 ± 0.40 b
PK	n.a	9.44 ± 0.41 b	12.80 ± 0.48 a	10.24 ± 0.43 b
AST	n.a	4.05 ± 0.26 b	6.21 ± 0.31 a	4.29 ± 0.28 b
ALT	n.a	0.35 ± 0.02 b	0.51 ± 0.02 a	0.37 ± 0.02 b
GDH*	n.a	63.50 ± 4.32	61.01 ± 5.06	59.06 ± 4.53
LDH	n.a	38.93 ± 1.62 b	68.83 ± 1.90 a	40.47 ± 1.70 b
MDH	n.a	13.59 ± 0.71 b	23.77 ± 0.84 a	8.22 ± 0.75 b
IDH	n.a	1.93 ± 0.12 ab	2.30 ± 0.14 a	1.56 ± 0.12 b
CS	n.a	1.63 ± 0.07 a	1.73 ± 0.07 a	1.05 ± 0.07 b

Table V-5: Enzyme activities (mean ± standard error) in the liver of GH-transgenic and non-transgenic Atlantic salmon (*Salmo salar*). All data are expressed as $\mu mol \cdot min^{-1}$ mg $protein^{-1}$, except for * which is in $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$; $N \geq 8$. Enzymes were assessed at 20 °C. PEPCK, phosphoenolpyruvate carboxykinase; G6PDH, glucose-6-phosphate dehydrogenase; see Table V-3 for list of other enzyme abbreviations. Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) between non-transgenic and transgenic salmon.

	Non-transgenic 4 month	Transgenic 4 month	Non-transgenic 7 month	Transgenic 7 month
Protein	100.07 ± 4.12 ab	83.20 ± 2.92 b	96.88 ± 3.37 ab	97.89 ± 3.19 a
PEPCK*	12.68 ± 1.09	15.05 ± 0.77	16.31 ± 0.89	14.65 ± 0.84
PK	0.40 ± 0.05 b	0.59 ± 0.03 ab	0.68 ± 0.04 a	0.54 ± 0.04 ab
AST	1.13 ± 0.15	1.56 ± 0.11	1.65 ± 0.13	1.41 ± 0.12
ALT	0.71 ± 0.09	0.82 ± 0.06	0.58 ± 0.07	0.65 ± 0.07
GDH	0.37 ± 0.04	0.31 ± 0.03	0.24 ± 0.03	0.26 ± 0.03
LDH	4.76 ± 0.54	4.95 ± 0.38	5.97 ± 0.44	5.00 ± 0.42
MDH	8.85 ± 1.19 ab	11.43 ± 0.84 ab	12.39 ± 0.97 a	7.89 ± 0.92 b
IDH	0.14 ± 0.01 ab	0.16 ± 0.01 a	0.18 ± 0.01 a	0.13 ± 0.01 b
G6PDH *	67.34 ± 0.53	82.89 ± 0.38	71.57 ± 0.43	76.25 ± 0.41

Figure V-1: PCR analysis of the GH gene copies from fin clips of GH-transgenic and non-transgenic Atlantic salmon (*Salmo salar*) A). The sequence of the primers used to amplified GH was 5'GCT CTT CAA CAT CGC GGT CA3'(F) and 5'ATA TGG AGC AGC TTC AGG AC3'(R). The PCR conditions were 92 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, for 32 cycles. The primers anneal to the Chinook salmon GH cDNA within the EO-1α transgene producing a 207 bases fragment. In addition, the primers also anneal within the endogenous Atlantic salmon GH DNA sequence, producing fragments of 798 and 1150 bases The PCR amplification and subsequent detection of this GH fragment indicates that the sample came from a transgenic fish. Lanes 1, DNA ladder; 2, 3, fin clip from GH-transgenic salmon; 4, 5, fin clip from non-transgenic salmon; 6, negative control (H_2O); 7, positive control. B) PCR analysis of GH mRNA transcripts in white muscle myosatellite cells extracted from GH-transgenic and non-transgenic Atlantic salmon (*Salmo salar*). The PCR primer used (see Table V-1) anneals to the ocean pout antifreeze promoter, explaining the fact that it doesn't amplify any of the endogenous GH gene. Lanes 1, 2 and 5, satellite cells extracted from transgenic salmon; 3, 4, satellite cells extracted from non-transgenic salmon; 6, positive control; 7, negative control (H_2O).

A



B

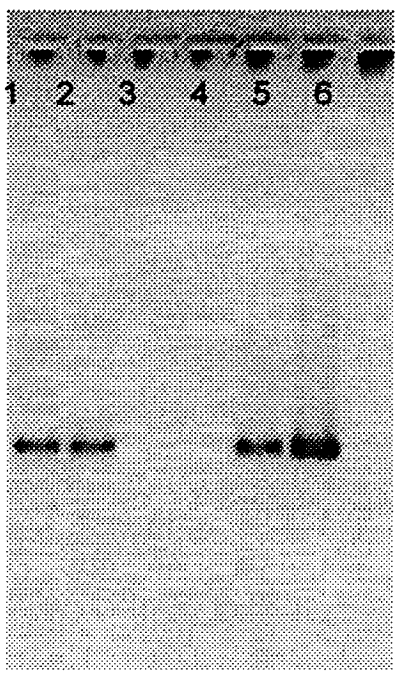


Figure V-2: Pyruvate kinase activity in GH-transgenic and non-transgenic salmon white muscle. N ≥ 8 (mean + standard error); *, indicates that the bars beneath the lines are significantly different from one another (Tukey-Kramer, $\alpha < 0.05$).

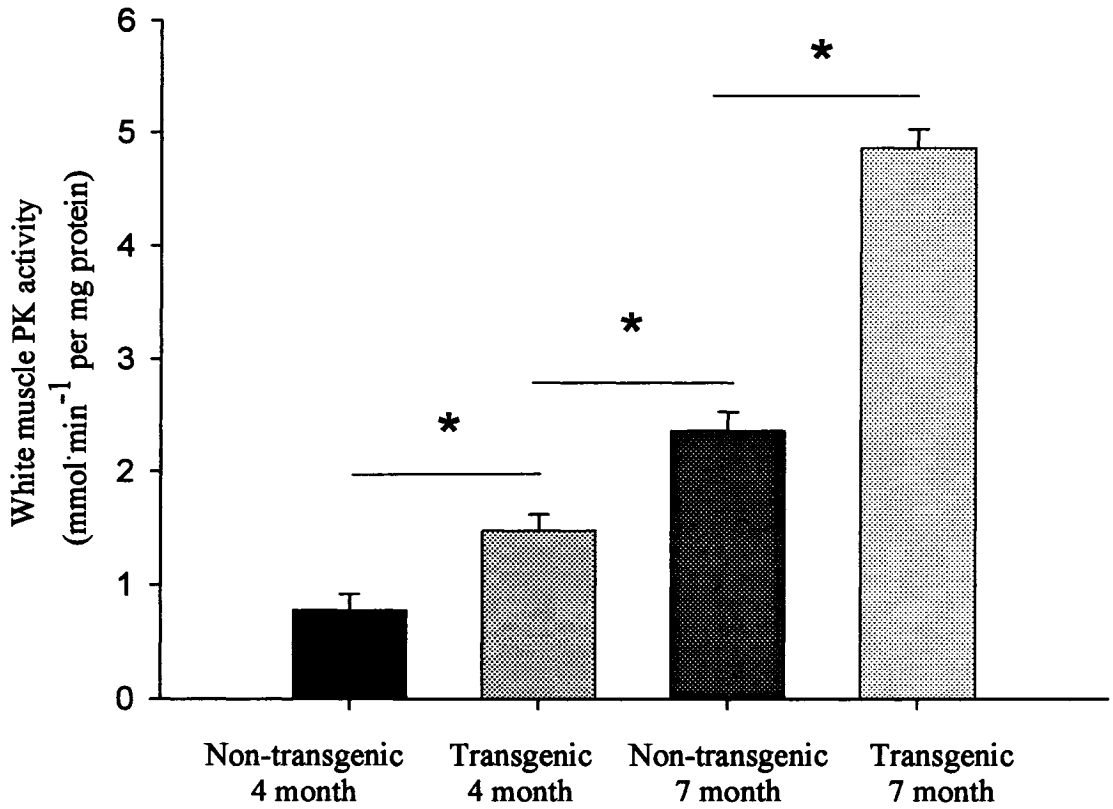


Figure V-3: Citrate synthase activity in GH-transgenic and non-transgenic salmon liver. N ≥ 8 (mean + standard error); *, indicates that the bars beneath the lines are significantly different from one another (Tukey-Kramer, $\alpha < 0.05$).

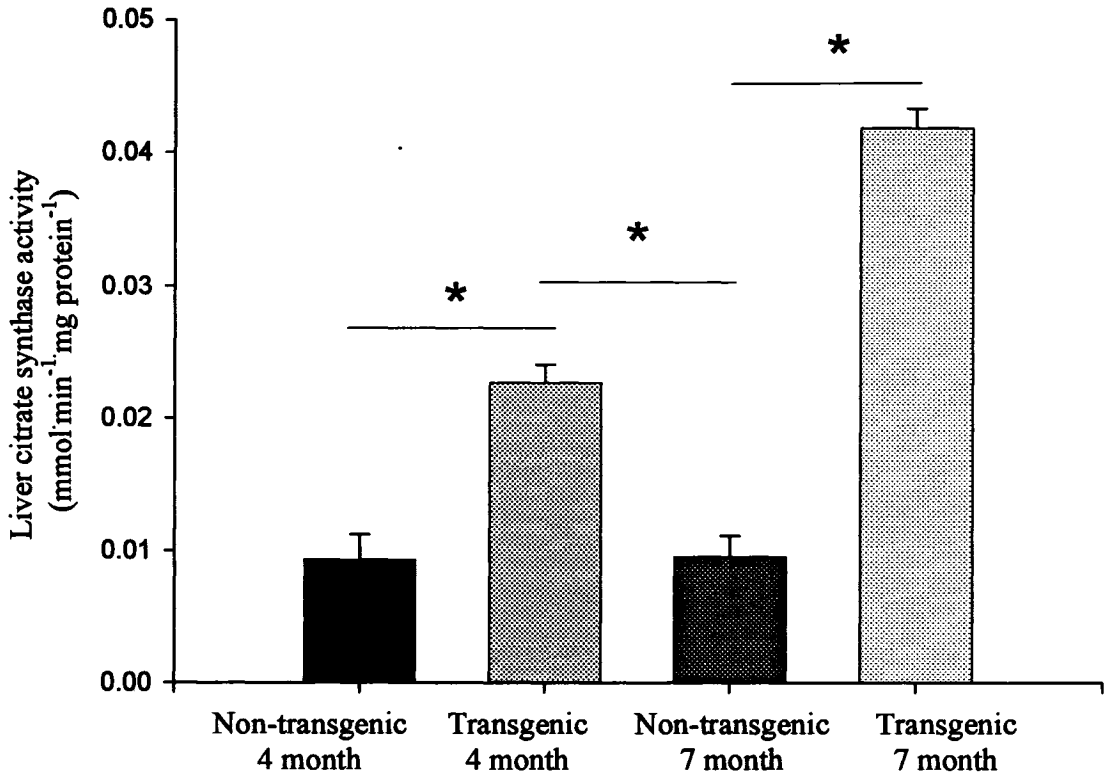


Figure V-4: White muscle myosatellite cell proliferation *in vitro*. ●, Myosatellite cells extracted from 4 month old GH-transgenic salmon; ○, myosatellite cells extracted from 4 month old non-transgenic salmon;▼, myosatellite cells extracted from 7 month old non-transgenic salmon. N = 3 (mean ± standard error); *, indicates significant differences between transgenic and non-transgenic fish at 4 months (Tukey-Kramer, $\alpha < 0.05$); a, indicates significant differences between transgenic salmon at 4 months and non-transgenic salmon at 7 months.

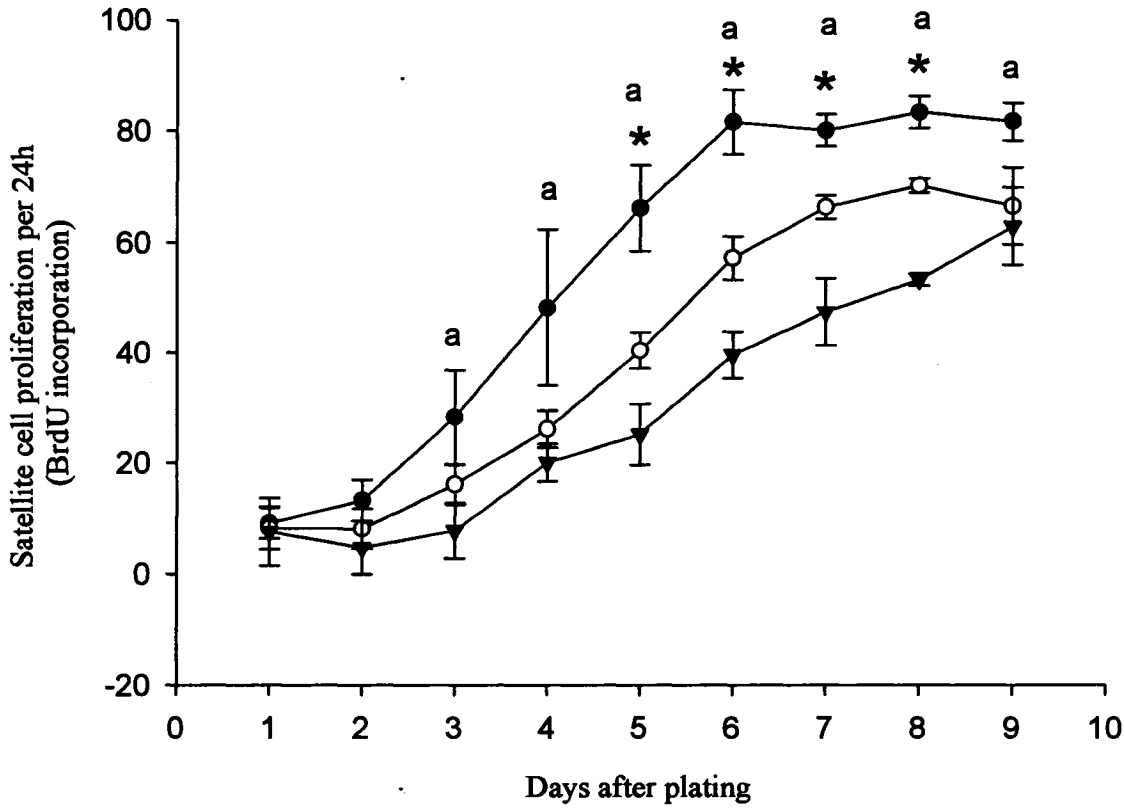


Figure V-5: Non-transgenic salmon white muscle myosatellite cell proliferation *in vitro* incubated with growth hormone at 10 $ng \cdot L^{-1}$ (GH10) and 50 $ng \cdot L^{-1}$ (GH50). N = 3 (mean + standard error); *, significantly different from control (Tukey-Kramer, $\alpha < 0.05$).

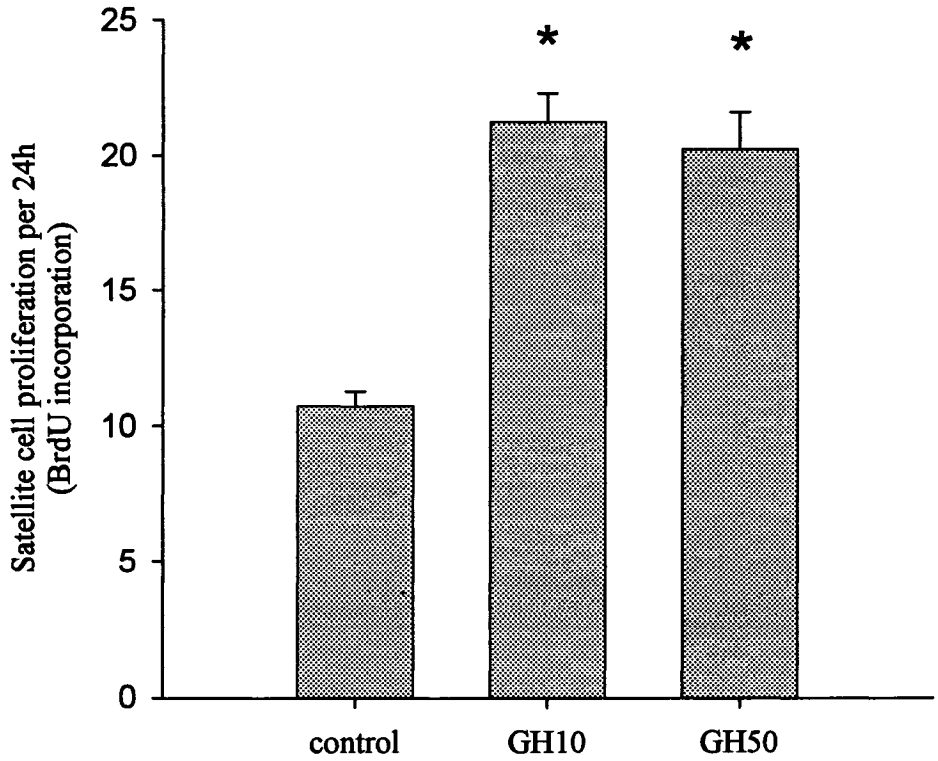
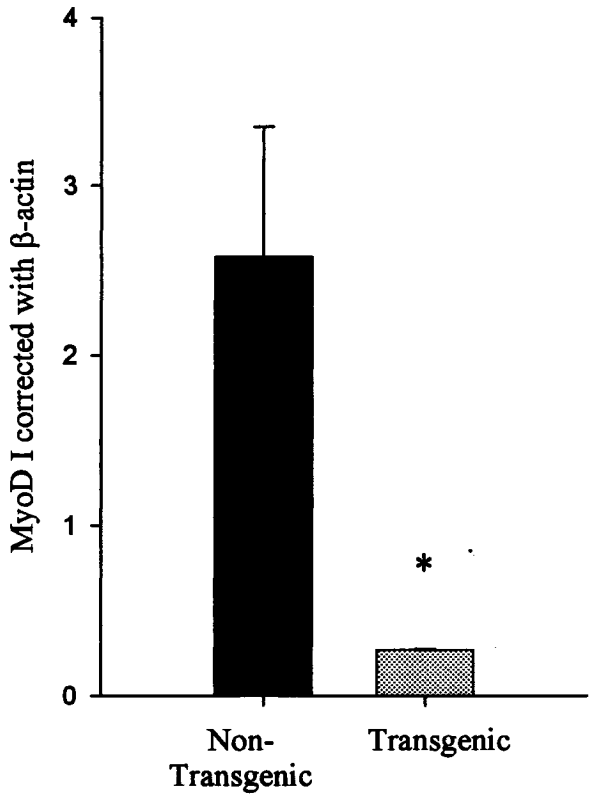
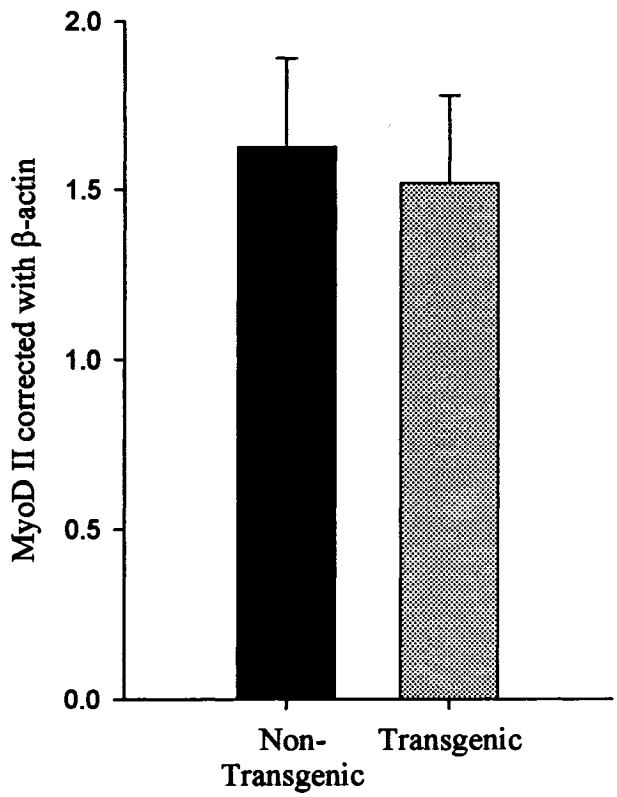


Figure V-6: Relative expression of A) MyoD I, B) MyoD II and C) myogenin mRNA in GH-transgenic and non-transgenic Atlantic salmon in white muscle myosatellite cells 6 days after plating. N=3 (mean + standard error); *, indicates significant difference compared with the non-transgenic fish (Tukey-Kramer, $\alpha < 0.05$). Relative band intensities (gene of interest compared with β-actin) were determined from the densities of the resulting gel bands.


MyoD I corrected with β-actin
4
3
2
1
0
*
Non-Transgenic
Transgenic


MyoD II corrected with β-actin
2.0
1.5
1.0
0.5
0.0
Non-Transgenic
Transgenic

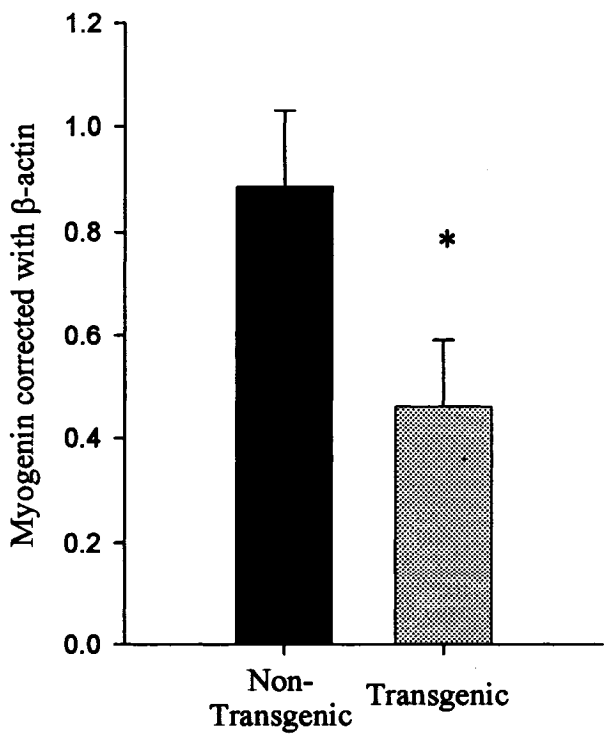

Myogenin corrected with β-actin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Non-Transgenic
Transgenic

Figure V-7: Relative expression of MyoD I mRNA in white muscle of GH-transgenic and non-transgenic Atlantic salmon at 4 and 7 months of age. $N \geq 8$ (mean + standard error); *, indicates that the bars beneath the line are significantly different (Tukey-Kramer, $\alpha < 0.05$). See Fig. V-6 for the definition of Relative Expression.

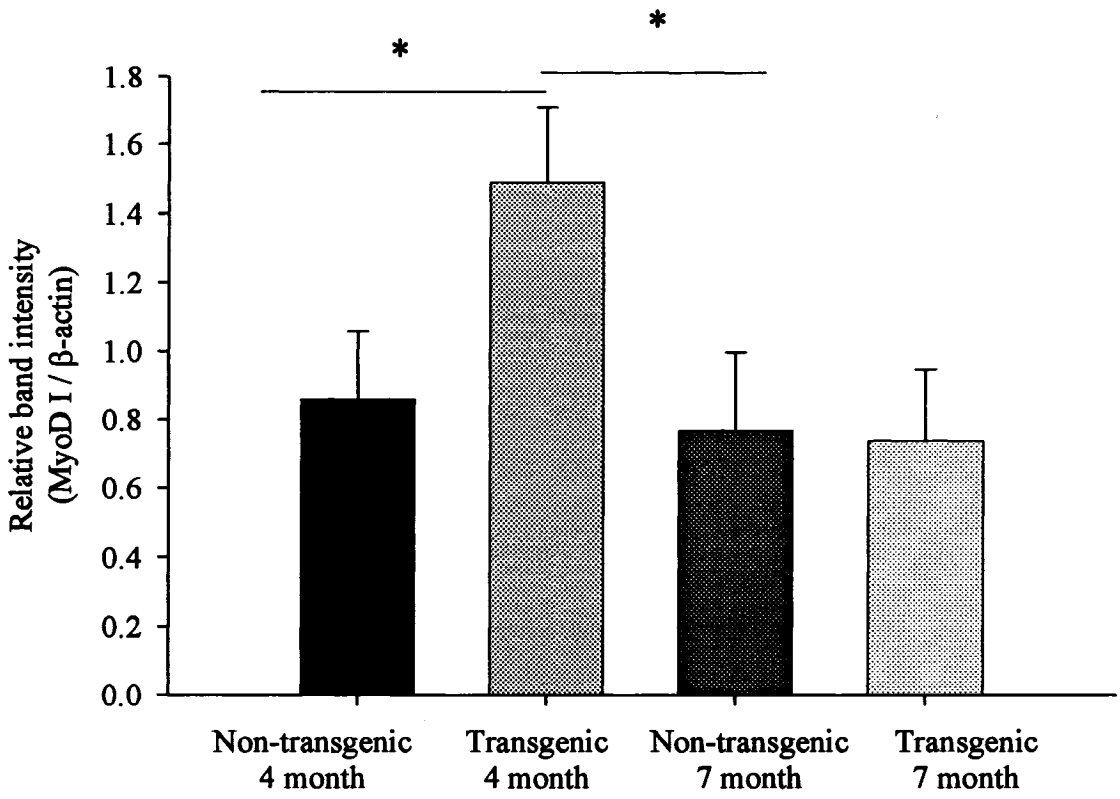


Figure V-8: Relative expression of myogenin mRNA in white muscle of GH-transgenic and non-transgenic Atlantic salmon at 4 and 7 months of age. $N \geq 8$ (mean + standard error); *, indicates that the bars beneath the line are significantly different (Tukey-Kramer, $\alpha < 0.05$). See Fig. V-6 for the definition of Relative Expression.

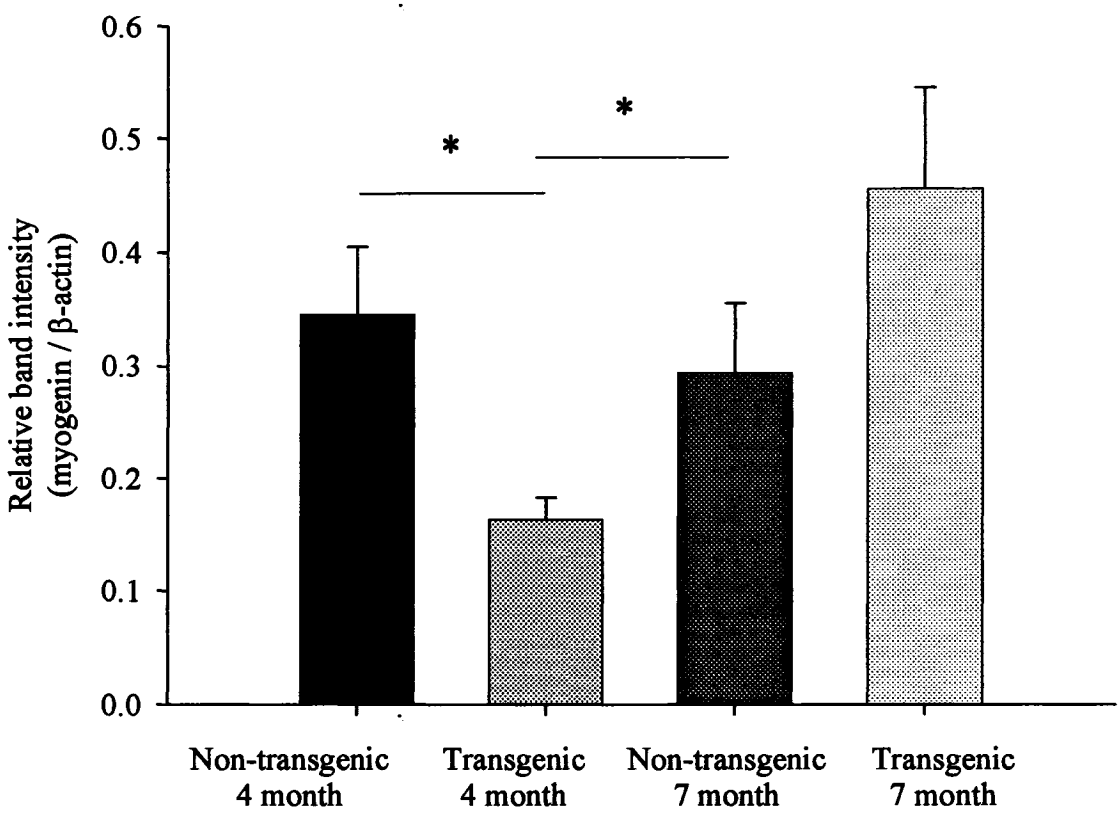


Figure V-9: Relative expression of A) MyoD I and B) MyoD II mRNA in red muscle of GH-transgenic and non-transgenic Atlantic salmon at 4 and 7 months of age. $N \geq 8$ (mean + standard error); *, indicates that the bars beneath the line are significantly different (Tukey-Kramer, $\alpha < 0.05$). n.a,: red muscle could not be sampled from control salmon at 4 months due to the small size of the fish. See Fig. V-6 for the definition of Relative Expression.

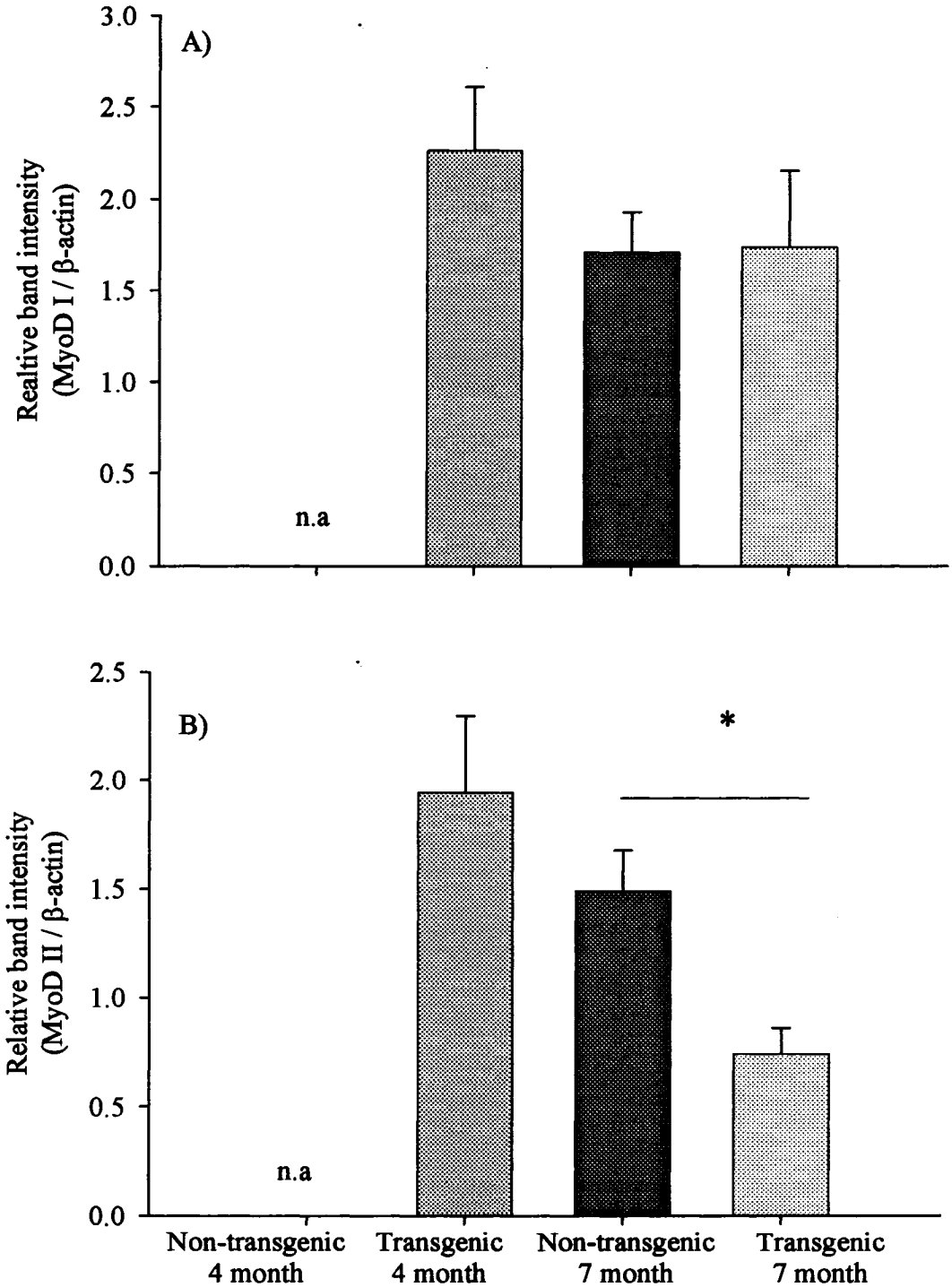
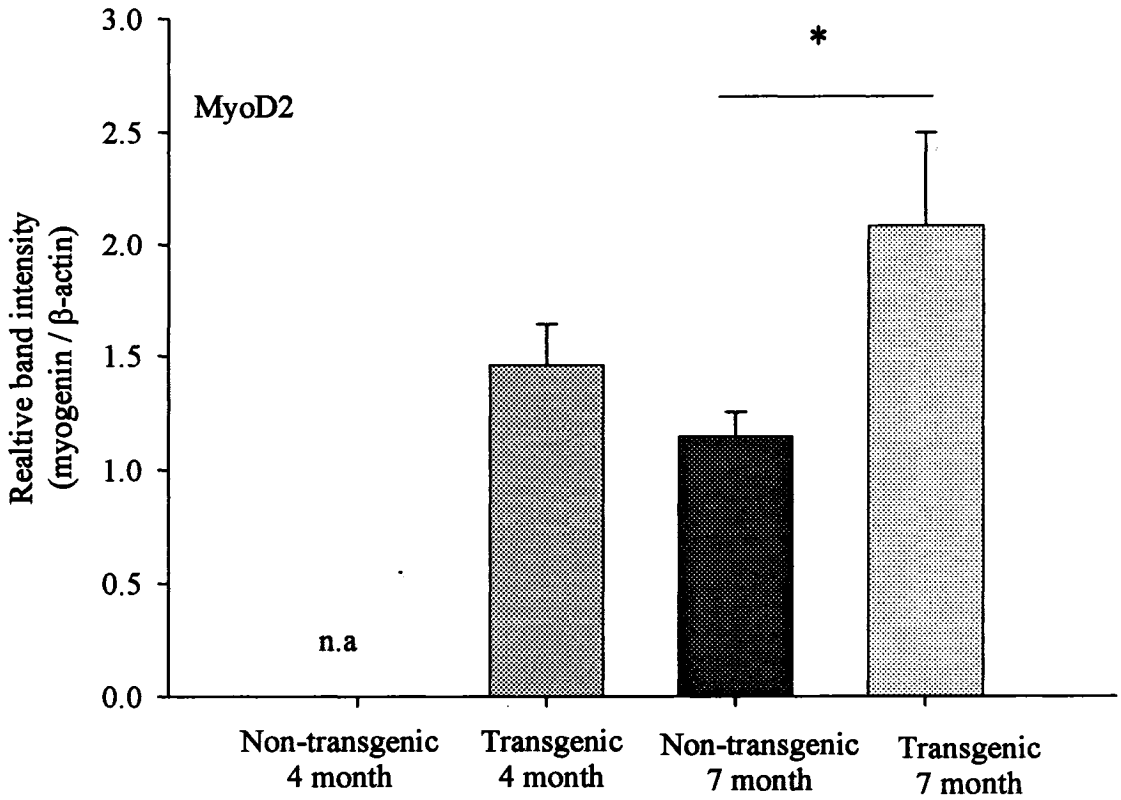

A)
Realtive band intensity
(MyoD I / β-actin)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
n.a
B)
Relative band intensity
(MyoD II / β-actin)
2.5
2.0
1.5
1.0
0.5
0.0
n.a
*
Non-transgenic
4 month
Transgenic
4 month
Non-transgenic
7 month
Transgenic
7 month

Figure V-10: Relative expression of myogenin RNA expression in red muscle of GH-transgenic and non-transgenic Atlantic salmon at 4 and 7 months of age. $N \geq 8$ (mean + standard error); *, indicates that the bars beneath the line are significantly different (Tukey-Kramer, $\alpha < 0.05$). n.a,: red muscle could not be sampled from control salmon at 4 months due to the small size of the fish. See Fig. V-6 for the definition of Relative Expression.



CHAPTER VI: EFFECTS OF β_2-ADRENERGIC AGONISTS ON MYOGENESIS AND TISSUE METABOLISM IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

Abstract

The two β_2-adrenergic agonists (β-AA) clenbuterol (CLEN) and ractopamine (RACT) were fed to juvenile rainbow trout *Oncorhynchus mykiss* for 12 weeks at two different times (January to April 2002, called spring; and, November 2002 to February 2003, called winter) and using food of different lipid contents (11 vs 16%). Myogenesis was assessed in red and white muscles and enzyme activities as an index of metabolism were measured in skeletal muscle, liver and kidney. Muscle myosatellite cells were extracted from white muscle to assess proliferation using the bromodeoxyuridine (BrdU) assay. Feeding RACT and CLEN at 10 ppm had minor effects on body parameters. However, plasma amino acids, total protein and lactate decreased, and liver and white muscle total protein increased in CLEN-treated fish following the spring exposure. CLEN and RACT affected liver, red and white muscle protein metabolism, glycolysis and citric acid cycle enzyme activities. CLEN- and RACT-feeding significantly increased white muscle myosatellite cell proliferation and CLEN significantly increased white muscle MyoD and myogenin mRNA expression. Myogenin was also increased in red muscle following CLEN-exposure. These results demonstrate that the amount of fat contained in the diet had a significant impact on the affects of CLEN- and RACT-feeding in rainbow trout. The small differences and contradictory changes observed on body parameters and on metabolic enzyme activities suggest that β_2-AA feeding has a limited effect on rainbow

trout metabolism. However, this study did show a significant impact of β_2-AA on white and red muscle composition and myogenesis. Further studies are needed to determine whether these agonists would affect fish filet quality or positively affect the aquaculture of this species.

Introduction

The adrenergic system, characterized by the catecholamine hormones adrenaline and noradrenaline, integrates and modulates many aspects of vertebrate metabolism and muscle growth (myogenesis), including those in fish. β_2-Adrenergic agonists (β_2-AA; synthetic catecholamine analogues that bind to and signal through the β_2-adrenergic receptor) increase muscle growth (Maltin *et al.*, 1992; Navegantes *et al.*, 2001) and act as repartitioning agents in domestic feed mammals by re-directing nutrients from adipose tissue to muscle (Roberts and McGeachie, 1992). β_2-AA also increase weight gain, feed utilization, lean body mass and protein accretion in mammals. However, studies in fish using β_2-AA are limited and restricted to reports on residue distribution (Brambilla *et al.*, 1994) and effects on body composition (Mustin and Lovell, 1992; Webster *et al.*, 1995; Vandenberg *et al.*, 1998). Lortie *et al.* (2004) did report a significant increase in fractional protein synthesis rates in whole protein, myofibrillar protein and sarcoplasmic soluble protein fractions of red (RM) and white (WM) muscles from rainbow trout (*Oncorhynchus mykiss*) fed 40 ppm ractopamine (RACT) and clenbuterol (CLEN) for 30 days.

The primary role of adrenergic innervation is to regulate the tone of smooth muscle associated with blood vessels and to increase the performance of the heart and of the respiratory system, but a specific metabolic role also exists. Effects of β_2-AA on individual

metabolic enzymes have yet to be investigated, but catecholamine hormones do act on tissue carbohydrate and lipid metabolism (Fabbri *et al.*, 1998). Catecholamines are also implicated in the response of animals to stressors including exercise, but also in catabolic reactions such as trauma and injury (Finley *et al.*, 2004). As catecholamines promote the break down of glycogen and fat, they are classified as catabolic hormones (Lundberg, 2005). However several studies demonstrate that catecholamine agonists have anabolic effects on protein metabolism (Navegantes *et al.*, 2001). Byrem *et al.* (1998) reported an increase in muscle amino acid uptake and enhanced muscle protein accretion in cattle following hind-limb infusion of the β_2-AA cimaterol. β_2-AA also have a preventive effect on muscle proteolysis and degradation, possibly linked to changes in calpastatin mRNA levels (Navegantes *et al.*, 2000, 2002). Myosatellite cells (MyC) are responsible for skeletal muscle growth (Fauconneau and Paboeuf, 2000). To better understand muscle growth, it is critical to understand the processes of MyC proliferation and differentiation. The factors responsible for these processes in MyC include a family of myogenic regulatory factors (MRFs), among them MyoD and myogenin, that are implicated in the conversion of quiescent MyC into mitotically active myoblasts, and ultimately the proliferation and differentiation of these myoblasts to muscle fiber types (Olson and Klein, 1994). These transcription factors control muscle specific genes, including the myosin heavy chain gene (Wheeler *et al.*, 1999), that are characteristic of the different muscle fiber types. In fish, MyoD is generally associated with proliferation of MyC, whereas myogenin characterizes differentiation of MyC into muscle cells (Watabe, 2001). During development of rat skeletal muscle, MyoD and myogenin proteins accumulate differentially in slow and fast muscle types (Hughes *et al.*, 1993, 1997), suggesting that MyoD and myogenin may regulate slow and fast myosin expression. Previous studies reported that β_2-AA and in

particular CLEN affects MyoD expression in muscle (Delday and Maltin, 1997; Jones *et al.*, 2004) and induces the transition of slow-type I to fast-type II fibers (Jones *et al.*, 2004), possibly by signaling through MyoD since changes in muscle fiber types were associated with altered levels of MyoD expression (Hughes *et al.*, 1993; Kraus and Pette, 1997).

Altering the concentrations of MRFs in muscle may also affect muscle metabolism. For example, over-expression of myogenin in transgenic mice was correlated with an increase in oxidative enzymes but no changes in myosin heavy chain expression (Hughes et al., 1999).

The use of β_2-AA was shown to increase the accretion of skeletal muscle protein in domesticated feed animals (Mersmann, 1998; Mills, 2000). RACT is currently commercially used in swine production, and was tested in rainbow trout (Vandenberg and Moccia, 1998; Lortie *et al.*, 2004), and channel catfish (*Ictalurus punctatus*) (Mustin and Lovell, 1992) but effects were less dramatic than in other vertebrates. Moreover the effective use of β_2-AA in an aquaculture situation requires better knowledge of how they may impact fish muscle myogenesis and metabolism.

The hypothesis of Chapter VI is that β-agonists will increase rainbow trout growth rate by acting on myogenesis and metabolic enzymes. The predictions are that rainbow trout fed clenbuterol and ractopamine will have a higher expression of myogenic factors, higher muscle satellite cell proliferation and higher metabolic rate that sham fed rainbow trout. To better understand the potential effects of β_2-AA on fish muscle, I examined the effects of diet sprayed with 10 ppm RACT or CLEN, two β_2-AA from the group of phenethanolamines, on rainbow trout (~6 g at the start of the experiment) intermediary metabolism, muscle growth and myosatellite cells (MyC). Two different exposure periods

were completed, one from January to April 2002 (called spring) and the other from November 2002 to February 2003 (called winter). After 12 weeks of exposure, body parameters were determined and tissues collected for enzyme and metabolite assays. Additional fish were used for MyC extraction to characterize *in vitro* proliferation.

Material and Methods

Experimental animals and body parameters

Juvenile rainbow trout (*Oncorhynchus mykiss*) (~6 g) were obtained from Linwood Acres Trout Farm (Campbellcroft, ON). Fish were held in the University of Ottawa Aquatic Care Facility in small 110-115 L fiberglass stock tanks supplied with flowing, aerated, and dechloraminated City of Ottawa tap water of 13 ± 1°C and a 12L:12D photoperiod. Fish were divided into three groups of 100 fish per tank. Throughout this two week acclimation period, fish were hand-fed to satiation (~2 % body weight) every day on a commercial diet (Purina Trout Chow).

The feeding experiment was repeated twice from January to April 2002 (called spring) and from November 2002 to February 2003 (called winter). During the spring exposure, trout were fed a commercial diet containing 41% crude protein (minimum), 11% fat (minimum), 3.5% crude fiber (maximum), 1% calcium (actual), 0.85% phosphorus (actual), 0.45% sodium (actual), 6800 IU·kg^{-1} vitamin A (minimum), 2100 IU·kg^{-1} vitamin D (minimum), 80 IU·kg^{-1} vitamin E (minimum), and 200 IU·kg^{-1} vitamin C (minimum). Trout exposed during the winter period received the same food except that the fat content was 16 rather than 11%. Fish were fed for 12 weeks one of the following three

experimental diets: commercial diet sprayed with 10 ppm RACT, 10 ppm CLEN or carrier (SHAM). Commercial trout pellets were carefully and evenly sprayed with carrier that consisted of 25 ml 95% ethanol solution containing the antioxidant ascorbic acid (0.1 $mg \cdot ml^{-1}$) and either10 ppm of CLEN or RACT; SHAM food was treated only with the carrier. Analyses of the CLEN-treated food revealed an agonist concentration similar to that spread on the food (Lortie *et al.*, 2004). Fish were ~6 g at the beginning of the experiment and ~30 g after 12 weeks.

After 10 to 12 weeks of feeding, 12 fish per group were sacrificed to collect samples of blood, liver, red and white muscles and kidney for analysis of metabolic enzymes and metabolites; liver and visceral lipid were removed and weighed to calculate a hepatosomatic index (HSI = [(liver mass) • $(body\ mass)^{-1}$] x 100) and lipid somatic index (LSI = [(fat mass) • (body mass)-1] x 100). Small pieces of red and white muscles were used for RNA extraction for myogenic studies. All tissues were frozen on dry ice and stored at -80 °C until used. The remaining trout were used for the extraction of myosatellite cells (MyC) to characterize *in vitro* proliferation of cells extracted from β-AA-exposed fish and to characterize proliferation and differentiation of MyC from control fish exposed *in vitro* to the β-agonists.

Myosatellite cell isolation and culture

Myosatellite cells were isolated from trout white muscle following the winter exposure period using the procedures of Fauconneau and Paboeuf (2000) described in Chapter III.

Myogenesis

RNA was extracted from frozen red and white muscle pieces using 1 ml TRIzol per 65 to 85 mg of tissue according to the manufacturer's instructions (Invitrogen, Life Technologies, Burlington, ON, Canada). Myogenesis was assessed by the presence of MyoD I, MyoD II and myogenin mRNA in total RNA isolated from the muscle as described in Chapter III. Specific primers were developed using Primer 3 software (Whitehead Institute, Cambridge, MA) for MyoD I and MyoD II using the sequences available in Rescan and Gauvry (1996) and GenBank for myogenin, 18S, elongation factor 1α (ElF-1α) and β-actin. The primer sequences and accession numbers are noted on Table VI-1.

MyoD I, myogenin, 18S, ElF-1α and β-actin were amplified for 27, 27, 13, 24 and 22 cycles, respectively for white muscle tissue mRNA. MyoD I, MyoD II, myogenin, 18S, ElF-1α and β-actin were amplified for 26, 27, 26, 13, 24 and 20 cycles for red muscle tissue mRNA. The PCR conditions are decribed in Chapter III. The bands obtained were quantified using the Quantity One computer program (Bio Rad) and the data are expressed using relative band intensities of the gene of interest and ElF-1α expressed in the same tissue. ElF-1α was used as a control gene, but it is important to note that the results were the same whether 18S, ElF-1α or β-actin were used as a comparator transcript.

Enzymes activities

Eight rainbow trout from each experimental group were used for enzyme analyses. Enzyme activities were measured under saturating substrate conditions using standard procedures (Moon and Mommsen, 1987) as previously described in Chapter III.

Plasma metabolites

Plasma lactate and glucose were measured enzymatically using lactate dehydrogenase (LDH) and hexokinase plus glucose-6-phosphate dehydrogenase, respectively, as described in Chapter II.

Clenbuterol measurements

Plasma samples were assayed for CLEN using a specific EIA kit (Neogen, Lexington, KY, U.S.A.) according to the manufacturer's instructions and as previously reported (Lortie *et al.*, 2004). No similar commercial kit is available for estimates of RACT.

Statistics

Statistical analyses were performed using SYSTAT (version 10). Normality was tested using Shapiro-Wilk; all data were normaly distributed. One-way analysis of variance (ANOVA) was performed on each parameter measured. When the one-way ANOVA showed significant effects, multiple mean comparisons were made using the Tukey-Kramer comparison. Simple correlations were performed on enzymes and fish mass and SYSTAT was used to assess levels of significance.

Results

The RACT and CLEN feeding experiments were repeated twice with the same trout food but containing different fat contents (11% in spring; 16% in winter) and at two different seasons (spring and winter); however temperature and photoperiod remained

identical between the two experiments. There were no significant differences in body mass or length or hepatosomatic index (HSI) following CLEN-, RACT- or SHAM-feeding regardless of diet or season (Table VI-2). However, liver mass in the spring experiment was significantly ($p<0.01$) lower in CLEN-treated and lower but not significantly in RACT-treated fish. In the winter exposure, liver mass was significantly lower ($p<0.01$) in CLEN- and RACT-treated fish compared to SHAM-fed trout. Condition factor (CF) was significantly higher ($p<0.01$) in β_2-AA compared with SHAM-fed trout following the spring experiment; this difference was not apparent in the winter with the high-fat diet (Table VI-2). In both experiments the lipid somatic index (LSI) was significantly lower ($p<0.01$) in CLEN-fed trout, showing that the fat content of the diet had no effect on the capacity to use body fat in the CLEN-treated trout. It is interesting to note that the LSI is generally higher during the spring than the winter experiment even though the fat content of the winter diet was higher.

CLEN was detected in the plasma of all CLEN-fed (16.47 ± 6.44 nM, $n = 10$) but not SHAM-fed (0.47 ± 0.09 nM, $n = 10$) trout and 4 weeks after the CLEN-diet was stopped (recovery group) (0.62 ± 0.20 nM, $n = 10$). Plasma lactate content was reduced with CLEN-feeding compared with the other two groups in the spring (Fig. VI-1A) but no differences were noted among groups following the winter experiment (SHAM: 3.31 ± 0.24 nM; CLEN: 3.36 ± 0.17 nM; RACT: 3.43 ± 0.23 nM). In a similar manner, plasma amino acids were lower in CLEN-fed compared with SHAM- and RACT-fed trout (Fig. VI-1B); plasma amino acid concentrations were not measured following the winter experiment. CLEN-feeding also decreased plasma total protein in both experiments (Fig. VI-1C, VI-1D). Plasma glucose levels were similar among groups after β_2-AA feeding in the spring experiment (SHAM: 1.15 ± 0.11 mg·ml^{-1}; CLEN: 1.01 ± 0.16 mg·ml^{-1}; RACT:

1.25 ± 0.08 mg·ml^{-1}; n = 10), but a small non-significant increase was noted in the winter experiment with CLEN- and RACT-feeding (SHAM: 0.99 ± 0.05 mg·ml^{-1}; CLEN: 1.23 ± 0.09 mg·ml^{-1}; RACT: 1.31 ± 0.17 mg·ml^{-1}, n = 10).

Liver and white muscle (WM) protein contents were significantly higher in CLEN-fed trout in the spring experiment (Table VI-3A, VI-5A) and liver mass was decreased with β_2-AA treatment in both the spring and winter samplings (Table VI-2). As a result enzyme activities are expressed in the liver as activities (μmol•min^{-1}) per total liver mass, whereas in skeletal muscle and kidney as activities (μmol•min^{-1}) per g tissue weight. Total hepatic lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and malate dehydrogenase (MDH) activities were higher in the spring CLEN-fed group compared with either the RACT- or SHAM-fed fish (Table VI-3A). In the winter experiment, liver LDH, phosphoenolpyruvate carboxykinase (PEPCK), ALT, glutamate dehydrogenase (GDH) and isocitrate dehydrogenase (IDH) activities were higher in the SHAM-fed group compared with either the CLEN- or RACT-fed fish. However, liver MDH activity was increased in CLEN- and RACT-fed fish compared with the SHAM fish (Table VI-3B). In the spring experiment, kidney LDH activities were lower in SHAM-fed fish compared with CLEN- and RACT-fed fish (Table VI-4) as was MDH activity but only compared with the RACT-fed fish (Table VI-4). White muscle (WM) enzymes were not significantly different after the spring experiment between β_2-AA and SHAM-fed fish (Table VI-5A). However, after the winter experiment, LDH and MDH activities were lower and ALT and GDH activities were higher in CLEN- and RACT- compared with SHAM-fed trout. IDH activity was lower in WM of CLEN-compared with SHAM-fed trout (Table VI-5B). In the spring, pyruvate kinase (PK) and IDH were higher in red muscle (RM) of RACT-fed compared with SHAM- and CLEN-fed trout (Table VI-6A). LDH and aspartate aminotransferase

(AST) activities in the winter experiment were higher and ALT and MDH activities lower in SHAM- compared with CLEN- and RACT-fed and GDH higher in SHAM-compared with CLEN-fed trout (Table VI-6B).

The proliferation capacity of WM myosatellite cells (MyC) extracted from CLEN-fed trout was greater when compared with the SHAM fish between 3 and 7 days after plating and greater in RACT-fed trout at 6 and 7 days after plating (Fig. VI-2). There were no differences between the numbers of MyC per g of muscle between groups (SHAM: $3.7 \pm 1.1 \times 10^6$ cell•g muscle^{-1}; CLEN: $3.3 \pm 0.5 \times 10^6$ cell•g muscle^{-1}; RACT: $3.4 \pm 0.4 \times 10^6$ cell•g muscle^{-1}).

Relative myogenin mRNA expression in WM was lower in RACT-fed trout in the spring experiment but higher in CLEN- and RACT-fed fish compared with the SHAM in the winter (Fig. VI-3A, VI-3B). MyoD I relative expression was higher in WM of CLEN-fed fish compared with the SHAM in the spring experiment (Fig VI-4A); no differences were noted among groups in the winter (Fig. VI-4B). Myogenin relative expression was significantly higher ($p<0.01$) in RM from the RACT- compared with the SHAM-fed group in the spring experiment (Fig. VI-5B); however no differences were noted among groups in the winter experiment (Fig. VI-5B). Relative MyoD I and II mRNA expression did not differ among the three groups in either the spring or winter experiment (MyoD I, spring: SHAM: 1.14 ± 0.18; CLEN: 1.77 ± 0.53; RACT: 1.05 ± 0.16; winter: SHAM: 1.08 ± 0.26; CLEN: 0.87 ± 0.06; RACT: 1.26 ± 0.21) (MyoD II, spring: SHAM: 1.60 ± 0.25; CLEN: 1.41 ± 0.16; RACT: 1.12 ± 0.12; winter: SHAM: 0.90 ± 0.21; CLEN: 0.74 ± 0.07; RACT: 0.94 ± 0.14).

Discussion

This study provides support for CLEN and RACT, two β_2-AA, acting on rainbow trout myogenesis and metabolism and activating white muscle (WM) myosatellite cells (MyC) proliferation in the absence of significant changes in body mass, length or HSI between SHAM- and β_2-AA fed rainbow trout. Other studies previously reported no significant differences in body parameters after β_2-AA feeding experiments in rainbow trout (Vandenberg and Moccia, 1998; Vandenberg *et al.*, 1998; Lortie *et al.*, 2004), blue catfish (*I. fructalis*) (Webster *et al.*, 1995), and pigs and cattle (Beermann, 1993). Our study is, however complicated by the fact that the two feeding experiments were undertaken at two different seasons (spring and winter) and with diets which differed in fat content (11 and 16%). Separating a seasonal effect (even if fish were juveniles and temperatures and photoperiods were identical between the two periods) from a food effect is not possible in this experimental design.

In our experiment, liver mass was lower in RACT- and CLEN-fed trout in both experiments compared with the SHAM fish. A reduced liver mass was previously reported for pigs fed cimaterol (Mersmann, 1987) and RACT (Aalhus *et al.*, 1990), and rats fed CLEN (Reeds *et al.*, 1988) and salbumatol (Wariss *et al.*, 1990). The decrease in liver mass with β_2-AA feeding implicates the transfer of glycogen and lipid reserves from the liver to build muscle mass (Vandenberg *et al.*, 1998). However no significant decrease in liver mass has previously been reported in fish fed β_2-AA.

The increase in condition factor (CF) in CLEN- and RACT-fed trout compared with the SHAM fish following the spring exposure must be interpreted with caution as it represents primarily a decrease in CF of the SHAM fish rather than an increase in CF in the

β_2- AA fish. However, it is interesting to note that β_2-AA fed fish, which were fed the same amount of food per body mass and maintained under the same conditions, did not exhibit this decreased CF. There was no significant change in CF following the winter β_2-AA feeding experiment, where trout received 16% rather than 11% fat in their diet. Other studies reported no differences in CF and liver mass following β_2-AA feeding in rainbow trout (Vandenberg and Moccia, 1998; Lortie *et al.*, 2004).

Both experiments showed that CLEN-feeding decreased LSI, supporting CLEN acting as repartitioning agent. It is interesting that even through the lipid content of the winter feed was higher, the LSI was lower (not significantly, but nonetheless lower) for trout fed this diet. The decrease in LSI was significant only for fish fed with CLEN. This result suggests a higher lipolytic activity of CLEN-fed trout compared with the RACT-group; unfortunately no lipolytic enzyme activities were estimated in this study. Previous reports did find a lipolytic effect of CLEN in WM of Lister rats (Reeds *et al.*, 1988), channel catfish (Mustin and Lovell, 1992) and blue catfish (Webster *et al.*, 1995). Overall the effects of RACT- and CLEN-feeding on rainbow trout body parameters and growth suggest that β_2-AA have a similar but a reduced effect on the trout compared with avian and mammalian models. The reduced effects of β_2-AA in fish are probably not due to the desensitization or down-regulation of muscle β_2-adrenoceptors (AR) as previous studies by Lortie *et al.* (2004) reported that feeding rainbow trout CLEN or RACT (40 ppm) for 30 days did not affect trout muscle β_2-AR density or affinity.

CLEN was detected in the plasma of all CLEN-fed fish after 12 weeks of β_2-AA feeding. As expected, no CLEN was detected in the plasma of SHAM trout. It is interesting to note that one month of recovery after 12 weeks of CLEN feeding is adequate to clear

plasma CLEN. Brambilla *et al.* (1994) also recorded that the CLEN content was significantly decreased in liver (~95%), muscle (~95%) and skin (~97%) following 30 days of recovery after 21 days of 5 ppm CLEN-feeding in rainbow trout. These data are important when the use of β_2-AA feeding is considered in an aquaculture situation in order to ensure no CLEN remains in tissues before sending the fish to market.

Cathecholamines are known to affect whole animal carbohydrate and lipid metabolism and catecholamine administration to vertebrates generally induces higher glucose production (Wright *et al.*, 1989) as well as lipolysis and oxygen consumption (Ensinger *et al.*, 1994). Adrenaline which is a full β_2-AA, increases metabolic rate, carbohydrate metabolism and blood flow to skeletal muscle of healthy human volunteers (Ensinger *et al.*, 1994). However, my experiment reports no difference in plasma glucose concentrations following feeding diets containing CLEN and RACT to trout. Geisser *et al.* (2004) also reported no effects of dopexamine, another β_2-AA, on plasma glucose concentration in healthy human volunteers. Some adrenergic agonists do result in hyperlactaemia (Clutter *et al.*, 1980; Galster *et al.*, 1981). It is interesting to see that only CLEN had an effect on plasma metabolite concentrations (lactate, amino acids and total proteins) and mostly during the spring or low fat-feeding experiment. Total protein content was increased in liver and WM only in trout following the spring experiment and only with the CLEN-treatment. It was reported that select β_2-AA such as CLEN and fenoterol (Emery *et al.*, 1984) increase body protein contents in the laboratory rat. β_2-AA action is usually associated with an increase of skeletal muscle and cardiac muscle mass (Reeds *et al.*, 1986). CLEN-feeding to laboratory rats and farm animals generally prevents the deterioration of muscle by stimulating protein synthesis and a long lasting reduction in degradation (Bricout *et al.*, 2004).

This study is the first to estimate metabolic enzyme activities in fish fed β_2-AA. However, the data are difficult to interpret because of the experimental design, which included a repetition of the β_2-AA feeding at different seasons and with diets containing different fat contents. The two experiments showed different effects of β_2-AA on liver and skeletal muscle enzyme activities. Higher dietary lipid composition certainly modulates enzyme activities in fish tissues and may interfere with the effects of β_2-AA on metabolism. It was previously reported that high dietary lipid content in mammals inhibits the activities of the glycolytic enzyme PK (Jump and Clarke, 1999; Fanelli *et al.*, 1993) while increasing PEPCK activities (Shimeno *et al.*, 1995). However, in trout the exact opposite result occurred in liver enzymes when comparing enzymes activities in spring with winter (low vs high lipid content) in SHAM fish liver. Chapters II and III demonstrated that season and specifically photoperiod, even when water temperatures are stable, influence fish metabolism (Levesque *et al.*, 2005a,b). However, it is hard to relate the findings from our cod experiment, where the photoperiod was natural, to this study, where the photoperiod was constant. The differences between the winter and spring experiments here probably reflect effects of both season and lipid content of the diet.

In trout, the spring experiment showed increased in some enzyme activities in the liver only following CLEN-feeding (LDH, glycolytic; ALT, amino acid metabolic enzyme; MDH, an oxidative enzyme) compared with the SHAM fish. An increase in LDH activity was also found in kidney tissue in the spring experiment in both the CLEN- and RACT-fed fish which may be related to the lower plasma lactate content at least in the CLEN-fed fish. The increase in LDH implies an enhanced capacity for lactate gluconeogenesis as previously reported in rat (Vestergaard *et al.*, 1994; Tappy *et al.*, 1995; Geisser *et al.*, 2004). However, in the winter experiment, activities of several enzymes tested decreased in

the liver in both RACT- and CLEN-fed compared with the SHAM fish, including LDH. LDH activity also decreased in WM following the winter feeding and a tendency to decrease in the spring, was reported by Vestergaard *et al.* (1994) in muscle of young Friesian bulls treated with cimaterol. In WM from the winter experiment, ALT and GDH increase in CLEN- and RACT-fed fish compared with the SHAMs. Other studies also report a release of alanine and glutamine from skeletal muscle following an adrenaline infusion and β_2-AR signaling in rat skeletal muscle (Rosdahl *et al.*, 1998). It is clear from my study that changing the fat content of diet and/or the season has a marked affect on the effects of β_2-AA on rainbow trout intermediary metabolism. The repartitioning effects of β_2-AA could explain the fact that I observed larger differences in enzymes activities between the SHAM and β_2-AA fed fish in the winter (when the lipids were higher in the diet) compared to the spring experiment.

CLEN was more effective than RACT in increasing myosatellite cell (MyC) proliferation *in vitro*. Previous studies also reported a significant effect of CLEN on MyC proliferation in mice (Roberts and McGeachie, 1992) and muscle fiber hypertrophy in Lister rats (Maltin *et al.*, 1992), although nothing was reported on an effect of RACT on MyC proliferation capacities. This study also reports the effects of β_2-AA on myogenin and MyoD mRNA expression, two myogenic regulatory factors (MRFs) implicated in MyC determination and proliferation and in myoblast differentiation, respectively. The effects of CLEN on MRFs expression have yet to be clearly defined. An increase in MyoD mRNA levels (Hughes *et al.*, 1993; Mozdziak *et al.*, 1998) or no effect (Maltin *et al.*, 1992; Delday and Maltin, 1997) was reported for CLEN administration in rodents. My study reports an increase in relative MyoD mRNA expression in WM following the spring experiment and a tendency to increase in the winter when trout were fed CLEN. In addition, WM myogenin

mRNA expression increased following the winter experiment with both CLEN- and RACT-feeding indicating an effect of CLEN on muscle cell proliferation in rainbow trout and possibly an enhanced sensitivity of the muscle cells to differentiation by RACT and CLEN in the winter. This result is consistent with the fact that administration of the β_2-AA cimaterol stimulates hypertrophy of skeletal muscle (Reeds *et al.*, 1986; Zeman *et al.*, 1987) and the previous report of an increase in MyoD protein levels in female rats fed CLEN at 2 ppm for four weeks (Bricout *et al.*, 2004). The differences between the spring and winter experiments may be associated with the different food used as a vehicle (see earlier in the Discussion) and the fact that CLEN and RACT work as repartitioning agents. In my study, there were no differences in MyoD I and II relative mRNA levels in RM which is not in agreement with the shift from slow to fast muscle fibers in rat soleus muscle following CLEN-feeding (Picquet *et al.*, 2004) or the induction of MyoD expression in this same model following β_2-AA feeding (Bricout *et al.*, 2004). However there was a difference in relative myogenin mRNA expression in RM following RACT- and CLEN-feeding in the spring and a tendency for the same difference in the winter, showing that CLEN increases RM cells proliferation and RACT decreases proliferation compared with SHAM-fed fish.

Differences have been reported to exist between the actions of CLEN and RACT are reported in rainbow trout, including the down-regulation or desensitization of hepatic β_2-AR found in CLEN- but not RACT-fed rainbow trout (Dugan *et al.*, 2003). The β_2-AA RACT contains two chiral carbons and exists as four stereoisomers (Smith *et al.*, 1993; Ricke *et al.*, 1999), which all have more-or-less similar effects on fish body mass and composition (Ricke *et al.*, 1999). This could explain the similar but lower effect of RACT on most of the parameters studied in this experiment.

This study reports the effects of CLEN and RACT feeding on rainbow trout myogenesis and metabolism. The fact that there were no differences in body mass or length suggests that the use of β_2-AA in rainbow trout aquaculture may be of limited benefit. However some parameters, such as the condition factor, the decrease amount of fat in the body cavity, the altered metabolic enzyme activities and the higher proliferation and differentiation capacity of MyC extracted from WM indicate that CLEN and to a lower extent RACT, do modify WM. Furthermore, given that these effects differ as a result of changes in diet composition and/or season, further analyses are needed before one can conclude whether these agents would impact positively on the aquaculture industry. This study further highlights the fact that even though fish WM β_2-AR are pharmacologically similar to the muscle β_2-AR in mammals, differences do exist in their downstream metabolic and myogenic actions between these two groups of vertebrates.

Table VI-1: Sequence of the primers used to amplify the genes of interest, the reference for this sequence and product size amplified.

Gene amplified	Gene bank sequence	Forward primer	Reverse primer	Product size (bases)
MyoD I	Rescan and Gauvry, 1996	5'CAACTGCTCAGACGGAATGA3'	5'GCTATGGAACCATCCTCCG3'	249
MyoD II	Rescan and Gauvry, 1996	5'TGAAGAGATGCACGTCGAAC3'	5'TCTTGCGGACGTGCACGTTG3'	230
myogenin	Z46912	5'TGAAGAGATGCACGTCGAAC3'	5'TCTTGCGGACGTGCACGTTG3'	232
β-actin	AJ438158	5'CGCCGCACTGGTTGTTGACA3'	5'GCGGTGCCCATCTCCTGCT3'	674
18S	AF243428	5'GGAGGTTCGAAGACGATCAG3'	5'CTCAATCTCGTGTGGCTGAA3'	438
EF1α	CF753072	5'CATTGCCAAGCGAACCATTGC3'	5'CCTTCAGCTTGTCCAGCAC3'	184

Table VI-2: Condition factor at the beginning (CF (i)) and at the end (CF (e)) of the feeding period, liver mass, hepatosomatic index (HSI (e)) and lipid somatic index (LSI (e)) (mean ± SE) at the end of the spring and winter experiments. CF, HSI and LSI were calculated by the following equations [(body mass, g)•(body length, cm)$^{-3}$] x 100, [(liver mass)•(body mass)$^{-1}$] x 100, [(mass of body cavity lipids)•(body mass)$^{-1}$] x 100. **, indicates significant differences compared to the SHAM of the same sampling ($\alpha = 0.01$).

Treatment	Exposure	n	Mass (g) (e)	Length (cm) (e)	Liver mass (g) (e)	Cf (i)	Cf (e)	HSI (e)	LSI (e)
Sham	I	12	28.10 ± 1.2	13.80 ± 0.21	0.56 ± 0.03	1.55 ± 0.05	1.06 ± 0.04	1.15 ± 0.19	1.84 ± 0.09
	II	10	24.6 ± 0.75	12.68 ± 0.13	0.55 ± 0.03	1.28 ± 0.06	1.21 ± 0.03	2.34 ± 0.18	1.50 ± 0.15
Clenbuterol	I	12	26.78 ± 1.33	12.41 ± 0.33	0.44 ± 0.03 **	1.23 ± 0.05	1.41 ± 0.07 **	1.39 ± 0.09	1.42 ± 0.10 **
	II	10	22.81 ± 1.46	12.12 ± 0.30	0.49 ± 0.04	1.18 ± 0.03	1.26 ± 0.02	2.14 ± 0.14	0.91 ± 0.12 **
Ractopamine	I	12	29.57 ± 1.28	12 99 ± 0.25	0.45 ± 0.03 **	1.34 ± 0.16	1.36 ± 0.03 **	1.12 ± 0.13	1.65 ± 0.11
	II	10	20.32 ± 1.58	11.56 ± 0.31	0.40 ± 0.04 **	1.19 ± 0.02	1.29 ± 0.03	1.98 ± 0.18	1.26 ± 0.12

Table VI-3: Total enzyme activities and total protein (mean ± standard error) in the liver of rainbow trout fed β-agonists from January to April 2002 (spring; A) and November 2002 to February 2003 (winter; B) for 12 weeks. All enzymes activities are expressed as μmol•min^{-1} per total liver mass and protein as mg•g tissue^{-1}; N ≥ 8. Enzymes were assessed at 20 °C. Alanine aminotransferase (ALT); aspartate aminotransferase (AST); glutamate dehydrogenase (GDH); glucose-6-phosphate dehydrogenase (G6PDH); isocitrate dehydrogenase (IDH); lactate dehydrogenase (LDH); malic enzyme (ME); malate dehydrogenase (MDH): phosphoenolpyruvate carboxykinase (PEPCK); pyruvate kinase (PK). Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) among groups.

Liver A		SHAM	CLEN	RACT
	Protein	85.86 ± 3.60 b	102.03 ± 3.58 a	85.80 ± 4.70 b
Glycolysis	PK	5.94 ± 0.56	8.67 ± 1.29	7.85 ± 0.68
	LDH	52.65 ± 6.43 b	72.74 ± 6.32 a	50.72 ± 4.48 b
Gluconeogenesis	PEPCK	0.64 ± 0.03	0.75 ± 0.07	0.62 ± 0.05
Amino acid metabolism	ALT	6.89 ± 0.78 b	10.43 ± 1.34 a	9.99 ± 0.93 ab
	AST	13.42 ± 0.98	14.42 ± 1.17	13.32 ± 1.29
Oxidative enzymes	GDH	10.25 ± 0.90	13.33 ± 1.36	11.02 ± 0.74
	MDH	86.93 ± 6.60 b	110.41 ± 6.05 a	89.58 ± 5.79 b
	IDH	4.01 ± 0.33	4.42 ± 0.42	4.48 ± 0.36
	ME	1.78 ± 0.16	2.09. ± 0.12	1.64 ± 0.16
Penthose phosphate pathway	G6PDH	7.64 ± 0.59	7.50 ± 0.65	7.06 ± 0.82

Liver B		SHAM	CLEN	RACT
	Protein	74.44 ± 1.47	72.88 ± 3.04	75.75 ± 2.72
Glycolysis	PK	9.23 ± 0.93	7.15 ± 0.63	7.54 ± 1.00
	LDH	105.59 ± 7.77 a	29.47 ± 2.69 b	25.85 ± 3.47 b
Gluconeogenesis	PEPCK	0.32 ± 0.01 a	0.20 ± 0.02 b	0.21 ± 0.02 b
Amino acid metabolism	ALT	9.58 ± 0.62 a	4.09 ± 0.47 b	3.87 ± 0.48 b
	AST	12.10 ± 0.31	12.98 ± 1.57	13.60 ± 1.65
Oxidative enzymes	GDH	9.01 ± 0.57 a	5.95 ± 0.64 b	4.94 ± 0.56 b
	MDH	82.83 ± 2.81 b	183.88 ± 18.75 a	182.12 ± 25.98 a
	IDH	4.13 ± 0.19 a	2.82 ± 0.31 b	2.37 ± 0.36 b
	ME	1.08 ± 0.05	0.80 ± 0.08	0.83 ± 0.13
Penthose phosphate pathway	G6PDH	9.02 ± 0.95	8.41 ± 0.77	6.74 ± 0.87

Table VI-4: Enzyme activities (mean ± standard error) in the kidney of rainbow trout fed β-agonists in the spring for 12 weeks. All enzyme activities are expressed as $\mu mol \cdot min^{-1} \cdot g\ tissue^{-1}$ and protein as $mg \cdot g\ tissue^{-1}$; $N \geq 8$. Enzymes were assessed at 20 °C. See Table VI-3 for the list of abbreviations. Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) among groups.

Kidney A		SHAM	CLEN	RACT
	Protein	54.30 ± 3.35	61.35 ± 1.95	54.12 ± 2.89
Glycolysis	PK	15.02 ± 0.49	14.37 ± 0.54	14.19 ± 0.41
	LDH	38.83 ± 0.76 b	42.79 ± 1.24 a	42.33 ± 1.48 a
Gluconeogenesis	PEPCK	0.812 ± 0.041	0.912 ± 0.096	0.84 ± 0.06
Amino acid metabolism	ALT	17.58 ± 1.43	22.37 ± 2.99	20.76 ± 1.93
	AST	30.08 ± 12.68	32.55 ± 2.66	32.341 ± 2.25
Oxidative enzymes	GDH	29.33 ± 0.87	27.83 ± 2.71	27.30 ± 1.65
	MDH	19.28 ± 1.52 b	24.06 ± 0.61 a	22.03 ± 0.83 ab
	IDH	7.35 ± 0.32	7.61 ± 1.72	7.82 ± 0.55
	ME	0.603 ± 0.02	0.63 ± 0.04	0.67 ± 0.03
Penthose phosphate pathway	G6PDH	12.24 ± 1.08 a	9.76 ± 0.97 b	11.38 ± 1.16 ab

Table VI-5: Enzyme activities (mean ± standard error) in white muscle of rainbow trout fed β-agonists in the spring (A) or winter (B) for 12 weeks. All enzyme activities are expressed as $\mu mol \cdot min^{-1} \cdot g\ tissue^{-1}$ and protein as $mg \cdot g\ tissue^{-1}$; $N \geq 8$. Enzymes were assessed at 20 °C. See Table VI-3 for the list of abbreviations of the enzymes. Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) among groups.

WM A		SHAM	CLEN	RACT
	Protein	34.82 ± 1.55 a	39.97 ± 1.13 b	39.24 ± 1.13 ab
Glycolysis	PK	40.44 ± 4.32	41.98 ± 1.93	36.34 ± 3.24
	LDH	525.28 ± 37.11	506.16 ± 22.67	486.81 ± 29.72
Amino acid metabolism	ALT	1.03 ± 1.15	0.96 ± 0.07	1.03 ± 0.07
	AST	15.49 ± 0.99	15.05 ± 1.36	13.98 ± 1.21
Oxidative enzymes	GDH	1.38 ± 0.13	1.28 ± 0.07	1.35 ± 0.11
	MDH	100.26 ± 6.82	85.43 ± 3.32	93.80 ± 8.44
	IDH	2.58 ± 0.25	2.36 ± 0.14	2.27 ± 0.15
	ME	0.87 ± 0.04	0.83 ± 0.03	0.77 ± 0.04

WM B		SHAM	CLEN	RACT
	Protein	29.69 ± 1.35	31.53 ± 0.74	31.87 ± 1.24
Glycolysis	PK	39.93 ± 3.15	33.67 ± 2.09	39.36 ± 2.01
	LDH	219.96 ± 18.81 a	64.33 ± 1.27 b	75.53 ± 4.59 b
Amino acid metabolism	ALT	0.68 ± 0.02 b	1.23 ± 0.03 a	1.43 ± 0.06 a
	AST	6.81 ± 0.19	6.98 ± 0.28	7.59 ± 0.39
Oxidative enzymes	GDH	0.37 ± 0.03 b	2.63 ± 0.13 a	3.02 ± 0.19 a
	MDH	78.05 ± 3.04 a	27.45 ± 2.43 b	34.59 ± 3.49 b
	IDH	3.21 ± 0.27 a	2.33 ± 0.14 b	2.93 ± 0.19 ab
	ME	0.53 ± 0.02	0.49 ± 0.01	0.56 ± 0.03

Table VI-6: Enzyme activities (mean ± standard error) in red muscle of rainbow trout fed β-agonists in the spring (A) or winter B) for 12 weeks. All enzyme activities are expressed as $\mu mol \cdot min^{-1} \cdot g\ tissue^{-1}$ and protein as $mg \cdot g\ tissue^{-1}$; $N \geq 8$. Enzymes were assessed at 20 °C. See Table VI-3 for the list of abbreviations of the enzymes. Different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) among groups.

RM A		SHAM	CLEN	RACT
	Protein	31.55 ± 1.50	37.48 ± 2.58	33.19 ± 1.30
Glycolysis	PK	35.30 ± 3.05 b	39.48 ± 3.25 ab	39.20 ± 4.69 a
	LDH	233.41 ± 22.48	269.27 ± 29.57	239 55 ± 26.54
Amino acid metabolism	ALT	1.16 ± 0.12	1.14 ± 0.04	1.53 ± 0.17
	AST	27.86 ± 2.51	27.84 ± 3.61	29.58 ± 3.25
Oxidative enzymes	GDH	2.91 ± 0.25	2.39 ± 0.11	2.84 ± 0.25
	MDH	185.27 ± 17.70	175 65 ± 16.26	236.25 ± 38.31
	IDH	20.63 ± 2.29 ab	14.51 ± 2.16 b	24.28 ± 1.82 a
	ME	1.73 ± 0.12	1.56 ± 0.08	1.76 ± 0.15

RM B		SHAM	CLEN	RACT
	Protein	25.99 ± 0.95	25.59 ± 0.77	25.96 ± 0.82
Glycolysis	PK	45.06 ± 3.85	37.78 ± 2.06	41.93 ± 2.50
	LDH	227.85 ± 13.03 a	115.66 ± 7.55 b	130.93 ± 7.40 b
Amino acid metabolism	ALT	1.18 ± 0.06 b	2.85 ± 0.19 a	2.98 ± 0.22 a
	AST	33.62 ± 1.93 a	10.50 ± 0.78 b	11.84 ± 1.25 b
Oxidative enzymes	GDH	3.78 ± 0.22 a	2.92 ± 0.21 b	3.03 ± 0.17 ab
	MDH	144.08 ± 7.47 b	263.63 ± 12.81 a	285.70 ± 25.08 a
	IDH	8.38 ± 0.46	7.17 ± 0.34	8.27 ± 0.76
	ME	0.92 ± 0.05	0.84 ± 0.04	0.95 ± 0.06

Figure VI-1: A) Lactate, B) amino acids and C) total protein in plasma of rainbow trout following the feeding of β-adrenergic agonists in the spring for 12 weeks; D) total protein in plasma of rainbow trout, following the winter exposure. N = 10 (mean + standard error); *, indicates significant differences compared with SHAM trout (Tukey-Kramer, $\alpha < 0.05$).

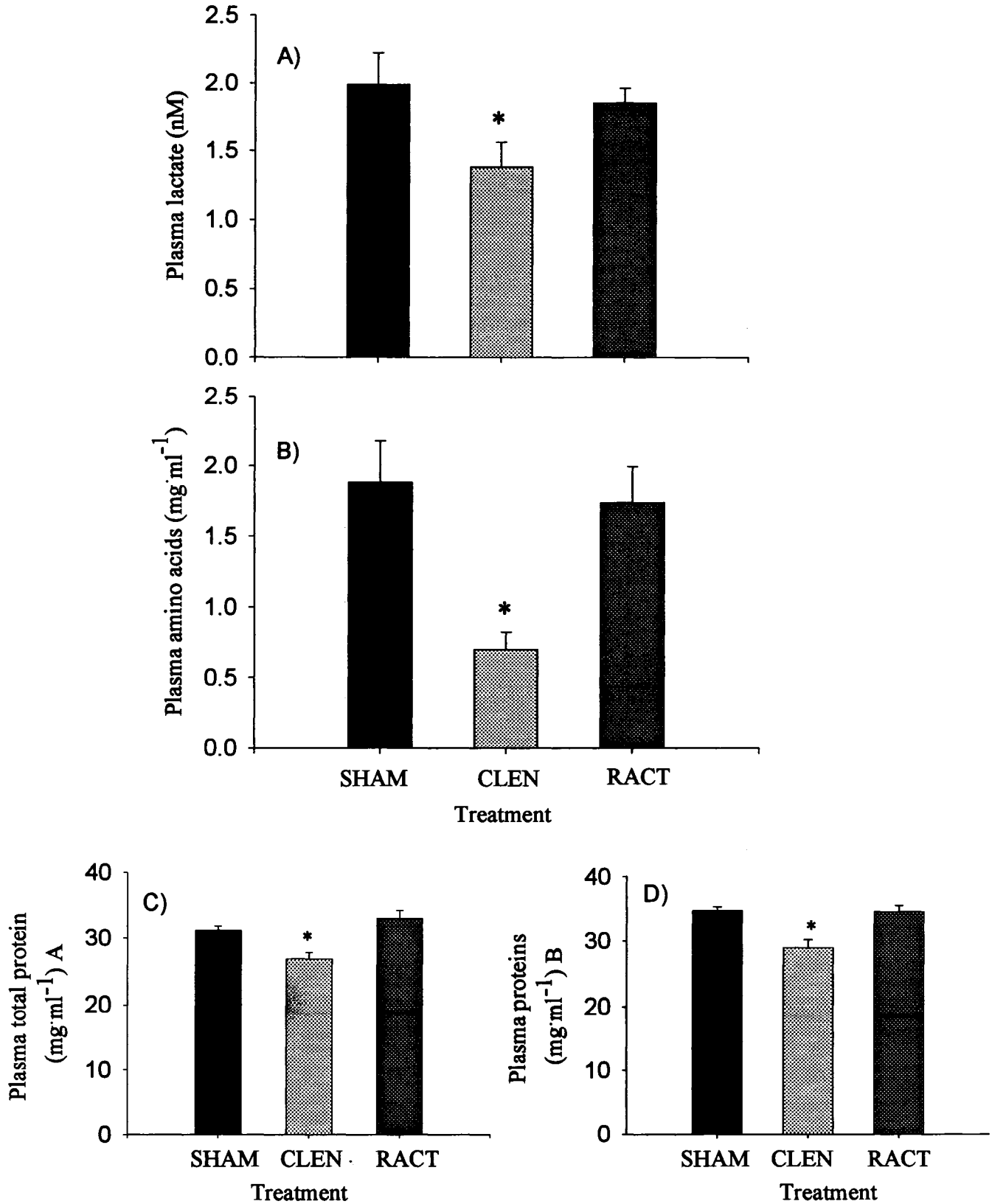

A)
Plasma lactate (nM)
2.5
2.0
1.5
1.0
0.5
0.0
*
B)
Plasma amino acids (mg·ml-1)
2.5
2.0
1.5
1.0
0.5
0.0
*
SHAM
CLEN
RACT
Treatment
C)
Plasma total protein (mg·ml-1) A
40
30
20
10
0
*
SHAM
CLEN
RACT
Treatment
D)
Plasma proteins (mg·ml-1) B
40
30
20
10
0
*
SHAM
CLEN
RACT
Treatment

Figure VI-2: Rainbow trout white muscle myosatellite cell proliferation *in vitro* from the winter exposure. ●, Myosatellite cells extracted from SHAM-fed trout; ○, myosatellite cells extracted from clenbuterol (CLEN)-fed trout;▼, myosatellite cells extracted from ractopamine (RACT)-fed trout. N = 3 (mean ± standard error); *, indicates significant differences among SHAM- and CLEN-fed trout (Tukey-Kramer, $\alpha < 0.05$); a, indicates significant differences between SHAM- and RACT-fed trout.

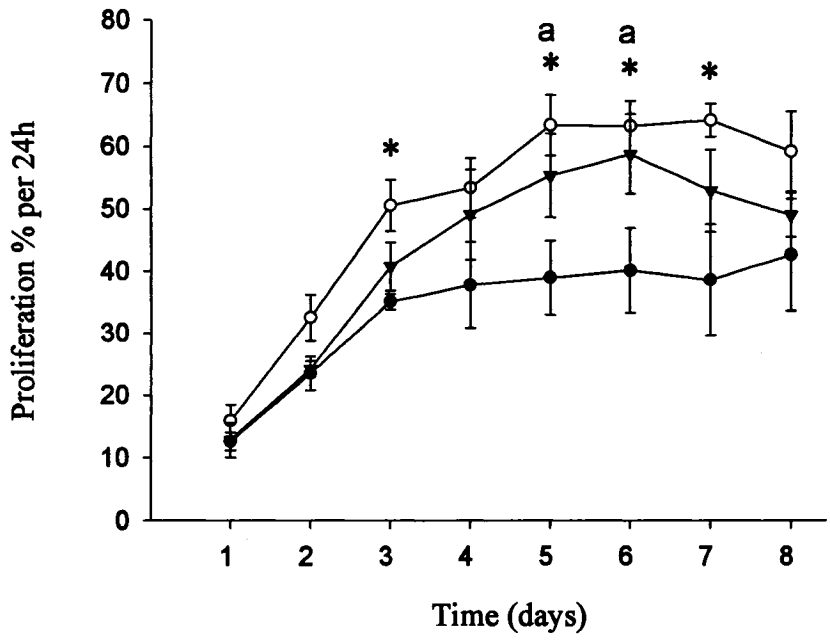


Figure VI-3: Relative expression of white muscle myogenin mRNA in the spring (A) and winter (B) following the feeding of rainbow trout ractopamine (RACT), clenbuterol (CLEN) or the vehicle (SHAM) for 12 weeks. N = 8 (mean + standard error); different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) among groups. Relative band intensities (gene of interest compared with ElF-1α) were determined from the densities of the resulting gel bands.

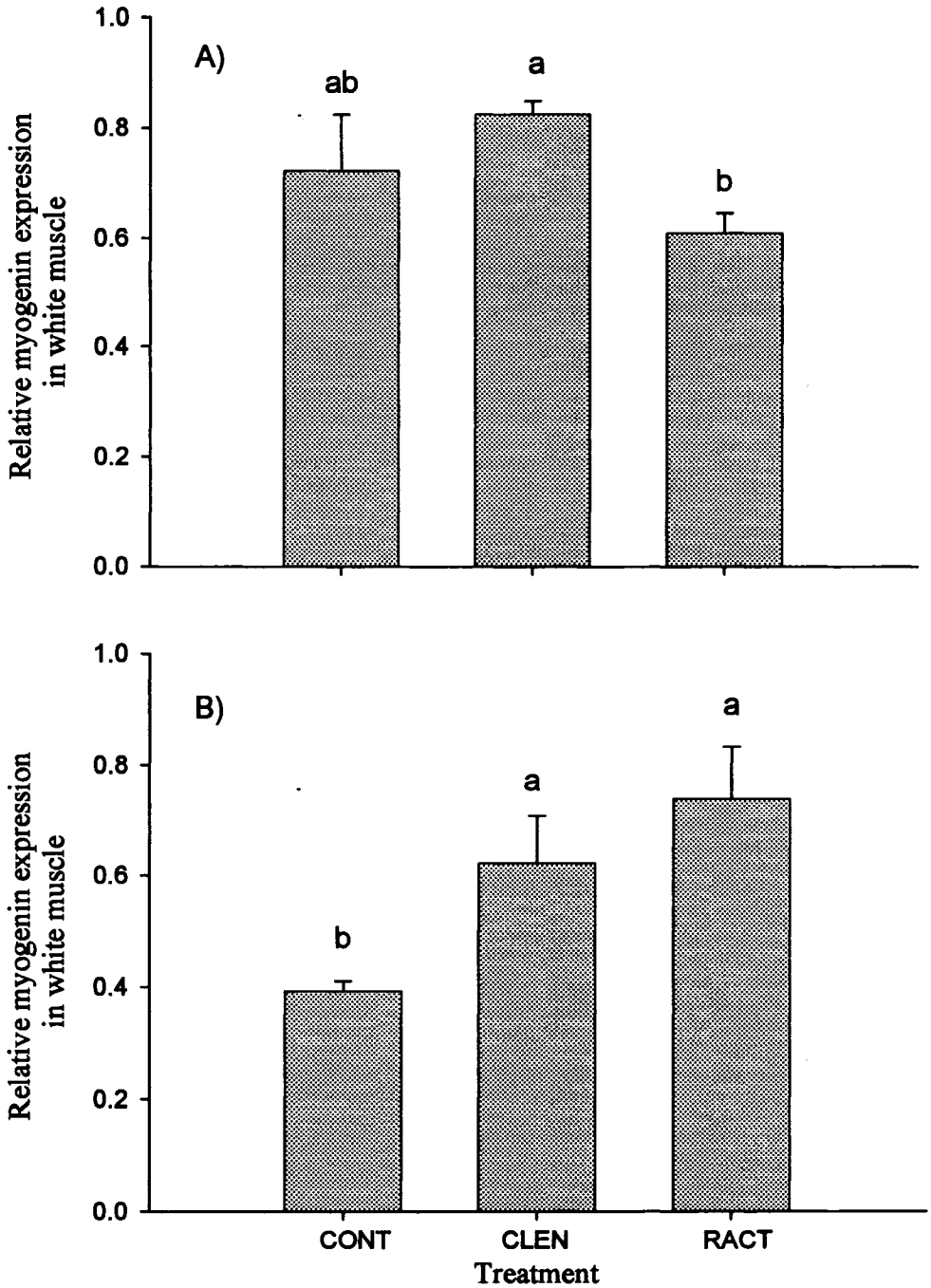

A)
B)
Relative myogenin expression in white muscle
1.0
0.8
0.6
0.4
0.2
0.0
ab
a
b
a
a
b
CONT
CLEN
RACT
Treatment

Figure VI-4: Relative expression of white muscle MyoD I mRNA in the spring (A) and in the winter (B) experiments in rainbow trout fed ractopamine (RACT), clenbuterol (CLEN) or the vehicle (SHAM) for 12 weeks. N = 8 (mean + standard error); different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) among groups. Relative band intensities (gene of interest compared with β-actin) were determined from the densities of the resulting gel bands.

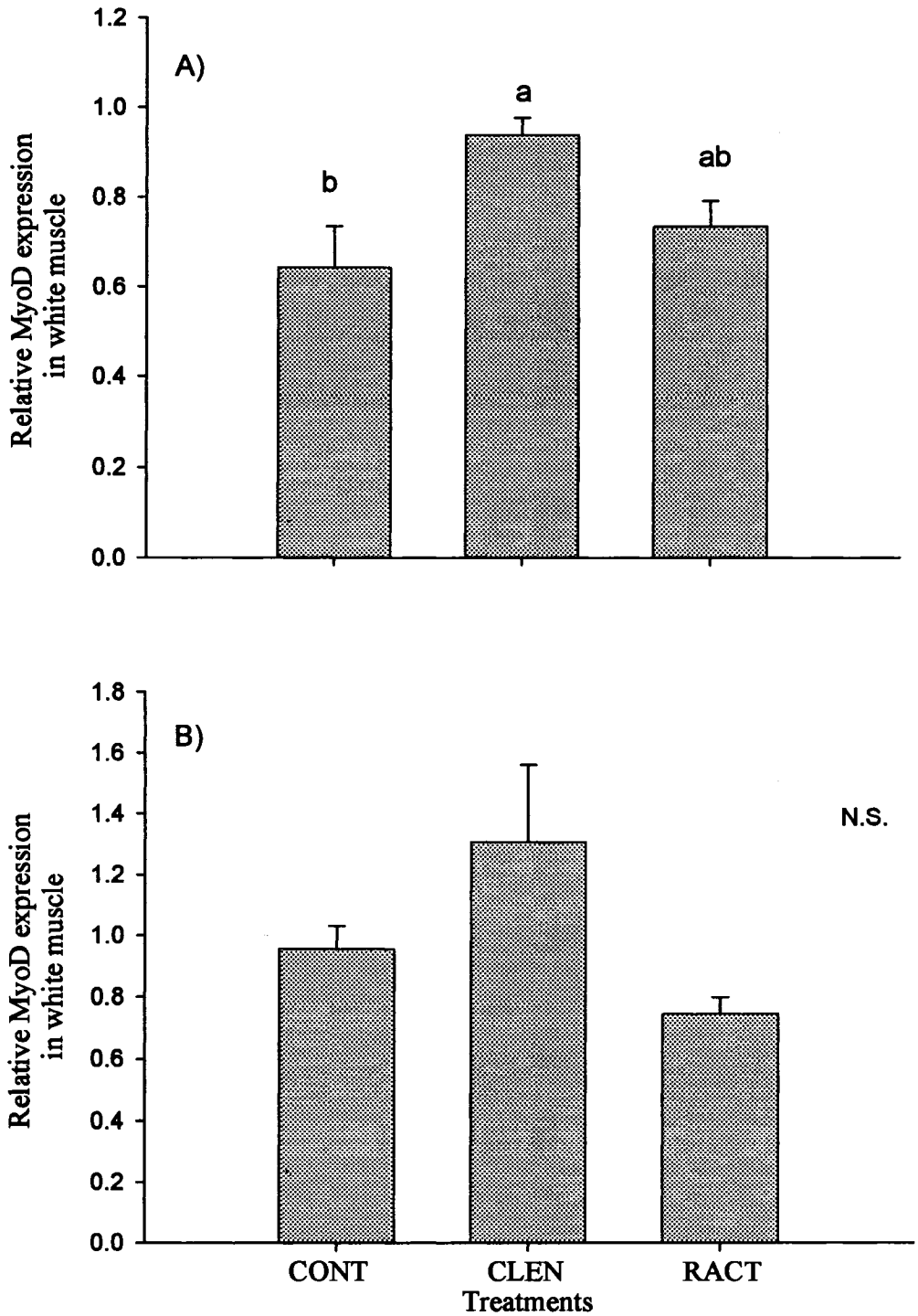
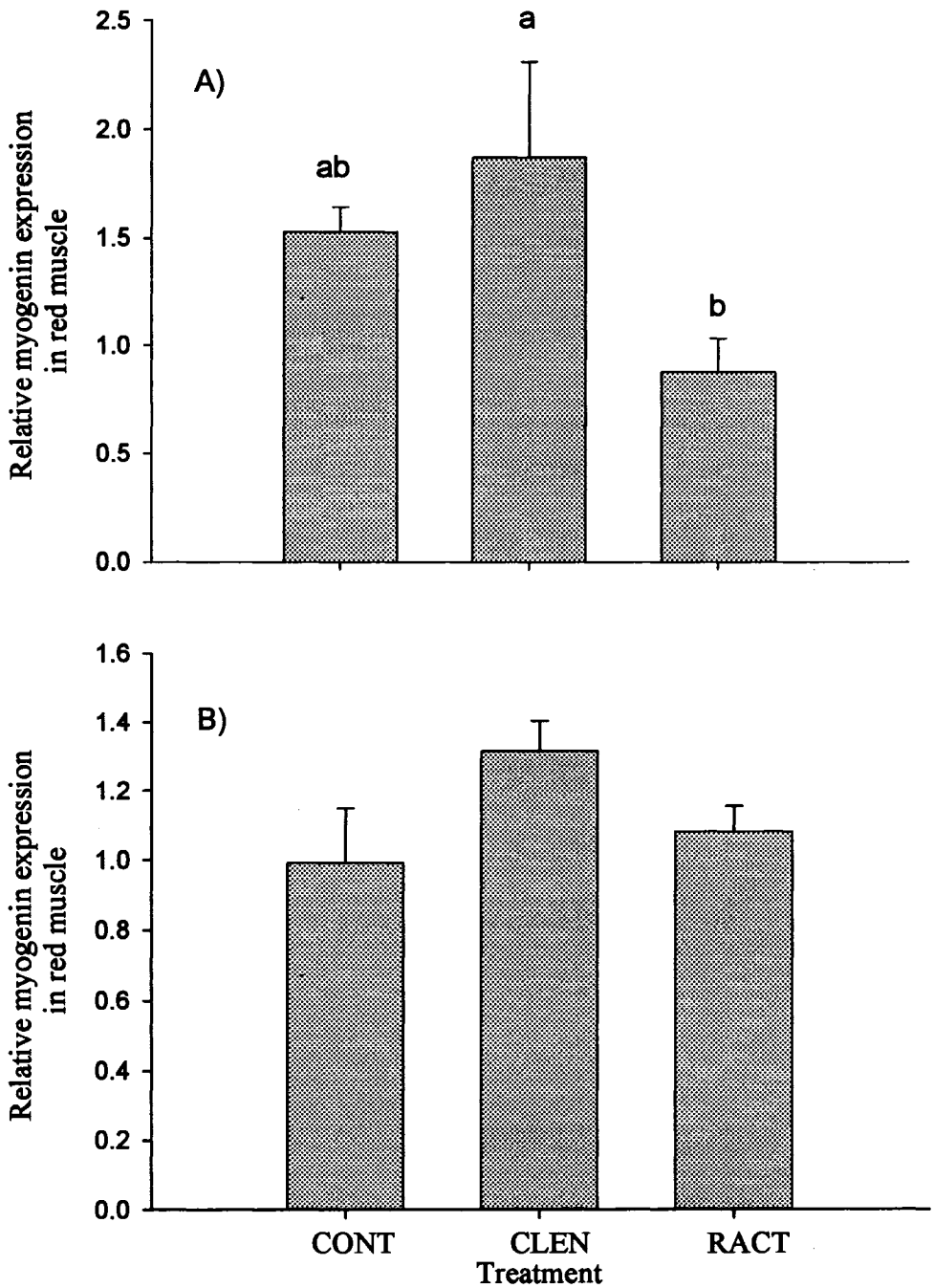

A)
Relative MyoD expression in white muscle
1.2
1.0
0.8
0.6
0.4
0.2
0.0
b
a
ab
B)
Relative MyoD expression in white muscle
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
N.S.
CONT
CLEN
RACT
Treatments

Figure VI-5: Relative expression of red muscle myogenin mRNA in the spring (A) and winter (B) experiments in rainbow trout fed ractopamine (RACT), clenbuterol (CLEN) or the vehicle (SHAM) for 12 weeks. N = 8 (mean + standard error); different letters indicate significant differences (Tukey-Kramer, $\alpha < 0.05$) among groups. Relative band intensities (gene of interest compared with β-actin) were determined from the densities of the resulting gel bands.


A)
Relative myogenin expression in red muscle
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
b
B)
Relative myogenin expression in red muscle
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CONT
CLEN
RACT
Treatment

CHAPTER VII: GENERAL CONCLUSIONS

This thesis provides new information on fish myogenesis and metabolism and the relation between fish growth, myogenesis and tissue metabolism. I employed different fish models [cod (*Gadus morhua*), rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*)] and different experimental treatments [temperature, transgenic fish for GH and β_2-adrenergic agonists feeding (β_2-AA)].

First, temperature is well known to have a marked impact on metabolic and morphological parameters in fish including cod (Pelletier et al., 1993, 1995; Guderley et al., 2001). Moreover, it is important to evaluate the effects of environmental parameters on fish growth and white muscle physiology from an aquaculture point of view. As described in the Introduction, aquaculture is one potential solution to feed our growing population and to protect the diversity of marine species. Temperature can be critical when considering using open water or in land-based aquaculture techniques. Chapters II and III allowed me to examine the importance of photoperiod and internal cycles on cod morphology and physiology. After 18 months exposure, morphological parameters (such as liver mass and growth rate, at least after one year) and physiological parameters (such as liver protein and glycogen and liver and muscle enzymes) were more affected by photoperiod than temperature. These data revealed that an increase in body mass is not accompanied by an increase in tissue metabolism. Only liver enzymes were included in mass and length multiple regression and contributed only a minor percentage to the overall change. This result indicates that when considering Atlantic cod, even if keeping them at a high temperature continuously results in a higher final growth, having them at ambient

temperature and photoperiod doesn't affect significantly most of the morphological and physiological parameters measured in Chapters II and III.

Another important finding in my thesis is the different results found between Atlantic cod exposed to ambient and constant temperatures (Chapters II and III) and rainbow trout acclimated to low and high temperatures (Chapter IV). The temperature experiment with trout was performed to supplement the Atlantic cod results as it was not possible to evaluate the expression of myogenic factors in the cod experiment. High and constant water temperatures increased juvenile cod and rainbow trout growth rates. I found no significant alterations in liver and white muscle (WM) metabolism in cod; however, there was a significant increase in WM enzyme activities in trout exposed to low temperatures as previously demonstrated in the literature (Johnston and Wokoma, 1986; Guderley and Gawlicka, 1992; Battersby and Moyes, 1998; Lannig *et al.*, 2003). This means that rainbow trout (a freshwater fish) metabolism is more sensitive to temperature than to internal cycles, compared with cod (a marine fish). These differences between cod and trout may come from the different photoperiod regimes used in both experiments. Both groups of trout were exposed to a constant photoperiod (12L:12D) and both Atlantic cod groups were exposed to an ambient photoperiod. Moreover, rainbow trout were acclimated for 4 months which is not representative of a natural thermal acclimatization (Sänger and Stoiber, 2001) such as in the cod experiment. Additional studies are needed to determine the effects of photoperiod on rainbow trout to determine if photoperiod or temperature is the more important factor in altering enzyme activities.

My results provided significant new information on myogenesis in adult fish. As reported in Chapter I, most fish growth is indeterminate, meaning they will grow either by hypertrophy (increased size of existing fiber) or hyperplasia (formation of new muscle

fibers) throughout their lives. Myosatellite cells (MyC) are muscle stem cells responsible for muscle growth. MyC in mammals and especially in fish are not fully characterized. Knowing more about muscle growth and especially MyC proliferation and differentiation will be important for aquaculture and also possibly for applications in mammalian muscle illnesses. There are few data available on adult fish myogenesis. My results show that myogenin (proliferation) and MyoD (myoblast determination and differentiation) were affected by temperature, the GH-transgene and β_2-AA feeding.

Also, I had the chance to work with GH-transgenic salmon. This model was really the best I could find for myogenic studies as GH transgenic salmon grow up to 5-times faster than their non-transgenic siblings. These studies indicated that the basis for these higher growth rates are not simply higher metabolism or myogenic growth factors, although higher proliferation capacity in the GH-transgenic salmon is at least part of the explanation.

It is clear from Chapter IV, V and VI that metabolism and myogenesis, at least in rainbow trout and Atlantic salmon, do not follow similar patterns and are not responding the same way to fish growth. For example, in rainbow trout exposed to two temperatures (Chapter IV), myogenin and MyoD mRNA expression measured in WM was higher in warm (17°C) than cold (5°C) acclimated trout. However, metabolic enzyme activities measured in WM were generally lower in fish acclimated to the higher compared with the lower temperature. This result is explained by a compensatory effect on WM metabolic enzyme activities in fish exposed to low temperatures, whereas there is no compensatory effect at the myogenic level. An opposite response of myogenesis and metabolism is also found in rainbow trout exposed to β_2-AA (Chapter VI) where oxidative enzymes were generally decreased and myogenin increased in WM, at least in the winter experiment. In the experiment with GH-transgenic Atlantic salmon (Chapter V), metabolic enzymes in

WM were correlated with body mass while myogenic factor expression (MyoD and myogenin) in WM was more affected by the presence of the transgene than the body mass of the fish. These results clearly show that an increase in growth rate is generally accompanied by an increase in myogenic factors (MyoD and myogenin) at least in WM. However, these results also demonstrate that an increase in growth rate (which was obtained in cod and trout exposed to high temperatures and Atlantic salmon made transgenic for GH) is not absolutely accompanied by an increase in tissue metabolic enzyme activities.

My results also show that the several treatments used had different effects on WM and red muscle (RM) myogenesis. First, proliferation and differentiation of Atlantic salmon skeletal muscle does not happen simultaneously but seems to alternate between RM and WM at least at 4 and 7 months of age. In rainbow trout acclimated to a low temperature, myogenin was decreased in WM but increased in RM, and in trout fed β_2-AA there were generally no effects of CLEN and RACT feeding on myogenin and MyoD expression in RM in either experiment. This result indicates that differences exist in energy allocation for growth purposes between RM and WM. The changes in enzyme activities with the treatments used were not different between RM and WM in trout acclimated to different temperatures or fed β_2-AA. However, enzymes activities in WM are negatively correlated with fish growth in Atlantic salmon independent of the presence of the GH transgene, and RM enzymes activities are lower in GH-transgenic compared with non-transgenic salmon. These results are certainly due to the differences between WM (fast muscle) and RM (slow muscle) physiology. RM has higher mitochondria content and higher concentrations of oxidative enzymes and is used for slow and sustained swimming. However, WM is used for burst swimming and works better under anaerobic conditions. WM has for most fish a

higher value in terms of aquaculture and it would be interesting to find treatments that favour WM growth over that of RM.

My studies with myosatellite cell (MyC) proliferation and differentiation revealed that further work remains to be done with these cells in order to fully understand their mechanisms of proliferation and differentiation. MyC are difficult to maintain in the lab and to achieve reproducible results. However, my results did elucidate some factors that may influence fish MyC activation including GH, IGF-I and FGFb which have a direct *in vitro* effect on MyC proliferation in both Atlantic salmon and rainbow trout. I discovered that β_2-AA increased MyC proliferation in trout when administrated *in vivo* in the food but not in a culture situation.

These results taken together contribute to an increased knowledge of fish muscle growth and metabolism and may be useful in the future to optimize aquaculture feeding regimes. However, other experiments need to be done to clarify the differences between RM and WM growth and to test other compounds possibly implicated in MyC activation.

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