GROWTH AND MYOSIN HEAVY CHAIN EXPRESSION IN THE WHITE MUSCLE OF JUVENILE WALLEYE (*Sander vitreus*)

By

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Abstract

Walleye are an important recreational and commercial fish species that are distributed over an expansive geographic range across North America. However, its palatable white flesh and appeal to anglers have lead to declines in natural populations throughout Canada and the United States. These declines have prompted the idea that aquaculture may serve as a means of satisfying consumer demands and decreasing pressure on wild stocks. While culture programs exist for walleye, little is known about the growth physiology of walleye in a culture system. The goals of this thesis, therefore, were to develop a molecular marker that could be used to rapidly assess growth in juvenile walleye, and to make improvements to culture practices that will optimize growth.

To begin, we examined the relationship between growth and the expression of the myosin heavy chain gene (MyHC) in the white muscle of juvenile walleye. The coding region of MyHC from the fast skeletal muscle of walleye was amplified using a full length cDNA. Growth was then characterized using traditional measurements of growth (length, weight and condition factor), as well as MyHC protein concentration and MyHC mRNA levels. Both MyHC mRNA and protein expression were highly correlated with faster growth in juvenile walleye. Over shorter time scales, the MyHC mRNA marker was sensitive enough to detect impacts of fasting that could not be detected using traditional measurements of growth.
Next, MyHC mRNA quantification was applied to an aquaculture setting. Feed training is an important bottleneck during juvenile walleye culture that often leads to mortalities and cannibalism. These experiments showed that the brief fasting period during the diet switch from plankton to commercial pellet feed caused a significant decrease in MyHC mRNA levels. Furthermore, the success of feed training in terms of survivorship and growth potential increased significantly for larger fish.

The final section of this thesis examined how acute and chronic temperature exposure impacted MyHC mRNA and protein expression. Results showed that the nature of the heat stress can significantly affect the MyHC response. These findings are important as the temperature stresses induced in these studies are common during the summer months in southern Ontario.
Co-authorship

Chapter 2: Myosin heavy chain mRNA as a marker for the rapid assessment of muscle growth in juvenile walleye (*Sander vitreus*).

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Comments: This project was designed collaboratively by R.S. Dhillon, Dr. Y.S. Wang and Dr. B.L. Tufts. R.S. Dhillon was responsible for carrying out all lab and field experiments and all sample collections. Phylogenetic analyses were performed by A.J. Esbaugh. All experimental analyses and writing were done by R.S. Dhillon.
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TABLE OF CONTENTS

Title ................................................................................................................................. i
Abstract ........................................................................................................................... ii
Co-Authorship ............................................................................................................... iv
Acknowledgements ........................................................................................................ v
List of Tables .................................................................................................................. x
List of Figures ............................................................................................................... xi
List of Abbreviations ..................................................................................................... xiii

Chapter 1. Introduction and literature review ......................................................... 1
  Assessing growth ........................................................................................................ 1
  Myosin: phylogenetics and evolution of the MyHC gene ........................................ 5
  The MyHC isoforms ............................................................................................... 9
  Class II Myosin ...................................................................................................... 10
  Structure and function ............................................................................................ 12
  Myogenesis and MyHC expression ...................................................................... 13
  The application of MyHC as a molecular tool ...................................................... 16

Literature cited ........................................................................................................... 18

Chapter 2. Myosin heavy chain mRNA as a marker for the rapid
assessment of muscle growth in juvenile walleye (Sander vitreus).... 27

Abstract .................................................................................................................... 28

Introduction ............................................................................................................... 30

Materials and Methods ............................................................................................ 32
Animals and sampling protocol ................................................................. 32
One-week food deprivation experiment ....................................................... 32
24 hour fasting experiment ........................................................................ 33
75-day in-pond growth experiment ........................................................... 33
Tissue collection and RNA and protein isolation ......................................... 34
Sequence determination ............................................................................. 34
Phylogenetics ............................................................................................. 35
Quantitative real-time RT-PCR ................................................................. 37
Protein sample preparation, SDS-PAGE and western blotting .................... 38
Statistical analysis ...................................................................................... 39
Results ........................................................................................................ 40
Sequence determination and phylogenetics ................................................. 40
Tissue distribution ...................................................................................... 41
One-week food deprivation experiment ..................................................... 41
24 hour fasting experiment ........................................................................ 41
75-day in-pond growth experiment ............................................................ 42
Discussion .................................................................................................. 42
Acknowledgments ...................................................................................... 47
Literature Cited .......................................................................................... 48

Chapter 3. Expression of myosin heavy chain mRNA during juvenile walleye (Sander vitreus) feed training ......................................................... 67
Abstract ..................................................................................................... 68
Introduction ................................................................................................ 69
Methods ................................................................................................................................. 72

Animals and early life-stage conditions ................................................................. 72
Feed training protocol ........................................................................................................ 72
Testing for a possible myosin mRNA response .................................................. 73
Size and feed training ...................................................................................................... 73
Age and feed training ....................................................................................................... 74
Tissue collection and RNA isolation ........................................................................... 74
Sequence development and quantitative real-time RT-PCR .................................... 75
Statistical analysis ........................................................................................................... 76

Results ................................................................................................................................. 76

Size and condition ........................................................................................................... 77
Survivorship ...................................................................................................................... 77
Feed training and myosin expression ........................................................................... 78

Discussion .......................................................................................................................... 78

Acknowledgments ........................................................................................................... 84

Literature cited .................................................................................................................. 84

Chapter 4. Effect of heat stress on the heat shock response and
myosin heavy chain in juvenile walleye (Sander vitreus) .............................. 94

Abstract ............................................................................................................................. 95

Introduction ......................................................................................................................... 96

Methods ............................................................................................................................... 98

Animal acquisition and holding ................................................................................... 98

Series 1: Acute temperature increase ....................................................................... 99
List of Tables

Table 2.1. Real-time RT PCR and primer-walking sequences for myosin heavy chain (MyHC). ................................................................. 53

Table 2.2. Growth rate of juvenile walleye over 75 d in low, medium and high feed treatments............................................................................... 55

Table 4.1. Primers used for quantitative real time PCR of Hsp 70 and MyHC genes. ...................................................................................... 117
List of Figures

**Figure 1.1.** Structure of the rat embryonic MyHC gene (adapted from Goldspink et al. 2001). ................................................................. 26

**Figure 2.1.** Walleye Myosin heavy chain sequence. ........................................ 56

**Figure 2.2.** Phylogenetic analysis of walleye MyHC and other myosin II isozymes. ........................................................................................................... 58

**Figure 2.3.** Myosin heavy chain (MyHC) content in white, red and cardiac muscle of *S. vitreus*. .............................................................................................................. 59

**Figure 2.4.** Growth indices and MyHC mRNA content of juvenile *S. vitreus* over the 17 d experiment. ........................................................................................................ 60

**Figure 2.5.** White muscle content of MyHC mRNA over a 24 h period in *S. vitreus*. .......................................................................................................................... 63

**Figure 2.6.** The effect of growth on MyHC protein and mRNA content in the white muscle of *S. vitreus*. ................................................................. 64

**Figure 3.1.** The effect of the feed training protocol on MyHC mRNA content in the white muscle of *S. vitreus*. ................................................................. 88

**Figure 3.2.** Weight and condition factor of juvenile walleye (*Sander vitreus*) during feed training. ................................................................. 89

**Figure 3.3.** Cumulative mortality rate among all three treatment groups for juvenile walleye (*Sander vitreus*). ................................................................. 91
Figure 3.4. White muscle content of MyHC mRNA over a 15 d period in *S. vitreus*.................................92

Figure 4.1. The effects of acute temperature increase on Hsp 70 mRNA content..................................................118

Figure 4.2. Temperature profile during the acute temperature increase and recovery..................................................119

Figure 4.3. Temperature profile during the acute temperature increase and recovery, MyHC mRNA and protein levels in response to heat shock. .................120

Figure 4.4. Temperature profile during the chronic temperature exposure; and Hsp 70 mRNA and protein levels in response to heat shock. .........................121

Figure 4.5. Temperature profile during the chronic temperature exposure; and MyHC mRNA and protein levels in response to heat shock. .........................122
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>FTEn</td>
<td>feed training experiment n</td>
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<td>GH</td>
<td>growth hormone</td>
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<td>Hsp</td>
<td>heat shock protein</td>
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<td>IGF</td>
<td>insulin-like growth factors</td>
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<td>K</td>
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<td>kDa</td>
<td>kilo Dalton</td>
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<td>LT</td>
<td>total length</td>
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<td>LMM</td>
<td>light meromyosin</td>
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<td>M</td>
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<td>MP</td>
<td>maximum parsimony</td>
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<td>myogenic regulatory factors</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<td>MyHC</td>
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Chapter 1. Introduction and literature review

Growth is an important aspect in assessing the overall health of a populations of fishes, individual fish, and related industries that rely on fish (commercial fishing, aquaculture, tourism, etc.). However, there are numerous ways of assessing growth, from traditional and direct measurements such as length, weight and condition factor, to proximate analyses such as protein accretion, to more novel techniques that measure gene expression. In this introduction, I will first review several methods of assessing growth in fishes. Next, I will introduce myosin as a candidate gene for describing growth in vertebrates which will be the basis for this thesis. The evolution of the myosin gene is briefly discussed, followed by a closer examination of class II myosins. I will describe the structure and function of myosin heavy chain with details regarding myogenesis and expression patterns. Finally, I will discuss the potential application of molecular markers such as MyHC for the aquaculture industry.

Assessing growth

As mentioned above, there are several ways to measure growth, each with positives and negatives associated with the method. Here I present four examples of techniques that have been used to assess growth, both in basic and applied

The underlying assumption of the study of condition suggests that a heavier fish at a given length is a healthier fish. The most widely used condition factor developed is the Fulton’s condition factor (K) which is $K = xWL^{-3}$, where $x$ is a scaling constant dependent on the unit of measure (Jones et al. 1999). However, this model assumes isometric growth which often is not the case (Cone 1989). Various models have been proposed as an alternative to better satisfy the ideal of a high condition fish. For example, the equation $M = BL^2H$, where $M$ is mass, $B$ is a regression constant, $L$ is fork length and $H$ is height, has been used to explain condition factor over a large range of sizes in Atlantic and chinook salmon (Jones et al. 1999). Such models take into account the considerable variation in girth between species, and within species at different life history events. For example, Jones et al. (1999) suggest that migrating salmon may trade-off weight in order to reduce energy use during swimming (reduce drag). However, these new models have only been tested on salmonids, and may have limited use in fish with eel-like shapes such as walleye or pike. Furthermore, condition factor may not provide the resolution for potential changes in growth that various RNA analyses may provide (Cech and Moyle 1991).

Back calculation is a technique that uses various hard parts of the fish (otoliths, scales, fin rays, spines or opercula) to infer length at earlier ages of the
individual. Otoliths have been examined to determine daily patterns in growth (Panella 1971, Struhsacker and Uchiyama 1976). Somatic growth is reflected in the deposition of daily rings, with wider rings being deposited in faster growing fish. The deposition of daily increments can be affected by factors such as temperature (Mosegaard et al. 1988), photoperiod (Dale 1984) and feeding (Clemmeson and Doan 1996). However, at very high growth rates or very slow growth rates, it is very difficult to obtain an accurate resolution reflective of changes in somatic growth (Clemmeson and Doan 1996). Furthermore, scale and fin measurements are susceptible to resorption (thereby the possibility of underestimating age) and occlusion (Francis 1990). Geffen (1983) has also demonstrated that after emergence, many salmonids do not deposit otolith bands in daily intervals.

In the past it was shown that a relationship exists between the ratio of RNA to DNA and growth rates (Buckley 1982, Dortch et al. 1984, Buckley 1984). Ribonucleic acid (RNA) is primarily involved in protein synthesis and will vary with physiological status, while DNA is used as an index of cell number. DNA is insensitive to changes in environmental conditions (Dortch et al. 1984). In a study correcting for temperature, Buckley (1984) demonstrated that the RNA:DNA ratio explained 92% of the variability in growth rate for sand lance (Ammodytes sp.), cod (Gadus morhua), and haddock (Melanogrammus aeglefinus). The terms hypertrophy and hyperplasia can be used to describe the
change in the size and the number of muscle DNA (Peragon et al. 2001). An increase in the size of DNA units is representative of hypertrophy, while increased in number is associated with hyperplasia. RNA concentrations have been positively associated with growth (Bastrop et al. 1992, Ferguson 1992, Mathers et al. 1993). However, the use of RNA:DNA ratios are not without limitations. For instance, the relationship of RNA:DNA can be affected by age (Jobling 1988), sex (Chicharo et al. 1988) and diurnal patterns (Rooker and Holt 1996), thus must be controlled for in any study.

The white muscle is the largest tissue in fish, and the location for most protein accumulation. While protein turnover is much slower in muscle than in tissue such as liver, this turnover still accounts for up to 30% of the synthesized tissue in cod (Houlihan et al. 1988). Combined with a very efficient uptake of amino acids (low fractional rate of protein synthesis), the linear relationship between protein synthesis and growth has been described in cod (Houlihan et al. 1988). In their examination of growth rate and protein accretion (measuring fractional rate of turnover, synthesis and degradation), Peragon et al. (2001) concluded that whole body growth rate and white muscle protein accretion were closely related. However, these patterns may decrease with the age of the fish, when growth rates decrease. Early work by Millward et al. (1973) suggests that in addition to changes in RNA content, changes in RNA efficiency may affect protein synthesis rates in skeletal muscle during fasting experiments. In their study, rats
were fasted and re-fed with either a protein-rich diet or a protein free diet. Long-
term fasting was predominantly altered by RNA concentration, but changes in
efficiency are important over short-term fasting periods.

*Myosin: phylogenetics and evolution of the MyHC gene*

Myosin is a highly conserved motor and structural protein that is involved
in numerous motile activities such as cytokinesis, vesicular transport, and cellular
locomotion (Weiss et al. 1999). Perhaps its most noted role is as a sarcomeric
motor protein responsible for muscle contraction. Myosin is the most abundant
protein in muscle, comprising as much as 25% of the whole organism protein
pool (Baldwin and Haddad 2001) and up to 50% of the muscle protein pool
(Watabe and Ikeda 2006). The high commercial value and the high abundance of
white muscle make myosin heavy chain (MyHC) an ideal protein for growth
studies. The myosin molecule is a hexameric molecule, comprised of two myosin
heavy chains (approximately 220 kDa) and four myosin light chains
(approximately 20 kDa). However, it includes a range of motor proteins that have
been classified based on function and activity. Goldspink (2001) and Hardy
(2001) both ascertained that growth can be reduced to the myofibrils, and
therefore, the myosin gene. However, gene and protein expression of myosin has
not been widely tested in fish. Furthermore, the limited number of studies in the
literature present quite disparate results (Overturf and Hardy 2001, Biga et al.
2004, Johansen and Overturf 2006). In the following paragraphs, I will introduce myosin in a phylogenetic context and suggest the class II fast forms of myosin as the optimal form for monitoring gene expression to assess growth.

Until recently, MyHC was categorized in 18 different classes based on the sequence of the conserved head domain as well as tail domain structure. The myosin superfamily consists of the familiar class II myosins which are involved in muscle contraction, as well as unconventional myosins that are distinguishable by the sequence of the tail domain (Berg et al. 2001). These unconventional myosins are important in that they make up the majority of myosin genes in humans (Berg et al. 2001), in particular in non-muscle cells (Bement et al. 1994). Myosin II are characterized by their ability to form filaments via the alpha-helical tail in parallel along the filament length (Sellers 2000). Most of the skeletal forms of myosin that partially make up class II are found on chromosome 17 in mouse, stickleback and humans.

Six novel classes established recently (Foth et al. 2006), primarily developed from investigations in unicellular eukaryotes such as Plasmodium and Toxoplasma gondii in the Apicomplexa phylum. Myosins are thought to have diversified very early in eukaryotic evolution. All myosins were likely derived from a single protein through gene duplication and diversification. The eukaryotic ancestor already possessed three different types of myosin, represented in the first case by class I (Foth et al. 2006). The second type is
represented by classes V and XI, and the third type of myosin in the eukaryotic ancestor is represented by the extant classes IV, VII, X, XII/XIV, XV, and XXIII (Foth et al. 2006). Yeast (S. cerevisiae) contain only 5 genes from 3 myosin classes (I, II and V) (Berg et al. 2001). Thirteen myosin genes are known in Drosophila which are distributed among 8 different myosin classes. C. elegans is known to have 17 myosin genes: 9 conventional and 8 unconventional. As with many species, the function of many of the unconventional myosins are unknown (Berg et al. 2001). However, some deductions can be made regarding function: class III myosins have been associated with the retina in flies, horseshoe crabs and humans. C. elegans, which lack eyes, also lack class III myosins. Other myosin classes are very restricted in phylogenetic distribution, such as class XIV which have been identified in parasites Toxoplasma gondii and Plasmodium falciparum). It is thought that these myosins may be associated with specific movements such as the gliding motility of Toxoplasma (Heintzelman and Schwartzman 1997 and Berg et al. 2001).

Interestingly, plants contain many myosin genes for the purposes of actin-based transport (Berg et al. 2001). However, plants lack class I and II myosins. All known myosin genes in Arabidopsis thaliana belong to classes VIII and XI. Given this and the presence of class I and II in yeast, it is possible that class I and II evolved after divergence of plants, or lost from the plant lineage (Berg et al. 2001).
The human genome consists of approximately 40 myosin genes that belong to approximately 12 different classes. In humans, there are 15 conventional class II myosins which include skeletal and cardiac myosins. The remaining 25 myosin genes that belong to the unconventional class of myosin include more recent discoveries of members associated with deafness, blindness, and organelle transport in humans. Class II myosin can be divided into two fiber types: type I (slow) and II (fast). These genes are found within a 500 kb segment on chromosome 17 (Weiss et al. 1999) and include MyHC IIa and MyHC IIx/d, the human gene most similar to the walleye gene isolated in the present thesis.

Myosin has undergone large lineage-specific radiations, as demonstrated by the number of class II myosins found in metazoans. While *Drosophila* possess only one conventional myosin II gene, *C. elegans* (9 of 17) and many vertebrates (15 of 40 in humans) possess many more. In this way, worms and vertebrates have developed diversity in muscle function by propagating a diversity of myosin heavy chain genes.

Differences in myosin sequences greater than 50% likely manifest themselves in significantly different ATPase rates and sliding filament velocities (Berg et al. 2001). However, when looking at the human and mouse MyHC genes, the conservation of order, orientation and spacing suggest that these were the product of gene duplications that occurred prior to divergence of these two species 75-110 million years ago (Sellers 1999). The arrangement of MyHC genes
along the chromosome does not reflect temporal expression patterns, as embryonic genes are separated by adult genes. However, the arrangement does represent sequence homology, with more similar sequences (adult forms) oriented more towards the centre of the chromosome (Sellers 1999).

The MyHC isoforms

Recent molecular techniques have enabled identification of the many myosin isoforms. In mammalian skeletal muscle, these forms have been classified by expression at particular developmental stages, and by the white muscle and red muscle forms, and can be distinguished by ATPase activities (Watabe 2002). Only the class II isoforms form filaments and use ATPase to promote contraction (Goldspink 1998, Weiss et al. 1999, and Watabe 2006). Class II (discussed in more detail below) distinguishes itself from the others in the myosin protein family by assembling into thick filaments (Cope et al. 1996, Desjardins et al. 2002). In humans and mouse, these genes are located in clusters on chromosomes 11 and 17, and are highly conserved despite divergence more than 75 million years ago (Watabe 2006). We draw our attention to Class II forms in growth assessment as these forms are what represent predominant form of myosin in the skeletal muscle of vertebrates.

Analogous MyHC isoforms responsible for contraction have been identified for fish. In carp (Cyprinus carpio), 29 different MyHC isoforms have
been identified as either cardiac or skeletal isoforms (Kikuchi et al. 1999). In torafugu (*Takifugu rubripes*), the chromosomal sites for skeletal isoforms are distributed on four loci, two of which correspond to locations of mammalian MyHC clusters (Watabe and Ikeda 2006). The other two MyHC clusters share properties with that of the zebrafish (*Danio rerio*) sequence. These results suggest that a gene duplication event created the clusters described in torafugu and zebrafish, and that these clusters are likely common among all teleosts (Watabe and Ikeda 2006).

**Class II Myosin**

The MyHC isoform chosen for measurement in this thesis belongs to the Class II group of myosin. Class II myosin, which are often referred to as conventional myosins, are a widely studied class of myosin which are expressed in striated muscle. The location and abundance of these proteins in white muscle make it an ideal candidate for investigating growth in fish. Class II myosin are sarcomeric, and are involved in skeletal and cardiac contraction. However, there are non-sarcomeric class II forms involved in smooth muscle contraction and non-muscle functions. Sarcomeric proteins are characterized by their ability to form fibers by assembling their tail regions into thick filaments (Reggiani and Bottinelli 2008). As previously mentioned, Class II include type I, II, embryonic, neonatal, alpha, beta (cardiac) and masticatory fibers. Type I are slow, oxidative fibers that are characterized by a slower hydrolysis of ATP. These oxidative fibers
are optimal for repetitive actions and sustained isometric force. In humans, these fibers are in high abundance in muscles such as the soleus that are involved in continual motion (walking) or standing. Type II fibers include fast, glycolytic fibers (IIb) and fast, oxidative and glycolytic (IIa). These fibers are designed for higher power output and fatigue quickly (Goldspink 1998). In mammalian systems, four class II are predominantly found in limb and trunk muscles: MyHC-1 slow (slow), MyHC 2a (fast, glycolytic and oxidative), MyHC 2d/x, and MyHC-2b (fast, glycolytic) (Watabe and Ikeda 2006, Reggiani and Bottinelli 2008). MyHC-alpha forms are expressed in cardiac muscle (primarily in the ventricles of bovine and humans) and in low abundance in some slow fibers. The beta form is most highly expressed in the atria. Finally, embryonic and neonatal MyHC isoforms are expressed in development and tissue regeneration in adults (Reggiani and Bottinelli 2008). Contributions of MyHC to growth have been demonstrated using both hypertrophic and hyperplasic frameworks. In addition to the increase in muscle cell sizes, the presence of small diameter MyHC isoforms in adult carp (Cyprinus carpio) suggest that hyperplasic growth is important for muscle growth in adults (Ennion et al. 1995). This sarcomeric protein in addition to the MyHC genes isolated from larger fibers demonstrate the indeterminate growth present in many species. The availability of these transcripts and those serving hypertrophic growth make MyHC an ideal candidate gene, as transcripts are often continually expressed throughout the lifespan of the fish.
Structure and function

The MyHC isoforms are large (approximately 6000 base pairs) yet maintain a high level of sequence homology (Goldspink et al. 2001). MyHC gene sequences of carp, human (*Homo sapien*), chicken (*Gallus gallus*) and rat possess similar intron and exon boundaries (Goldspink et al. 2001). However, the carp gene is half the size of MyHC genes in mammals and birds due to shorter introns (Gauvry et al. 1996, Goldspink et al. 2001). The protein itself can be divided into three major sub-domains: sub-fragment 1 (S1), sub-fragment 2 (S2) and light meromyosin (LMM). Sub-fragment 1, which makes up approximately 40% of the protein, is located on the N-terminal half of the myosin heavy chain, and forms the globular head. The S2 and LMM fragments are located on the C-terminal half with the S2 region situated closer to the N-terminus (Watabe 2002). These two fragments form the rod, which forms the thick filaments. The greatest degree of sequence homology of MyHC genes exist in the globular head region (S1) (Strehler et al. 1986) (Figure 1.1).

During contraction, the globular heads, which protrude from thick filaments, form cross-bridges with the actin-rich thin filaments. Each cross-bridge is composed of two S1 units, which is the site of ATP hydrolysis (Watabe 2002). The hydrolysis of ATP allows the myosin head to extend ahead along the microfilament and pull the filament through a power-stroke. ADP is released at the end leaving a vacant site on the myosin head for more ATP for the next cross-
bridge. As such, the ADP release cycle is the rate limiting step (Goldspink 1998). The energy required for contraction is directly related to ATPase activity, and this activity is dependent on the MyHC isoform (Reiser et al. 1985).

*Myogenesis and MyHC expression*

The red, slow twitch muscle and white, fast twitch muscle are located in physically distinct areas of the fish. Slow twitch muscle is typified by a thin, continuous band that runs along the lateral line above the fast twitch muscle. In zebrafish, differentiation may occur from the slow muscle fibers that originate from adaxial cells adjacent to the notochord and migrate to the lateral surface during development. As this migration occurs, myogenic cells differentiate into fast twitch muscle fibers (Devoto et al. 1996). However, trout models using labeled fast and slow MyHC isoforms indicate that the differentiation of fast twitch MyHC may occur prior to migration (Rescan et al. 2001).

The processes that facilitate muscle determination and differentiation appear common among all vertebrates, although they are not fully understood. The general steps are as follows: commitment of stem cells for myoblasts, proliferation, cell cycle exit, differentiation, migration, and fusion (Johnston 2006). Myogenic regulatory factors (MRFs) play a large role in committing cells to development and differentiation. MRFs loosely refer to a group of genes that include MyoD, myogenin, MEF2 and E protein that bind to the E-box sequences
in the regulatory regions of muscle genes, or A/T-rich regions of muscle promoters (Watabe 2001). During somite formation in carp, expression patterns that produce myosin were as follows: myf-5 is expressed initially during somite formation, followed by MEF2C and MyoD, then myogenin and MEF2A and finally MyHC (Watabe et al. 2001). Several MyHC genes are known to possess a CCAAT box where transcriptional factors bind to regulate gene transcription levels. A more common feature in the promoter region is a TATA box, which serves as a marker for RNA polymerase II for the start site of transcription (Goldspink et al. 2001). The E-box region (CAANTG) serves as a binding site for the transcription factors in the helix-loop-helix family (Goldspink et al. 2001). The regulatory and promoter regions of myosin are located on ATG start codon. Myosin gene expression is incorporated into myogenesis through a sensitivity to the myogenic regulatory factors that bind to positions at or near the promotor.

Muscle growth in fish can be hypertrophic (increase in fiber size) and hyperplasic (increase in fiber number). Unlike mammals, post embryonic growth in fish occur due to both the increase in muscle cell size (hypertrophy) and the formation of new fibers (hyperplasia). However, during juvenile growth, hypertrophy is the predominant form of growth. (Gauvry and Fauconneau 1996). Insulin-like growth factor-1 and -2 (IGF-1 and -2), growth hormone (GH) and myostatin have major roles in muscle hypertrophy. Growth hormone, IGF-1 and -2 act as pathway signals to regulate protein synthesis and degradation, and can

Endocrine control plays an important role in muscle growth (Rescan 2005). Thyroid hormone (T3) in particular can regulate transcriptional events related to MyHC levels (Baldwin and Haddad 2001, Goldspink et al. 2001). It is known in rat and human cardiac MyHCs that there are three at least two thyroid responsive elements (TREs) in the promoter region (Tsika et al. 1990). In many mammals, T3 is responsible for the switching of MyHC isoforms during development, from embryonic to fast adult forms of the gene (Baldwin and Haddad 2001).

The persistence of MyHC mRNA during development and growth make it an ideal marker for growth in fish. To date, the literature contains examples of how MyHC transcript content changes in response to various experimental manipulations (see Overturf and Hardy 2001, Biga et al. 2004, Johansen and Overturf 2006, Buckley et al. 2006). Recent advances in cDNA microarrays (Buckley et al. 2006) as well as real time PCR and western blotting techniques have provided much insight into gene profiling and the links between mRNA and protein levels. It is clear that MyHC expression is a sensitive indicator of the plasticity of muscle tissue based on examples in the literature. However, there are many contradictions in the current literature regarding how MyHC levels change
in fish muscle in response to experimental conditions (Overturf and Hardy 2001, Biga et al. 2004, Hevrøy et al. 2006, Johansen and Overturf 2006). This could be the result of differences in time scale, species, or life stage. The paucity of literature examining MyHC expression makes it difficult to understand how MyHC transcript and protein levels change under experimental conditions.

The application of MyHC as a molecular tool

The aquaculture industry has much to gain from widespread use of molecular markers as tools for monitoring growth. Aquaculture is a rapidly expanding industry that has grown an average of 8.8 percent a year, which is faster than any animal food-producing sector (FAO 2006). As the consumer base increases, expansion will demand more efficient culture practices and the incorporation of novel species for culture systems. To increase efficiency and develop protocols for novel species, the expression profiles for MyHC can be invaluable in enabling the rapid assessment of growth potential in fish muscle. Growth is paramount in an industry that depends on the white, fast twitch muscles achieving marketable sizes in as short a time frame as possible. Hyperplasic growth, a trait that only fish possess among all vertebrates, make muscle growth monitoring in fish a very unique and powerful measurement in fish culture. Furthermore, the highly conserved nature of the MyHC sequence simplifies its application in novel species.
Walleye (*Sander vitreus*) aquaculture has existed for many years (see Summerfelt 1996 for review). However, little is known regarding their optimal conditions for growth. Walleye is a freshwater perciid fish that ranges from the Northwest Territories to Alabama (Galarowicz and Wahl 2003). The decline of natural populations throughout North America has prompted the idea that aquaculture may serve as a means to satisfy consumer demand and decrease pressure on wild stocks. At present, however, little is known about the optimal conditions for growth in species such as walleye. Because of the rapid expansion and economic value of the aquaculture industry, experiments that could provide results in this area within a relatively short time frame would be ideal. Given their high potential economic value, rapid growth rate and large ultimate body size, walleye are an ideal study organism for the development of molecular tools to rapidly assess growth.

On this background, my thesis will have two main objectives: 1) to identify a MyHC isoform for the fast twitch muscle of juvenile walleye, and 2) to examine changes in the expression patterns of this gene in response to two variables that are very relevant to aquaculture. More specifically, I will examine the impact of nutrition levels and temperature change on MyHC mRNA and protein content, as well as growth. In addition to developing a powerful tool for aquaculture, these objectives address a major gap in the literature. To date, there is very little know about the combined MyHC mRNA and protein responses in fish to different
exogenous and endogenous influences. Successful production of marketable fish protein depends on rapid growth, and a better understanding of the responses of the major protein within the most marketable tissue (muscle) to different influences could provide very valuable information for a rapidly expanding industry. By addressing these issues, we will better understand the dynamic nature of muscle growth at the transcript level and can then apply this information to enhance aquaculture and alleviate pressures on world fisheries.

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Figure 1.1. Structure of the rat embryonic MyHC protein (adapted from Goldspink et al. 2001). The schematic illustrates the relative size of the subdomains: S1 (subdomain 1, or head region), S2 (subdomain 2), and LMM (light meromyosin or tail).
Chapter 2. **Myosin heavy chain mRNA** as a marker for the rapid assessment of muscle growth in juvenile walleye (*Sander vitreus*)

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Running title: MYOSIN HEAVY CHAIN mRNA AND GROWTH
Abstract

Over the years, a wide range of approaches has been used to assess growth in fish. Many of the traditional methods, however, are indirect and require long periods of time before growth becomes discernible. In this study, myosin, the major component of myofibrillar protein in the skeletal muscle, was evaluated as a molecular marker to monitor juvenile walleye growth using real time RT PCR. First, the coding region of myosin heavy chain from the fast skeletal muscle of walleye was amplified by long-distance PCR using a full-length cDNA. Phylogenetic analysis was used to determine the evolutionary relationship of this walleye myosin sequence to other vertebrate myosin sequences. Next, we established that this myosin isoform was most prevalent in the white muscle, compared to red and cardiac muscle. Experiments were then conducted to determine the efficacy of the walleye myosin marker as a rapid means to assess growth. First, we demonstrated that myosin mRNA and protein expression levels were higher in fish with greater changes in size (mm · d⁻¹). Next, the regulation of myosin was monitored under acute changes in feeding. Fish exposed to a one-week fasting period showed significant decreases in myosin mRNA levels by the end of the fast. The effect of feeding was also examined more closely over a 24
hour period after feeding, but our results show no significant change in myosin expression levels through this time period. This study indicates that myosin mRNA can provide a rapid and sensitive means of measuring relative growth rates in fish.
Introduction

Over the years, a wide range of methods has been used to monitor growth in fish. Traditionally, studies in this area have relied on information such as length and weight (Ricker 1979), condition factor and relative weight (Murphy et al. 1990), to assess growth. Proximate analyses such as protein and lipid content have also been used to evaluate growth potential (Weatherly and Gill 1983). More recently, growth has been correlated to the expression levels of different genes important in the regulation of muscle size. Expression patterns of genes involved in the GH/IGF-1 growth axis and the direct transcription of muscle proteins can provide a lot of insight into the nutritional status of fish and growth regulation (Black and Olson 1998, Duan 1998, Hornick et al. 2000, Kelley et al. 2001, Rescan 2001, Ayson et al. 2007). Recently, Johansen and Overturf (2005, 2006) quantified expression levels of myogenic regulatory factors (MRFs), myocyte enhancer factor 2 genes (MEF2), myostatin and several structural genes to demonstrate the impact of development and nutritional modifications on potential growth in rainbow trout Oncorhynchus mykiss (Walbaum). At present, however, this research has not thoroughly addressed how protein expression and fish size relate to gene expression.

Myosin heavy chain (MyHC) is an indicator that has been used in several studies to assess muscle development (see Johnston 2001 for review). Myosin
heavy chains account for the majority of myofibrils, which themselves account for
two-thirds of muscle protein synthesis (Mommsen 2001). Considering this, and
the unique property of fish to continue hyperplasic growth throughout their
lifespan, changes in the expression of myosin heavy chain may be an ideal
indicator to quickly monitor growth in fish. At present, however, the limited
amount of research has been focused on salmonids (Overturf and Hardy 2001,
Xie et al. 2001, Johansen and Overturf 2006) and there is still much to be learned
in this area.

These days, the ability to rapidly assess growth may be especially
important in aquaculture situations. In many parts of the world, aquaculture is
now viewed as the best means to meet the high demand for fish in the face of
collapsing wild fisheries. In North America, walleye *Sander vitreus* (Mitchell)
fisheries provide a classic example of this situation and many wild populations
have been decimated by over-harvest. At present, however, domestic walleye
culture is still in its infancy and there is still much to be learned in this area. In
view of this situation, a technique to rapidly assess growth in this species could
prove to be extremely valuable in order to evaluate the impact of novel rearing
protocols.

On this background, the purpose of the present study was to assess the use
of relative MyHC mRNA content as a means of monitoring growth in juvenile
walleye. The first objective was to determine the full sequence of the targeted
MyHC isoform and its phylogenetic relationship with that of other organisms. Next, the relative tissue distribution of the isolated MyHC isoform was determined in cardiac, red and white skeletal muscle. Two experiments were then performed to measure MyHC mRNA expression during long term and short term fasts. Finally, an end-point comparison was conducted between three different ponds that contained walleye growing at different rates to examine the relationship between long term growth, MyHC mRNA expression, and MyHC protein levels.

**Materials and Methods**

**Animals and sampling protocol**

For the two fasting experiments, juvenile walleye of 5.1 ± 0.8 g (mean ± SD) were collected from a local hatchery (Leonard Walleye Culture and Research, Hartington, ON) and held in 400 L recirculation tanks at Queen’s University (Kingston ON) for at least 2 weeks prior to experimentation. Fish were fed a commercial pellet diet (Corey Aquafeeds, Fredriction, NB) twice daily to satiation during this period. Temperature was maintained at ambient levels of 22 ± 1 °C.

**One-week food deprivation experiment**
Juvenile walleye \((n = 140)\) were divided into two recirculating tanks held at 22ºC and feed trained. Once it had been determined that all fish were consuming the pellet diet for at least two weeks and fish were of a uniform size, feed deprivation was initiated. After establishing baseline levels (days 0-3), feeding was discontinued for one week, during which time walleye \((n = 6)\) were sampled daily. After one week of nutrient deprivation, feeding resumed and the fish were again sampled daily for 7 days to monitor recovery.

24 hour fasting experiment

Fifty-four juvenile walleye were feed trained in a recirculation system (22 ºC) for 4 weeks prior to the experiment. When all fish had accepted the pellet diet and were of uniform size, an experiment to determine the effects of short term fasting on MyHC mRNA was conducted. This experiment began immediately after morning feeding, at which time 6 fish were sampled. This sampling protocol was continued every 4 h for the next 24 h.

75-day in-pond growth experiment

To examine MyHC mRNA levels in relation to MyHC protein levels and growth, an end-point comparison was performed on walleye reared in the hatchery that were growing at different rates. The three ponds were fertilized at different levels which consequently affected the size of juvenile walleye. The
ponds were categorized by the growth rates of the fish in their respective ponds (low, medium, and high). Total length ($L_T$) measurements were taken at four time points to determine changes in $L_T$ (mm·d$^{-1}$). After 75 d post hatch, thirty walleye were collected with a seine net from each of three ponds and terminally sampled on site.

*Tissue collection and RNA and protein isolation*

All fish were terminally anaesthetized in water containing 250 mg·µL$^{-1}$ tricaine methanesulfonate (MS-222, Sigma-Aldrich Corp., St. Louis MO) buffered with 500 mg·µL$^{-1}$ NaHCO$_3$ (Sigma-Aldrich Corp.). White, red and cardiac muscle were harvested and frozen in liquid nitrogen at -80 °C until they were processed for RNA and protein analyses. Tissues (0.05 g) were ground with mortar and pestle and total RNA was extracted from the muscle using a silica-gel-based membrane and centrifugation technique (Qiagen RNeasy Mini Kit, Qiagen, Valencia CA). Total RNA was then quantified with a SpectraMax Plus 384 (Molecular Devices, CA) plate spectrophotometer.

*Sequence determination*

The complete 5838 bp MyHC cDNA sequence was determined by primer walking (Robarts Research Institute, London, ON) (Table 2.1). To verify portions of this sequence, cDNA libraries were constructed from white skeletal muscle...
RNA from the caudal region of juvenile walleye (Norclone Biotech Laboratories, London ON). A 495 bp MyHC sequence was used to screen the library under high stringency conditions. Duplicate membranes were hybridized with a 32P dCTP-labelled probe overnight at 60ºC (Ready to Go DNA Labelling Beads, Amersham Biosciences, UK). Stringency washes were performed with SSC containing 0.1% SDS. Positive clones were isolated and plated for a secondary screening using a second 508 bp MyHC sequence. Positives from the second screening were cloned in *E. coli*. PCR was used to determine whether these positives contained the appropriate sized clone. PCR products of approximately 1000 bp in length were sent for sequencing (Norclone Biotech Laboratories).

**Phylogenetics**

A phylogenetic analysis of the amino acid sequence of fish myosin heavy chain (MYH) isoforms was also performed. The fish MYH sequences included isoforms from walleye, white croaker *Pennahia argentata* (Houttuyn) (AB039672), Chinese perch *Siniperca chuatsi* (Basilewsky) (AY454304), blackside hawkfish *Paracirrhites forsteri* (Schneider) (AJ243770) and zebrafish *Danio rerio* (Hamilton) (NM_152982), as well as zebrafish MYH6 and a cardiac MYH. This analysis also included: human *Homo sapiens* MYH 1 (NM_005963), 2 (NM_017534), 3 (NM_002470), 4 (NM_017533), 6 (NM_002471), 7 (NM_000257), 7b (NM_020884), 8 (NM_002742), 9 (NM_002473), 10 (NM_004244), 11 (NM_002474), 13 (NM_003802) and 14 (NM_024729);
mouse *Mus musculus* MYH 1 (XM_354615), 3 (XM_354614), 6 (NM_010856), 7 (NM_080728), 9 (NM_181327), 11 (NM_013607) and 14 (NM_028021); pig *Sus scrofa* MYH 2 (NM_214136); rabbit *Oryctolagus cuniculus* MYH 13 (AF212147); cow *Bos taurus* MYH 10 (NM_174834); and frog *Xenopus tropicalis* MYH (NM_001001244). It should be noted that mouse MYH 1 and 3 are predicted amino acid sequences based on genomic data. Alignment used for the phylogenetic analysis was performed by ClustalX (version 1.81). Phylogenetic hypotheses were constructed using both neighbour joining (NJ; Saitou and Nei 1987) and maximum parsimony (MP) as performed by PAUP* (beta test version 4.0b10; Swofford 2001). MP analysis consisted of a heuristic search with TBR branch swapping and ACCTRAN character state optimization enforced, and with random stepwise addition and 1000 random addition replicates. NJ was performed on a matrix of mean character distances. Support for nodes for both analytical procedures was performed using the bootstrap analysis with 1000 pseudoreplicates. All analyses were performed using Drosophila MYH as an outgroup to the vertebrate myosin heavy chain isoforms.

Gaps in sequence alignment were accounted for in three distinct series of analyses. In the first analysis, all possibly informative gaps were included and treated as missing data. In the second analysis, all gaps were removed, and in the third analysis, all gaps were treated as a distinct character state. The final analysis could only be performed using MP analysis. All subsequent trees were compared qualitatively for differences, with no major differences arising.
Quantitative real-time RT-PCR

To ensure 18S ribosomal rRNA expression was not affected by treatment, total RNA per gram of tissue was calculated for all samples. In all experiments, the total RNA \( \cdot \, \text{g}^{-1} \) was not significantly different between treatment groups. A one-step SYBR green PCR was performed on a Cepheid SmartCycler (Cepheid, CA) using the QuantiTect SYBR Green RT-PCR kit (Qiagen). Cycling conditions for the 18S control gene were as follows: 50\(^\circ\)C for 30 min (RT), 95\(^\circ\)C for 15 min (to activate HotStarTaq DNA polymerase), followed by 45 cycles of cDNA synthesis at 94\(^\circ\)C for 15 s, 56\(^\circ\)C for 30 s, 72\(^\circ\)C for 30 s, and 78\(^\circ\)C for 15 s with optics on. Finally, a melt curve was created using a 60\(^\circ\)C to 95\(^\circ\)C temperature ramp at 0.2\(^\circ\)C\,s\(^{-1}\). The conditions for the myosin target gene were very similar, however, during cDNA synthesis, the fourth stage was increased from 78\(^\circ\)C to 80\(^\circ\)C. Each PCR tube contained a total of 25 \( \mu\)l of PCR mixture, containing: 12.5 \( \mu\)l of Qiagen QuantiTect SYBR Green RT-PCR Master Mix (HotStarTaq DNA polymerase, QuantiTect SYBR Green RT-PCR buffer, dNTP mix including dUTP, SYBR Green 1, ROX passive reference dye, 5 mM MgCl\(_2\)), 2 \( \mu\)l each of 5 \( \mu\)M myosin forward and reverse primers (0.75 \( \mu\)l of 2.5 \( \mu\)M 18S primers), 0.25 \( \mu\)l RT mix (containing both Omniscript and Sensiscript reverse transcriptases), 5 \( \mu\)l of RNA template (0.2 ng/\( \mu\)l) and 3.25 \( \mu\)l of RNase-free water (5.75 \( \mu\)l in the 18S set-up). Prior to and following each experiment, a 10-fold serial dilution was run using control and experiment samples to assess PCR efficiency.
Western blotting analysis was used to quantify MyHC in the white muscle of juvenile walleye from the three ponds. The myofibrillar protein fraction was isolated by washing ground muscle in 500 µl of 25 mM KCl, 5 mM EDTA, 50 mM imidazole, 1 mM DTT, and 0.001% protease inhibitors (Sigma-Aldrich, St. Louis MO), and centrifuging at 1000 x g. This myofibrillar protein pellet was homogenized in 500 µl of buffer containing phosphate buffered saline (PBS: 10.1 mM NaH$_2$PO$_4$, 1.8 mM KH$_2$HPO$_4$, 136.9 mM NaCl, 2.7 mM KCl, pH 7.4), 1% Igepal CA-630, 0.5% $C_{24}H_{39}NaO_4$ (deoxycholic acid), 0.5% SDS, 10 mM HSCH$_2$CH$_2$OH (beta-mercaptoethanol), protease inhibitors (Sigma-Aldrich, St. Louis MO), and 0.5% Triton X-100, following a 60 s vortex and 45 min incubation in the buffer. Homogenates were centrifuged at 5000 x g for 10 min at 4 ºC. The supernatant was transferred to a centrifuge tube, and the above procedure was repeated with the pellet. This was to ensure the protocol extracted maximal and uniform levels of MyHC monomers for all samples. The total protein content of the supernatants was determined by the Bradford assay (Bio-Rad Laboratories, Hercules CA).

Protein samples were diluted to a concentration of 1 µg·µl$^{-1}$ in Laemmli’s buffer, boiled, and 15 µg of each sample was loaded onto a one-dimensional SDS polyacrylamide gel (7.5%). Running buffer consisted of 25 mM Tris, 0.192 M
glycine, and 0.1% SDS. To allow for comparisons between gels, an internal control was designated for each gel. Proteins were transferred in (50 V for 2 h at 4 °C) onto nitrocellulose membranes (Amersham Biosciences, UK) with a mini trans-blot cell (Bio-Rad Laboratories, Hercules CA) using a transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, and 0.1% SDS). Membranes were blocked overnight at 4 °C with a blocking solution consisting of 10% skim milk in PBST (PBS: 10.1 mM NaH$_2$PO$_4$, 1.8 mM KH$_2$HPO$_4$, 136.9 mM NaCl, 2.7 mM KCl, 0.01% Tween 20). To detect myosin, membranes were then probed with a 1:1000 dilution of the primary antibody MF20 (University of Iowa Developmental Studies Hybridoma Bank, Iowa City IA) and 5% skim milk in PBST for 1 h at room temperature, followed by 4 washes (1 x 15 min, 3 x 5 min) in PBST. An anti-mouse HRP-conjugated secondary antibody (Promega, Madison WI) diluted 1:2500 in 5% skim milk in PBST was then added and incubated at room temperature for 1 h followed by 4 washes (1 x 15 min, 3 x 5 min). Bands were visualized with the Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer, Boston MA). Band density was determined by measuring pixel intensity using AlphaImager software by Alpha Innotech (San Leandro CA).

Statistical analysis

Best-fit linear models were used to determine growth rate for the pond experiment. Significant differences in growth rate were determined using analysis of covariance (ANCOVA), with $L_T$ as the dependent variable and age of the fish as
the covariate. Body mass (g), condition factor, MyHC mRNA and protein expression are presented as means ± 1 standard error of the mean (SEM). A one-way analysis of variance (ANOVA) followed by a Tukey’s post hoc test was used to assess significant differences (P<0.05) in MyHC mRNA content and growth indices.

Results

Sequence determination and phylogenetics

Isolation and sequencing of the final cDNA product for walleye white muscle MyHC resulted in a complete coding region of 5838 bpc (EU769112; Fig. 2.1). Inclusion of this gene in phylogenetic analyses of the vertebrate myosin heavy chain family yielded well-supported trees for both neighbour joining (NJ; Fig. 2.2) and maximum parsimony analyses (data not shown). All analyses gave trees of near identical topology. As expected, the non-muscle MYH isoforms (9, 10, 11 and 14) grouped separately from the muscle specific isoforms, with cardiac muscle isoforms (6 and 7) forming a distinct group within the muscle group. Interestingly, the fish MYH isoforms formed a separate group from those of mammals, with the walleye MYH grouping closely with isoforms from chum salmon Oncorhynchus keta (Walbaum) and white croaker.


Tissue distribution

The tissue distribution of the MyHC sequenced from walleye was examined via real time RT PCR analysis of RNA (Fig. 2.3). This analysis showed that the MyHC heavy chain isoform used in this study was highly expressed in the white muscle, while levels in red and cardiac muscle were negligible.

One-week food deprivation experiment

Fish exposed to one week of food deprivation experienced no significant changes in body mass ($M$) as a consequence of fasting or re-feeding (ANOVA, $p>0.05$) (Fig. 2.4a). Similarly, the condition factor ($K$) of these fish did not differ significantly throughout the course of the experiment (ANOVA, $p>0.05$) (Fig. 2.4b). In contrast, by day 11 MyHC mRNA content decreased more than 5-fold below baseline levels established between days 1-3 (ANOVA, $p<0.05$) (Fig. 2.4c). A trend of higher MyHC mRNA expression was observed upon refeeding, however, this increase was not significant (ANOVA, $p>0.05$).

24 hour fasting experiment

No significant differences for MyHC mRNA levels were observed over the 24 h fasting period (ANOVA, $p>0.05$) (Fig. 2.5). Although there was a tendency
for MyHC mRNA levels to increase 8 h after feeding, and gradually decrease by the 24th h, these changes were not significant (ANOVA, p>0.05).

75-day in-pond growth experiment

The three ponds contained fish of significantly different final mass ($M_f$) (ANOVA, p<0.05) and growth rates ($G$) (ANCOVA, p<0.05), which as attributed to zooplankton density (Table 2.2). Juvenile walleye in the high growth pond grew at a rate 1.3-fold greater than those in the medium growth pond, and 3.6-fold greater than walleye in the low growth pond. In light of these differences, we examined white muscle MyHC expression. The concentration of MyHC protein was significantly different between all three ponds (high > medium > low) (ANOVA, p<0.05) (Fig. 2.6a). Levels of MyHC mRNA varied significantly among the three groups in a similar pattern as protein (ANOVA, p<0.05) (Fig. 2.6b). Specifically, MyHC mRNA content was 3.5-fold higher in the medium pond than the low pond. The high pond had a MyHC mRNA content level that was twofold greater than the fish in the medium pond.

Discussion

Although MyHC expression has previously been examined in relation to muscle development, few studies have examined the potential of MyHC as a marker for MyHC protein accretion and fish growth. Furthermore, the
contrasting results in the current literature indicate that more research is required to further our understanding of how the MyHC isoforms respond to factors that may alter growth. In the present study, a MyHC isoform that is highly expressed in the muscle of walleye was identified, and its utility as an indicator of growth in both field and laboratory experiments was assessed.

Using homology cloning techniques, the full coding sequence for a myosin isoform was amplified from the white muscle of walleye. This protein grouped closely with other fish MyHC isoforms in phylogenetic analyses of the vertebrate MyHC family. More specifically, the walleye MyHC protein grouped very closely with two myosin proteins isolated from the white muscle of chum salmon (Iwami et al. 2002) and white croaker (Yoon et al. 2000), respectively. The results of these analyses strongly suggest that the isolated protein is, in fact, the predominant myosin protein found in white muscle, and the integral component of muscle growth. Real-time PCR results reinforce this idea as the MyHC isoform used in this study is expressed almost exclusively in the white muscle of juvenile walleye (Fig. 2). It has been demonstrated that different MyHC isoforms are expressed in red and white skeletal muscle and can be characterized by the different functions, spatial distribution and developmental expression patterns of these muscles (Johnston 1982, Karasinski 1993, Gauvry and Fauconneau 1996, Xu et al. 2000, Rescan et al. 2001). Confident that the isolated protein was, in fact, the predominant white muscle MyHC isoform found in juvenile walleye, we
then proceeded with the next phase of the experiment, which involved assessing this protein as a molecular marker of tissue growth.

The five-fold decrease from baseline levels after 7 days of fasting illustrates the rapid transcriptional response of MyHC during periods of low food abundance. An equally rapid response was observed by Overturf and Hardy (2001) who examined the expression level of myosin in rainbow trout exposed to different diet regimes. Fish fed at different feed levels had higher MyHC mRNA concentrations, and switching diet from low feed levels to high feed levels and vice versa revealed a 1 week response time in changes in MyHC mRNA concentration. Fong et al. (1989) reported a decrease in myosin heavy chain mRNA after only 3 d starvation in the gastrocnemius muscle of Sprague-Dawley rats *Rattus norvegicus*. In contrast, Johansen and Overturf (2006) found little change in MyHC gene expression after 30 d of starvation in rainbow trout. However, the expression of Tmyogenin – an important activator of myosin transcription (Watabe 2001) – did decrease, suggesting a reduction in myotube hypertrophy. This response, in the absence of a myosin response, may be the result of the change in Tmyogenin not being large enough to affect myosin expression. Furthermore, the response of myosin to Tmyogenin may require longer periods of time than that used in the study (Johansen and Overturf 2006). Biga et al. (2004) also observed no changes in MyHC mRNA despite significant increases in MyHC protein following growth hormone (GH) treatment in rainbow trout. However, the fish used in the authors’ studies were approximately 300 g
(Johansen and Overturf 2006) and 500 g (Biga et al. 2004), compared to 160 g
fish used in Overturf and Hardy (2001) and the 6 g mean weight of fish used in
the present study. Size is an important variable when considering the effects of
starvation, as smaller fish have less energy stores and higher metabolic rates than
larger fish (Schmidt-Nielson 1984, Kieffer and Tufts 1998). We suggest that
decreases in MyHC mRNA may lead to lower protein synthesis and reduced
growth in juvenile walleye. White muscle makes up over half of the total mass of
fish. By reducing muscle growth during starvation, fish can alleviate the
substantial metabolic cost of protein synthesis (Mommsen 2001).

Further experiments were conducted to determine whether MyHC levels
would change after a single feeding session. In our study, MyHC mRNA content
remained unchanged over the 24 h fasting period (Fig. 4). Food deprivation for
24 h had a similar response in mice (Jagoe et al. 2002). Only after 48 h of fasting
did any of the myosin heavy chain isoforms decrease in the gastrocnemius
muscle. While daily patterns in MyHC mRNA expression have not been
investigated in fish, daily changes in growth regulators in the GH/IGF-I axis are
well studied (Fong et al. 1989b, Small 2005, Ayson et al. 2007). No significant
differences were measured in mRNA levels of growth hormone (GH) over a 24 h
cycle in the rabbitfish *Siganus guttatus* (Bloch), although there were tendencies
towards low levels during the day (Ayson et al. 2007). It is likely that the
transcriptional machinery requires a longer time to respond to fasting, especially
given that an upstream regulator of growth such as GH does not change in the 24
h time frame. Another factor to consider is the detection limits and sensitivity of the methods in the present and cited studies.

Our 75 d end-point investigation of fish growing at different rates in different ponds demonstrated that the expression of MyHC mRNA was positively related to both MyHC protein concentration in the white muscle (Fig. 5) and to length increases ($G$) (mm $\cdot$ d$^{-1}$) and body mass ($M_i$) (g) of juvenile walleye (Table II). Overturf and Hardy (2001) found similar results in the myosin transcripts of rainbow trout that received different levels of feed over a six-week trial. Cod Gadus morhua (Linnaeus) also increased MyHC mRNA expression upon receiving higher protein diets (von der Decken and Lied 1992). In contrast, several studies report no changes in MyHC mRNA during periods that affect growth (Biga et al. 2004, Johansen and Overturf 2006). However, in these studies it is unclear how protein expression or growth was affected. In our experiment, higher levels of mRNA were correlated with increased protein synthesis. Several studies support these findings using total protein or myofibrillar fractions. Anabolism induced by the $\beta$2-adrenergic agonist, ractopamine, which is orally administered to fish to promote muscle accretion, was found to significantly increase MyHC mRNA in (Salem et al. 2006) and myofibrillar protein in rainbow trout (Lortie et al. 2004). Hevrøy et al. (2006) observed a positive correlation between elevated MyHC gene expression and increased specific growth rate and white muscle total protein accretion in Atlantic salmon Salmo salar (Linnaeus). Myofibrillar protein synthesis in sand bass
Paralabrax nebulifer (Girard) decreased significantly after 23 d starvation (Lowery and Somero 1990). Our study demonstrates that higher expression of MyHC mRNA causes a proportional change in MyHC protein in walleye.

In summary, this study shows that mRNA expression of an isolated MyHC isoform can serve as a sensitive marker for muscle growth in walleye. Using molecular tools to monitor growth may be especially valuable for aquaculture research where it is important to quickly determine the optimal conditions for culturing different species and life stages. Understanding the response of the various MyHC isoforms to factors that may affect growth remains an exciting avenue for future research.

Acknowledgments

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Table 2.1. Real-time RT PCR and primer-walking sequences for myosin heavy chain (MyHC).

<table>
<thead>
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<th>Primer usage</th>
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Table 2.2. Growth rate of juvenile walleye over 75 d in three different pond treatments given different levels of fertilization to adjust zooplankton density: low, medium and high. Fish were terminally sampled on day 75. Growth rate was determined using a best fit linear model.

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<tr>
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Mean ± SD; N = 10; different superscripts (a-c) in the same column are significantly different (an ANCOVA was used to compare growth rates and an ANOVA was used to compare final weight; P < 0.05)
Figure 2.1.

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Figure 2.2. Phylogenetic analysis of walleye MyHC and other myosin II isozymes. The phylogenetic tree was constructed using neighbor joining analysis with support for nodes assessed using bootstrap analysis.
Figure 2.3. Myosin heavy chain (MyHC) content in white, red and cardiac muscle of *S. vitreus*. MyHC levels were corrected for loading differences using 18S rRNA and were expressed relative to a reference sample loaded during each PCR run. Values are mean ± 1 SEM, with statistically different groups indicated by different letters (ANOVA; N = 6; P < 0.05).
Figure 2.4 (a). Weight (g) of juvenile *S. vitreus* over the 17 d experiment.

Walleye were deprived of food through days 4 -11. Weight is shown as mean ± 1 SEM. Grey bars indicate period of food deprivation. Different letters denote significant differences (ANOVA; N = 6; P < 0.05).
Figure 2.4 (b). Condition factor of juvenile *S. vitreus* over the 17 d experiment. Walleye were deprived of food through days 4 -11. Condition factor is shown as mean ± 1 SEM. Grey bars indicate period of food deprivation. Different letters denote significant differences (ANOVA; N = 6; P < 0.05).
Figure 2.4 (c). MyHC mRNA content of juvenile *S. vitreus* over the 17 d experiment. Walleye were deprived of food through days 4-11. MyHC mRNA content are shown as mean ± 1 SEM. MyHC levels were corrected for loading differences using 18S rRNA and were expressed relative to a reference sample loaded during each PCR run. Grey bars indicate period of food deprivation. Different letters denote significant differences (ANOVA; N = 6; P < 0.05).
**Figure 2.5.** White muscle content of MyHC mRNA over a 24 h period in *S. vitreus*. Walleye were fed just prior to 10:00 h and were sampled every 4 h (N = 4). MyHC mRNA levels were corrected for loading differences using 18S rRNA and were expressed relative to a reference sample loaded during each PCR run. No significant differences were observed (ANOVA; N = 6; P > 0.05).
Figure 2.6. (a) The effect of growth on mRNA content in the white muscle of *S. vitreus*. Fish were reared for 75 d from hatch in low, medium, and high productivity ponds to control growth rates. MyHC mRNA levels were corrected for loading differences using 18S rRNA and were expressed relative to a reference sample loaded during each PCR run. Statistically different groups (Kruskal-Wallis nonparametric one-way ANOVA) were indicated by different letters (P < 0.05).
Figure 2.6 (b). The effect of growth on MyHC protein content in the white muscle of *S. vitreus*. Fish were reared for 75 d from hatch in low, medium, and high productivity ponds to control growth rates. Protein levels were corrected with an internal standard loaded in each gel. Values are mean ± 1 SEM. (N = 6), with statistically different groups (Kruskal-Wallis nonparametric one-way ANOVA) indicated by different letters (P < 0.05).
Chapter 3. Expression of myosin heavy chain mRNA during juvenile walleye (*Sander vitreus*) feed training

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Keywords: myosin, walleye, feed training, aquaculture
Abstract

The conversion to a pellet diet from natural food sources is one of the most critical stages of aquaculture. This is especially important in walleye aquaculture, where both starvation and cannibalism are common occurrences. To date, no study has examined the implications of the feed training response on muscle development of juvenile walleye, or how this response is modified by size and age of the juveniles. Our results show a significant decrease in the expression levels of myosin mRNA in juvenile walleye within three to five days from the start of the feed training regimen. The size of the fish at the beginning of the feed training regimen had a significant effect on myosin expression. Smaller fish had significantly higher mortality rates, larger variations in size at the end of feed training, and significantly lower levels of myosin expression than larger fish of equal age. Furthermore, smaller fish required more time for myosin expression levels to increase significantly after the acceptance of the commercial feed. The effect of juvenile age - or the initiation of feed training - on myosin mRNA expression was also examined. Fish feed trained in late September required no additional time to convert to the pellet diet, and experienced no decreases in MyHC mRNA content. We conclude that fish condition and life stage should be considered when initiating a feed training program for fish.
Introduction

Aquaculture is the fastest growing animal food production sector in the world (FAO 2006). Increasing market demand for fish coupled with decreases in wild stocks have resulted in pressure to expand aquaculture practices and introduce new technologies to increase productivity. Therefore, advances in the methods for monitoring of fish growth (Johnston 1999, Watabe 2001, Mommsen 2001) could have important implications for aquaculture. Traditionally, efforts to assess fish growth have included variables such as length, weight, condition factor, and protein synthesis and turnover. However, these methods can be inadequate for short-term changes in growth and metabolic condition. Recently, studies have examined expression patterns for genes responsible for growth over short and long periods of time (Buckley et al. 2006). However, relatively little is known about how these genes are modified under various conditions, particularly in applications of interest to the aquaculture industry.

Monitoring gene expression can be a powerful tool in aquaculture, as it provides a means of rapidly and accurately assessing growth. Early work by Fong et al. (1989) demonstrated that decreases in myofibrillar proteins were associated with decreases in the corresponding mRNA transcripts. Recent work in fish has demonstrated the plasticity of muscle growth to environmental factors such as feeding (Johnston 1999, Johansen and Overturf 2006, Hevrøy et al. 2006). Post-natal hypertrophy and hyperplasia make fish an ideal system to study the
regulation of muscle proliferation (Johansen and Overturf 2006). Myosin, in particular, is an important protein as it is the largest component of myofibrils, which themselves account for two-thirds of muscle protein synthesis (Mommsen 2001). Transcript levels for MyHC responded within 1 week to changes in protein content in the white muscle of rainbow trout (Overturf and Hardy 2001). In Chapter 1, I demonstrated how MyHC transcript levels respond to modifications in nutrient levels, and the positive relationship between MyHC mRNA and protein levels. Despite these findings, it is unclear how MyHC mRNA responds over a feed training regiment.

Due to declines in natural populations resulting from habitat loss and over-fishing, there is a tremendous economic potential associated with walleye aquaculture in North America. Current culture practices involve rearing fish in “grow-out” ponds until they are large enough to be transferred to tanks (see Summerfelt 1996 for review). Under this regime, juvenile walleye must switch diets from zooplankton found in the ponds to commercial pellets they are fed in tanks. This feeding transition is critical as walleye are susceptible to starvation during the feed training process. During this period, high mortality rates are often observed stemming from starvation or cannibalism. Juvenile walleye often resort to cannibalism when food resources are inadequate. At present, very little is known about the effects of feed training on growth, or whether feed training techniques can be improved to increase survivorship and growth.

The relationship between body size and metabolism has been well studied in fish (Kieffer and Tufts 1998, Navarro and Gutierrez 1995, Moyes and West
1995, Moyes and Lemoine 2005). As demonstrated with many species of fish, larger juvenile walleye are more capable of surviving periods of starvation than are smaller walleye (Jonas and Wahl 1998) due to greater total body energy (J/g wet weight) normally found in larger fish (Beamish 1964). In walleye aquaculture, larger sizes of fish for feed training are attained by providing healthy zooplankton populations in ponds or by commencing feed training later in the summer. To date, no research has been conducted to determine how the MyHC gene is affected during the feed training process. Furthermore, there has been no attempt to determine whether the potential negative impacts of feed training on MyHC can be reduced by using fish of different ages or sizes.

In this study, we analyze MyHC mRNA expression in the white muscle of juvenile walleye using 3 common feed training scenarios: 1) juvenile walleye from fertilized ponds trained early summer (FTE1); 2) juvenile walleye in unfertilized ponds trained early summer (FTE2); and 3) juvenile walleye feed trained in early fall (FTE3). In walleye aquaculture, pond fertilization is a common method for increasing zooplankton and prey biomass for juvenile walleye (Fox et al. 1989). The condition of these fish was examined during the pond rearing phase prior to being transferred to indoor tanks for feed training. We predict that the larger sizes of juvenile walleye in the fertilized ponds and those trained late summer will result in smaller changes in MyHC mRNA expression and body condition and higher survivorship during feed training.
Methods

Animals and early life-stage conditions

Juvenile walleye were hatched and placed in five different ponds at the Leonard Walleye Culture and Research facility (Hartington, ON). All stocked walleye were of identical age and all originated from eggs collected from the Bay of Quinte, Lake Ontario stock. Four ponds were fertilized with an organic fertilizer (ground hay) to promote zooplankton food base, as per Harding et al. (1992). Two of these ponds were used for the early summer feed training experiment (FTE1), and the other two were used for the late summer feed training experiment (FTE3). In the fifth pond, no organic fertilizer was added with the goal of producing smaller walleye due to reduced zooplankton populations (FTE2). Ponds were examined a minimum of twice a week to ensure walleye in fertilized ponds were of uniform in length and larger than the non-fertilized pond.

Before a feed training experiment, juvenile walleye were seined from the ponds and transferred to a 400 L tank in the recirculation system at Queen’s University (Kingston, ON). Temperature was maintained at 22 ± 2 °C, which was approximately equal to the pond temperature in late June and late August.

Feed training protocol

In general, the feed training process involved converting fish from a zooplankton diet of the ponds to a commercial pellet diet (Corey Aquafeeds,
Fredricton, NB) within tanks. The training process involved feeding fish small servings several times throughout the day until they became habituated to the pellets. This process typically lasts several days. Juvenile walleye were considered feed trained by visually determining whether all fish were consuming the pellets.

*Testing for a possible myosin mRNA response*

We first examined whether MyHC mRNA content was affected during the feed training process (Figure 3.1). Juvenile walleye were transferred to the indoor recirculation system (1.29 ± 0.21 g, N=100) at Queen’s University. Prior to transport, eight fish were sampled as pond controls. Once placed in the recirculation system, eight fish were sampled after 8 h. On day 2, prior to the morning feed, eight fish were removed and sampled, while the remaining fish were fed, once in the morning and once in the afternoon. This procedure was repeated for the entire 17 d experiment. At all sample times, fish were quickly removed from the water and terminally anaesthetized in water containing 250 mg·L⁻¹ 3-amino-benzoic acid ethyl ester (MS-222 Sigma-Aldrich Corp., St. Louis, Mo.) buffered with 500 mg·L⁻¹ NaHCO₃. Whole fish were then weighed and measured for length, and the white muscle was harvested and frozen in liquid nitrogen and stored at -80°C until the tissues were processed for RNA analysis.

*Size and feed training*

Next, we examined the impact of fish size during feed training, in terms of MyHC mRNA content, size and condition factor, and cumulative mortality. Two
hundred fish were collected from the fertilized ponds (100 from two fertilized ponds) for FTE1 and transferred to two tanks at Queen’s University (1.31 ± 0.20 g). For comparison between large and small walleye, another two hundred fish (FTE2) were collected from the no fertilizer pond as well (0.14 ± 0.02 g) and transferred to Queen’s University. The protocol upon collection was the same as above. The feed training program began the day the fish were placed into the recirculation system. The experiment ended after 15 days.

**Age and feed training**

To determine whether there was any advantage to either early summer fingerling feed training or late summer fingerling feed training, 200 juvenile walleye (2.33 ± 0.24 g) were seined from two fertilized ponds (100 walleye from each pond) and transferred to two tanks at Queen’s University in late August. The feed training program for this experiment also began the day the fish were placed into the recirculation system. The experiment ended after 15 days.

**Tissue collection and RNA isolation**

White muscle was carefully excised from the caudal region of the terminally anaesthetized walleye, and ground under liquid nitrogen using a mortar and pestle. Once ground, total RNA was extracted from 0.05 g of frozen tissue using a silica-gel-based membrane and centrifugation technique (Qiagen RNeasy Mini Kit, Qiagen, Valencia CA). Total RNA was then quantified with a SpectraMax Plus 384 (Molecular Devices, CA) plate spectrophotometer.
Sequence development and quantitative real-time RT-PCR

The MyHC amplicon was 147 bp long, and designed based on highly homologous regions of *O. mykiss* (accession no. Z48794), *N. coriiceps* (accession no. AJ24376), *D. rerio* (accession no. AF180893), and *O. keta* (accession no. AB024929). All sequences were obtained in GenBank and aligned using Clustal W. The MyHC amplicon was amplified by PCR at an annealing temperature of 58 ºC using the forward primer 5’-GAGAYGTTGGCAGACTKCAGG-3’ and the reverse primer 5’-TTTCCTTCCAGAGTCACG-3’. All PCR reactions involved an initial denaturation at 94 ºC for 30 s followed by 30 cycles of 94 ºC for 30 s; annealing temperature for 60 s; 72 ºC for 90 s, and ending with a final extension for 10 min at 72 ºC.

A one-step SYBR green PCR was performed on a Cepheid SmartCycler (Cepheid, CA) using the QuantiTect SYBR Green RT-PCR kit (Qiagen Inc., Mississauga, ON, Canada). Cycling conditions for the 18S endogenous control gene were as follows: 50 ºC for 30 min (RT), 95 ºC for 15 min (to activate HotStarTaq DNA polymerase), followed by 45 cycles of cDNA synthesis at 94 ºC for 15 s, 56 ºC for 30 s, 72 ºC for 30 s, and 78 ºC for 15 s with optics on. Finally, a melt curve was created using a 60 ºC to 95 ºC temperature ramp at 0.2 ºC/s. The conditions for the myosin target gene were very similar, however, during cDNA synthesis, the fourth stage was increased from 78 ºC to 80 ºC. Each PCR tube contained a total of 25 μl of PCR mixture, containing: 12.5 μl of Qiagen QuantiTect SYBR Green RT-PCR Master Mix (HotStarTaq DNA polymerase,
QuantiTect SYBR Green RT-PCR buffer, dNTP mix including dUTP, SYBR Green 1, ROX passive reference dye, 5 mM MgCl2), 2 μl each of 5 μM myosin forward and reverse primers (0.75 μl of 2.5 μM 18S primers), 0.25 μl RT mix (containing both Omniscript and Sensiscript reverse transcriptases), 5 μl of RNA template (0.2 ng/μl) and 3.25 μl of RNase-free water (5.75 μl in the 18S set-up). Prior to and following each experiment, a 10-fold serial dilution was run using control and experiment samples to assess PCR efficiency.

**Statistical analysis**

Myosin HC mRNA is presented as means ± 1 standard error of the mean (SEM). A one-way analysis of variance (ANOVA) followed by a Tukey’s post hoc test was used to assess significant differences (P<0.05) in MyHC mRNA content. Replicates were pooled for all MyHC mRNA content comparisons after it was determined that there were no significant differences between any of the within-run replicates (ANOVA, P > 0.38 in all cases).

**Results**

Juvenile walleye in the FTE1 group required 4 d before they began to accept a modified pellet diet. The same was true for the FTE3 group. In both cases, all fish within the two treatments converted to pellets on the same day. The FTE2 did not start consuming the pellet feed until day 8. Furthermore, not all
fish switched to pellets at the same time. Some had not converted by the end of the experiment (day 15).

Size and condition

All three feed training groups differed significantly in weight (FTE3 > FTE1 > FTE2) (Figure 3.2a). Interestingly, weights did not change significantly within FTE1 and FTE3 throughout the course of the experiment. In contrast, FTE2 did increase significantly in weight after 15 d (Figure 3.2a). This can largely be attributed to cannibalism, which was absent in the other two treatments. Five percent of the juveniles in FTE2 resorted to cannibalism by day 5. By day 15, it was estimated that up to 39% of the remaining fish were cannibals. The condition factor between the three groups differed significantly (FTE3 > FTE1 > FTE2) (Figure 3.2b). As was the case with weight, condition factor did not change significantly within the FTE1 and FTE3 groups throughout the course of the experiment, whereas the condition factor of FTE2 increased significantly by day 15.

Survivorship

The cumulative mortality reached 38% in FTE1 by day 8 (Figure 3.3). The mortalities in FTE1 slightly exceeded those of FTE2 early in the experiment between days 0 – 4. However, the cumulative mortality of FTE2 nearly doubled that observed in FTE1, reaching 75% by the end of the experiment (Figure 3.3).
Feed training and myosin expression

Myosin heavy chain mRNA levels decreased significantly from day 0 to day 4 in the FTE1 group, prior to accepting the pellet diet (Figure 3.4). After the fish accepted the pellet diet (day 4), MyHC levels exceeded the day 4 low and reached the day 0 baseline by day 11. After two weeks, MyHC mRNA levels were greater than baseline levels (Figure 3.4). In the FTE2 group, MyHC mRNA levels fluctuated over the first 11 d, however, these changes were not significant (Figure 3.4). The exception to this was day 2, where MyHC mRNA reached its lowest levels. On day 15, MyHC mRNA increased significantly, more than doubling levels observed prior to this sampling point. While feed training, FTE3 experienced no changes in MyHC mRNA content. However, MyHC mRNA levels did increase over their lowest levels from day 2 after 15 d. Baseline levels for FTE3 established on day 0 were not exceeded by the end of the experiment, as was the case for FTE1 and FTE2.

When the three groups are compared, baseline and final MyHC mRNA levels are highest in FTE1. Interestingly, baseline MyHC mRNA levels for FTE3 were significantly lower than FTE1 and similar to those found in FTE3. MyHC mRNA content was more responsive to starvation and growth, as changes were noted here that were not detected using weight or condition factor.

Discussion
Juvenile walleye feed training has long been a major bottleneck in the success of walleye aquaculture. During the feed training process, culture facilities may experience substantial mortality due to starvation and cannibalism (see Summerfelt 1996 for review). The main purpose of this study was to use MyHC mRNA as a molecular marker to rapidly assess how size and age of juvenile walleye would affect feed training success. The results of this study suggest that MyHC mRNA levels are sensitive enough to be significantly affected by the short starvation period during the feed training process. In addition, it was found that a higher condition factor had a significant impact on the success of the feed training regimen.

Periods of starvation shift pathways to derive energy from storage sources such as lipid stores or muscle, in the form of protein breakdown. During starvation, genes that promote muscle growth, such as MyHC, would also be expected to decrease in expression (Buckley et al. 2006, Overturf and Hardy 2001). The expected changes in MyHC mRNA content observed in FTE1 and, to a lesser extent, FTE3 were similar to patterns observed in rainbow trout (Overturf and Hardy 2001). In their study, diet was switched to a level between 0 and 100% satiation, after 6 weeks at a prescribed feeding level. As was the case in the present study, MyHC mRNA expression levels responded within a week to the increase or decrease in ration level. The decline observed in FTE3 was not significant, likely due to the fact that the starvation period was not long enough to substantially affect fish with a higher condition factor (Figure 3.2b). Hevrøy et al. (2006) also observed that changes in MyHC mRNA expression were positively
correlated with solubilized dietary protein levels in Atlantic salmon (*Salmo salar*). Increases in MyHC expression have been correlated with increases in muscle protein (Dhillon et al. Chapter 2, Hevrøy et al. 2006), suggesting that this marker can be used to assess muscle growth.

The pattern observed in the walleye from high productivity ponds trained in the early summer (FTE1) was not observed in fish from the low productivity ponds (FTE2) or those trained late summer (FTE3). The low MyHC mRNA content of FTE2 to begin the experiment was likely a result of the initial condition of the fish. Lower nutrient uptake may have caused MyHC mRNA content to be relatively low at the start of the experiment. Feed training would not change this condition while the fish are not accepting pellets. However, fluctuations would be expected should cannibalism or feed habituation take place. The large number of cannibals observed in FTE2 suggests that the increase in mRNA was likely not attributable to feed training. The robust initial condition of FTE3 would explain why MyHC mRNA content changed very little during feed training. Larger fish are more resistant to the effects of starvation (Jonas and Wahl 1998), and the present study shows that this is also reflected in MyHC mRNA content.

While studies have demonstrated decreases in MyHC mRNA expression in response to fasting (Overturf and Hardy 2001, Svanberg et al. 2000), conflicting results have also been observed. In white muscle tissue sampled from rainbow trout before and after a 30 d feed restriction, and after 4 and 14 days of refeeding, MyHC mRNA expression did not change upon refeeding (Johansen and Overturf 2006). The authors suggest that the two week refeeding period may have been
too short to detect changes in myosin. Myosin transcription is activated through several intermediates by Tmyogenin and MEF2A. Therefore, it is also possible that Tmyogenin and MEF2A content in muscle would have to be higher to significantly increase myosin expression (Johansen and Overturf 2006).

Interestingly, the FTE1 and FTE3 groups required the same length of time to accept the pellet diet. Both groups had significantly greater MyHC mRNA contents by the end of the experiment compared to their day 2 lows. Levels of MyHC mRNA were very high at day 15 for FTE2. As previously mentioned, however, this is likely due to cannibalism in the FTE2 group. Diet switches from low percentage satiation to higher percentage satiation caused similar results in rainbow trout (Overturf and Hardy 2001). The increase in MyHC content observed in all three groups can be explained by an acceleration of protein synthesis relative to protein degradation upon refeeding (Hornick et al. 2000). While increases in myosin were not detected, the higher levels of Tmyogenin and MEF2A in rainbow trout upon refeeding (Johansen and Overturf 2006) suggest a possible cellular mechanism for the increase in MyHC upon refeeding.

The overall condition of a fish will determine how it will cope with starvation. Weight, condition factor, and survivorship were all greatest in FTE3 and lowest in FTE2. Past experiments have demonstrated lower success in feed training smaller juvenile walleye compared to larger sizes (Cheshire and Steele 1972, Malison and Held 1996). The strong relationship between fish size and energy stores has been well studied (Henderson et al. 1988, Johnson and Evans 1990, Kieffer and Tufts 1998). Longer survivorship for fish with larger body sizes
during long-term starvation has been previously observed in pond-reared walleye (Jonas and Wahl 1998). Enhanced body condition can benefit juvenile walleye during feed training by allowing the fish to cope with starvation. Cargnelli and Gross (1997) described a positive allometric relationship between body size and energy reserves in bluegill. The authors found that the amount of lipid stored per gram of body weight was greater in larger bluegills (Lepomis microchirus) than smaller bluegills, allowing larger bluegills to emerge from winter in better condition. Similarly, we believe that, given the smaller sizes of walleye in FTE2, these fish were less tolerant of starvation induced by feed training, and resorted to cannibalism to alleviate this pressure. Cannibalism is common among piscivores, particularly during nutrient deficiency (Smith and Reay 1991). Poor nutritional state coupled with high intraspecific contact found in tanks would provide a high energy gain per unit effort, as predicted by optimal foraging theory, and thus encourage cannibalism (Cuff 1977, Smith and Reay 1991). The time selected to begin feed training during the first year of walleye growth is an important consideration for culture facilities. Our experiment demonstrates that timing has significant implications towards the survivorship and MyHC mRNA expression in juvenile walleye. Body condition, weight, survivorship were significantly greater throughout the experiment in juvenile fish trained in late September as compared to fish trained early July. In fish trained in late September, MyHC mRNA content did not change during the feed training process which is in contrast to the decreases observed in early summer fingerlings undergoing the same training regime. These findings suggest there are
advantages to commencing feed training in the fall. However, in situations such as stocking, the opposite has been found (Pratt and Fox 2003). In their study, Pratt and Fox (2003) observed higher survivorship and larger sizes in later years for summer fingerlings versus fall fingerlings. Furthermore, the authors cite the cost of raising walleye to the fall fingerling stage is higher. Based on their work, summer feed training may be more advantageous for long-term resource applications such as stocking. Even in these cases, however, our results indicate that this will be most effective if the fish are in the best possible condition at the time of stocking. Measures such as maximizing productivity in the ponds used for rearing early life stages would be one step to enhance juvenile walleye energy stores.

Juvenile walleye feed training represents a major bottleneck in walleye aquaculture. As shown in this study, success can be determined by body condition at the beginning of the feed training regimen and the time at which feed training is initiated. Sensitive markers for growth such as MyHC mRNA content provide insight as to what initiatives can be used to enhance culture practices, especially as new species and practices are brought online. Future research should incorporate markers such as Tmyogenin and MEF2A, which may be even more responsive to changes in culture conditions and would provide more insight about muscle gene expression for more fish species. In light of these findings, care should also be taken in culture facilities to optimize the condition of juvenile fish prior to feed training. Further studies should also use markers
such as MyHC to establish how various factors such as rearing temperature or nutrient content can modify short term growth.

Acknowledgments

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Figure 3.1. The effect of the feed training protocol on MyHC mRNA content in the white muscle of *S. vitreus*. MyHC levels were corrected for loading differences using 18S RNA and were expressed relative to a reference sample loaded during each PCR run. Values are mean ± 1 SEM, with statistically different groups indicated by different letters (ANOVA; N=6; P < 0.05).
Figure 3.2

Weight (g) of juvenile walleye (*S. vitreus*) during feed training. The experiments were conducted over a 15 d period, with day 0 being the first day of training. FTE1 and 3 required 4 d to switch to a pellet diet, whereas FTE3 required 8 d for at least some fish to consume pellets. Values are mean ± 1 SEM, with statistically different groups indicated by different letters (ANOVA; N = 20; P < 0.05).
Figure 3.2. Condition factor (B) of juvenile walleye (*S. vitreus*) during feed training. The experiments were conducted over a 15 d period, with day 0 being the first day of training. FTE1 and 3 required 4 d to switch to a pellet diet, whereas FTE3 required 8 d for at least some fish to consume pellets. Values are mean ± 1 SEM, with statistically different groups indicated by different letters (ANOVA; N = 20; P < 0.05).
Figure 3.3. Cumulative mortality among all three treatment groups for juvenile walleye (*Sander vitreus*). The experiments were conducted over a 15 d period, with day 0 being the first day of training. No mortalities were recorded for FTE3.
Figure 3.4. White muscle content of MyHC mRNA over a 15 d period in *S. vitreus*. MyHC levels were corrected for loading differences using 18S RNA and were expressed relative to a reference sample loaded during each PCR run. Different letters denote significant differences (ANOVA; N = 6; P > 0.05).
Chapter 4. Effect of heat stress on the heat shock response and myosin heavy chain in juvenile walleye (*Sander vitreus*)

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Abstract

In recent years, both wild and cultured fish may be exposed to higher water temperatures due to climate change. We used white muscle in juvenile walleye to examine the effects of heat stress on myosin heavy chain expression, a potential indicator of muscle growth. Walleye were subjected to acute (12 h) temperature increases from 22 °C to 34 °C, and chronic (144 h) temperature exposure at 28 °C. Heat shock protein (Hsp) 70 mRNA and protein expression was measured to determine the response and recovery to heat stress. Myosin heavy chain (MyHC) mRNA and protein levels were also quantified to see if there was a relationship between stress and growth responses. Acute and chronic temperature stress induced a significant increase in Hsp 70 mRNA expression at temperatures that could be experienced by walleye in natural environments and culture facilities. Under acute and chronic temperature stress, Hsp+Hsc protein levels increase, and subsequently return to baseline levels during the recovery phase or sustained temperature stress, respectively. MyHC mRNA expression did not change during acute heat shock, but increased significantly at the end of the recovery phase. During chronic temperature stress, MyHC mRNA decreased after 22 h of exposure to temperatures of 28 °C. MyHC protein levels in both experiments increased significantly shortly after maximum temperatures were reached, and decreased quickly during recovery from acute stress (42 h) or later in the chronic exposure period. This study shows that thermal stress affects both Hsp and MyHC expression in walleye, but these responses are largely determined by the nature of the heat stress.
Introduction

Natural populations of walleye (Sander vitreus) can be found from the Northwest Territories to Alabama (Galarowicz and Wahl 2003). A variety of factors, including the popularity of walleye as a sport fish and for consumption have led to dramatic declines in natural populations in both the United States and Canada. To alleviate pressure on natural populations, efforts have been made to develop aquaculture programs of this species (see Summerfelt 1996 for a review). For culture, walleye are typically introduced into small ponds and/or outdoor recirculation systems during the juvenile stage. Towards the southern end of their geographical range, these walleye are often exposed to high summer temperatures that may induce stress (Lester et al. 2004). However, very little is known about the relationship between heat stress and growth in walleye.

In contrast to these changes in mRNA, longer periods of time are required for changes in Hsp 70 protein levels to occur, but changes in protein are more sustained (Lund et al. 2003). Under similar thermal conditions, induction or repression of over 200 genes in the gill and white muscle of the goby (*Gillichthys mirabilis*) was observed (Buckley et al. 2006). At present, however, very little is known about the impact of extreme temperatures on other cellular processes.

The consequences of high concentrations of Hsp 70 have been demonstrated in several organisms. The induction of Hsp 70 requires large portions of the cell’s amino acid stores and protein synthesis machinery, and can repress other gene activation, protein synthesis and folding (McKenzie and Meselson 1977, Theodorakis and Morimoto 1987, Krebs and Feder 1997, Krebs and Feder 2003). Transgenic *Drosophila melanogaster* that contain extra copies of the Hsp 70 gene had higher survival immediately after heat stress. However, these flies also exhibited decreases in growth and higher adult mortality (Krebs and Feder 1997). Interestingly, there was no induction or repression of structural genes such as actin, tubulin, keratin or myosin in gobies exposed to heat stress (Buckley et al. 2006). The authors conclude that a stressor cascade, from heat stress to osmotic stress (as a secondary effect of heat stress), may be required to induce changes in muscle protein expression. However, it is difficult to determine whether this is a function of the magnitude or the duration of the heat stress.

The synthesis of muscle protein is regarded as an ideal marker for overall fish growth because protein synthesis in the muscle is representative of that of
the whole animal (Houlihan et al. 1993). As a result, myosin heavy chains (MyHC), which are the major component of myofibrils, can be used as an indicator of growth in fishes (Mommsen 2001). For example, MyHC mRNA levels responded within one week to changes in feeding level in rainbow trout (Oncorhynchus mykiss) (Overturf and Hardy 2001). MyHC protein levels have also been shown to be very sensitive to rbGH treatment (Biga et al. 2004).

A strategy to cope with the energetic costs associated with the heat shock response may be to conserve energy by limiting growth. In order to examine the potential relationship between heat stress and growth, this study quantified MyHC and Hsp 70 mRNA expression patterns and protein levels in the white muscle of juvenile walleye during acute and chronic temperature stress. The time frames and temperatures used in our study were intended to mimic natural warming events that may be experienced by walleye during summer months in culture facilities, or in the wild.

**Methods**

**Animal acquisition and holding**

Juvenile walleye (16.52 g ± 0.6) were obtained from the Ontario Ministry of Natural Resources White Lake hatchery and held at the Queen’s University Animal Care facility, located in Kingston, Ontario, Canada. Fish were maintained in 400 L flow-through tanks in dechlorinated Kingston city tap water on a 16:8 light:dark photoperiod. Water temperature was 22°C, and water pH was 7.6 ±
Fish were fed commercially purchased pellets on a ration of 2% body mass per day.

**Series 1: Acute temperature increase**

The first experimental series was performed to determine the temperatures at which the heat shock response is induced in walleye. Twenty fish, acclimated at 22ºC, were transferred to a recirculating tank maintained at a temperature of 22ºC for 48 h prior to experimentation. At the beginning of the experiment, four fish were transferred to an insulated recovery tank containing water from the experimental tank, to control for possible effects of handling on the heat shock response. After the 1 h period, these fish were sampled as outlined below. The water in the recirculating tank was then raised to 25ºC over a 1 h period and maintained at that temperature for 2 h. Four fish from this tank were then transferred to the recovery tank for 1 h prior to sampling. This procedure was repeated for 28, 31, and 34 ºC. For each sampling period, fish were anesthetized in water containing 250 mg·L⁻¹ 3-amino-benzoic acid ethyl ester (MS-222 Sigma-Aldrich Corp., St. Louis, Mo.) buffered with 500 mg·L⁻¹ NaHCO₃. White muscle samples were taken caudally and immediately frozen in liquid nitrogen. All samples were stored at -80ºC until they were processed for RNA and protein analysis.
Series 2: Acute temperature increase and recovery

A second experimental series was performed to determine the expression of Hsp and MyHC during recovery from heat shock. Fifty-two fish, acclimated at 22ºC, were transferred to a recirculating tank maintained at a temperature of 22ºC for 48 h prior to experimentation. At the beginning of the experiment (0 h), four fish were transferred to an insulated recovery tank, filled with water from the experimental tank, and sampled after 1 h. The remaining fish were exposed to a ramped increase in temperature from 22 to 32 ºC as follows: temperature was increased to 25ºC over a 1 h period and maintained at that temperature for 2 h. Temperature was increased to 28 ºC over the course of an hour, and maintained at this temperature for 2 h. Then, temperatures were increased to 32 ºC over 1 h, and maintained at this temperature for 2 h. Thus, the overall temperature profile for heat shock for Series 2 was similar to that of Series 1. Following 2 h of exposure at 32 ºC, water temperature was lowered back to 22 ºC over 7 h and fish were sampled at 18, 22, 30, 42, 66, and 90 h.

Series 3: Chronic temperature increase

Forty fish, previously acclimated at 22 ºC, were transferred to a recirculating tank maintained at a temperature of 22ºC for 48 h prior to experimentation. The tank was aerated and 50% of the water volume was replaced daily to ensure suitable water quality. Water in the recirculation tank was heated to 28 ºC over a 2 h period, and maintained at that temperature for 142 h. Four fish were sampled at the beginning of experiment (at 22 ºC), and
after the temperature had been at 28 °C for 0, 22, 46, 70, 94, 118, and 142 h, using the sampling protocols described above.

Quantitative real time PCR analyses

Total RNA was extracted using a silica-gel-based membrane and centrifugation technique (Qiagen RNeasy Mini Kit, Qiagen Inc., Mississauga, ON). Total RNA was then quantified with a SpectraMax Plus 384 (Molecular Devices, CA) plate spectrophotometer. First strand cDNA was synthesized from 100 µg of total RNA using Multiscribe Reverse Transcriptase as per the manufacturer's instructions (Applied Biosystems, Foster City, CA).

We amplified a 101 bp segment of the Hsp 70 gene using forward and reverse primers from a conserved region of the Hsp 70 genes of *O. mykiss* (accession no. AB062281) sequenced by Currie et al. (1999, 2000). However, the forward primer was modified to shorten the sequence for optimization for quantitative real time PCR (Table 1). The Hsp 70 cDNA fragment was amplified by PCR at an annealing temperature of 58 °C. The MyHC sequence was 147 bp long, and designed based on highly homologous regions of *O. mykiss* (accession no. Z48794), *N. coriiceps* (accession no. AJ243767), *D. rerio* (accession no. AF180893), and *O. keta* (accession no. AB024929). All sequences were obtained in GenBank and aligned using Clustal W. The MyHC cDNA fragment was amplified at an annealing temperature of 58 °C using the forward primer 5’-GAGAYGTGGCGAGACTKCAGG-3’ and the reverse primer 5’-TTTCCTTCCAGAGTCACG-3’. All PCR reactions involved an initial denaturation
at 94 ºC for 30 s followed by 30 cycles of 94 ºC for 30 s; annealing temperature
for 60 s; 72 ºC for 90 s, and ending with a final extension for 10 min at 72 º C.
The resulting PCR products were ligated into pDrive vectors (Qiagen Inc.,
Mississauga, ON) and sequenced by Robarts Research Institute(London, ON).
Gene expression data was obtained using quantitative real time PCR on an ABI
7500 Real Time PCR System (Applied Biosystems Inc., Foster City, CA). Gene
specific primers for real time PCR were designed based on the walleye sequence
determined here using Primer Express 3.0 software (Table 4.1). Reactions were
performed using 2 µl of cDNA, 5 µM of each primer for both Hsp 70 and MyHC
genes, 2x SYBR Green Master Mix (Applied Biosystems Inc., Foster City, CA) and
3.5 µl of RNase-free water (Qiagen Inc., Mississauga, ON, Canada) to a total
volume of 25 µl per well. Cycling conditions were as follows: 1 cycle at 95 ºC for
10 min, 40 cycles of 95 ºC for 15 s; 60 ºC for 30 s; and 72 º C for 1 min. At the end
of the reaction, a melt curve analysis was performed under the following
conditions: 95 ºC for 15 s, 60 º C for 1 min, and 95 ºC for 15 s. Prior to and
following each experiment, a 10-fold serial dilution was run using control and
experiment samples to assess PCR efficiency. The Hsp 70 and MyHC gene
expression data were normalized to the TaqMan 18S rRNA assay as per the
manufacturer’s instructions (Applied Biosystems, Inc., Foster City, CA).

**Protein sample preparation, SDS-PAGE and Western blotting**

Western blotting analysis was used to quantify Hsp 70 and MyHC protein
concentrations in the white muscle of juvenile walleye. The myofibrillar protein
fraction was isolated by washing ground muscle in 500 µl of 25 mM KCl, 5 mM EDTA, 50 mM imidazole, 1 mM DTT, and 0.001% protease inhibitors (Sigma-Aldrich, St. Louis, MO), and centrifuging at 1000 x g. This protein fraction was homogenized in 500 µl of buffer containing phosphate buffered saline (PBS: 10.1 mM NaH2PO4, 1.8 mM KH2HPO4, 136.9 mM NaCl, 2.7 mM KCl, pH 7.4), 1% Igepal CA-630, 0.5% C24H39NaO4 (deoxycholic acid), 0.5% SDS, 10 mM HSCH2CH2OH (beta-mercaptoethanol), protease inhibitors (Sigma-Aldrich, St. Louis, MO), and 0.5% Triton X-100, following a 60 sec vortex and 45 min incubation in the buffer. Homogenates were centrifuged at 5000 x g for 10 min at 4 ºC. The supernatant was transferred to a centrifuge tube, and the above procedure was repeated with the pellet. This was to ensure the protocol extracted maximal and uniform levels of MyHC monomers for all samples. The total protein content of the supernatants was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Protein samples were diluted to a concentration of 1 µg·µl-1 in Laemmli’s buffer, boiled, and 15 µg of each sample was loaded onto a one-dimensional SDS polyacrylamide gel (7.5%). Running buffer consisted of 25 mM Tris, 0.192 M glycine, and 0.1% SDS. To allow for comparisons between gels, an internal control was designated for each gel. Proteins were transferred in (50 V for 2 h at 4 ºC) onto nitrocellulose membranes (Amersham Biosciences, UK) with a mini trans-blot cell (Bio-Rad Laboratories, Hercules, CA, USA) using a transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, and 0.1% SDS). Membranes were blocked overnight at 4 ºC with a blocking solution consisting of 10% skim milk in
PBST (PBS: 10.1 mM NaH₂PO₄, 1.8 mM KH₂HPO₄, 136.9 mM NaCl, 2.7 mM KCl, 0.01% Tween 20). To detect myosin, membranes were then probed with a 1:1000 dilution of the primary antibody MF20 (University of Iowa Developmental Studies Hybridoma Bank, Iowa City, IA) and 5% skim milk in PBST for 90 min at room temperature, followed by 4 washes (1 x 15 min, 3 x 5 min) in PBST. An anti-mouse HRP-conjugated secondary antibody (Promega, Madison, WI) diluted 1:2500 in 5% skim milk in PBST was then added and incubated at room temperature for 90 min followed by 4 washes (1 x 15 min, 3 x 5 min). To detect Hsp 70, membranes were probed with a 1:5000 dilution of the Anti-Hsp70 Global primary antibody (Agrisera, Vännäs, Sweden) and a 1:2500 anti-rabbit HRP-conjugated secondary antibody (Promega, Madison, WI. Washes were performed as above. Bands were visualized with the Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer, Boston, MA). Band density was determined by measuring pixel intensity using AlphaImager software by Alpha Innotech (San Leandro, CA). These data were normalized to the average of the time zero individuals.

**Statistical analysis**

Protein and mRNA content for MyHC and Hsp 70 are presented as means ± 1 standard error of the mean (SEM). A one-way analysis of variance (ANOVA) followed by a Tukey’s post hoc test was used to assess significant differences (P<0.05) in MyHC and Hsp 70 mRNA and protein content.
Results

**Series 1: Acute temperature increase**

During acute temperature exposure, Hsp 70 mRNA content increased 45-fold at 28 °C, which was an increase of 6 °C over 8 h above 22 °C (Figure 4.1) (significant p < 0.05). After exposure at water temperatures of 32 °C, Hsp 70 mRNA concentrations increased 742-fold. Finally, after the 1 h increase from 32 °C and 2 h exposure at 34 °C, Hsp 70 mRNA increased 952-fold from baseline levels. Temperatures of 34 °C resulted in mortality in two fish after 1 h of exposure at 34 °C and the remaining two fish losing equilibrium.

**Series 2: Acute temperature increase and recovery**

The transcriptional response of Hsp70 was significant over the 9 h, 10 °C increase in temperature (Figure 4.2b). Specifically, Hsp 70 mRNA levels of fish exposed to the 32 °C heat shock exhibited an 800-fold induction over control levels after the 9 h temperature increase. As temperature was reduced and recovery initiated, Hsp 70 mRNA content decreased. The decrease was significant 33 h after peak heat shock levels (or 24 h after temperatures returned to control conditions (22 °C)).

The 9 h period of heat shock elicited a significant response (3.5-fold) in hsp+hsc 70 protein concentration (Figure 4.2c). These concentrations did not decrease significantly from peak levels until 48 h after temperatures returned to
initial conditions. However, mean levels did drop sharply, returning to levels similar to baseline levels 4 h after temperatures returned to 22 °C.

During the period of elevated temperatures (hours 0 - 18), MyHC mRNA content fluctuated, though these fluctuations were not significant (Figure 4.3b). Unexpectedly, MyHC mRNA content increased 3.4-fold 24 h after temperatures returned to 22 °C, and continued to rise to a high 5.3-fold greater than baseline concentrations.

MyHC protein concentration increased rapidly immediately following the acute heat shock (Figure 4.3c). MyHC concentration increased nearly 4-fold from 18 to 42 h. However, these values decreased sharply to baseline levels after 42 h (or 24 h after water temperatures returned to 22 °C).

**Series 3: Chronic temperature exposure**

As temperature increased from 22 °C to 28 °C over 2 h (Figure 4.4a), Hsp 70 mRNA increased 86-fold (Figure 4.4b). Interestingly, these levels decreased significantly after 22 and 46 h at 28 °C, and continued to drop, reaching baseline levels after 46 h. Baseline levels were maintained for the remainder of the 144 h experiment. The concentration of Hsp 70 protein increased gradually, reaching peak levels after 72 h – a 3-fold increase (Figure 4.4c).

During the first 24 h of the experiment (after 22 h at 28 °C), MyHC mRNA levels remained constant (Figure 4.5b). However, between 22 h and 46 h of exposure to high temperatures, MyHC mRNA content decreased significantly (3-fold). The transcript level remained low for the duration of the experiment. The
concentration of MyHC protein increased significantly during the first 24 h of the experiment (3.3-fold increase) (Figure 4.5c). After 22 h of exposure to water temperatures of 28 °C, protein concentration steadily decreased, returning to baseline levels after 144 h.

Discussion

The first goal of these experiments was to determine how different temperature stresses affect the heat shock response in walleye. Initial experiments showed that temperatures as low as 28 °C (a 6 degree increase over the acclimation temperature) will initiate a heat shock response in this species. The preferred temperatures for the Lake Ontario stock of walleye from the Bay of Quinte that was used in this experiment range between 18-22 °C and the upper lethal temperature for walleye in this region is thought to be around 31 °C (Kerr et al. 1997, Lester et al. 2004). Induction of Hsp 70 mRNA in the present experiments therefore occurs about 6-10 degrees above the preferred temperature range and about 3 °C below the upper lethal temperature. At present, temperatures in the natural environment where this stock originates from are normally around 25 °C during summer months, but temperatures in some areas of this water body can reach 30 °C (Chu et al. 2004, Lester et al. 2004). It is also noteworthy that many of the culture sites for juvenile walleye in Ontario exceed 28 °C during warmer summer periods. In all of these situations, it would be expected that temperatures would further increase as a result of
climate change. These results suggest that walleye living in the wild, or in culture in Ontario, may already be periodically exposed to temperatures that could initiate a heat shock response, and that this will likely occur more frequently in the future.

In the second series of experiments, an acute temperature stress of 32 °C caused a significant up-regulation in Hsp 70 mRNA and protein expression. During the recovery phase at normal temperatures, the levels of Hsp 70 transcripts rapidly decreased from peak values, although baseline levels were not achieved until the 78 h sample time. In comparison, Hsp/Hsc 70 protein concentration peaked at 32 °C, but required a longer period of time to decrease from peak values than mRNA values. In general, the responses of Hsp 70 mRNA and protein in walleye muscle to an acute heat stress were similar to patterns observed in the white muscle of goby (*Gillichthys mirabilis*; Buckley et al. 2006), and rainbow trout (*Oncorhynchus mykiss*; Lund et al. 2003, Viant et al. 2003). When brook trout were exposed to similar experimental regimes, Hsp 70 mRNA and protein levels in red blood cells followed much the same pattern after acute heat stress as that observed in this study (Lund et al. 2003). However, the time necessary for protein concentration to decrease from maximal values was much longer in walleye than trout. The fact that walleye muscle responds to heat stress in a similar fashion to that in other species is interesting because it suggests that the additional findings about the impact acute temperature stress on myosin may also be broadly applicable to many species.
Much less is known about the heat shock response during chronic temperature stress in fish. In juvenile walleye, Hsp 70 mRNA became significantly elevated during the 2 h temperature increase from 22 to 28 °C. After 46 h of chronic exposure to 28 °C, this variable returned to baseline levels. As expected, the Hsp 70 protein response was slower to develop compared to the Hsp 70 mRNA response. As would be expected, these results show that Hsp 70 mRNA is a more sensitive marker for heat stress than Hsp 70 protein, but Hsp 70 mRNA does not remain elevated during prolonged heat stress. These results are very similar to the findings of Lund et al. (2003) for brook trout and Buckley et al. (2006) for goby. Although protein levels remained elevated throughout the chronic temperature experiment, and also exhibit prolonged increases in some other species (Sanders et al 1992), it is likely that Hsp 70 protein levels would eventually return to baseline levels. The suppression of Hsp 70 mRNA transcripts should reduce Hsp 70 protein levels, despite the chronic thermal stress (DiDomenico et al. 1982). According to (Krebs and Feder 1997), sustained high concentrations of Hsp 70 protein can become deleterious by suppressing the synthesis of other proteins.

As expected, there are changes in MyHC mRNA and protein expression during heat shock. However, the patterns for these responses were quite different between the acute and chronic temperature experiments. Following acute temperature stress, MyHC mRNA levels became elevated during the recovery phase. In the same experiment, MyHC protein increased rapidly in the later stages of the temperature elevation and then decreased shortly thereafter. In
contrast, MyHC mRNA exhibited a rapid decrease during chronic temperature stress. During the chronic heat exposure, MyHC protein concentrations briefly increased, but then decrease for the remainder of the experiment. In both series of experiments, the observed changes in MyHC protein levels suggest that heat stress may have an impact on muscle growth in fish within a relatively short time frame. The increase in MyHC mRNA transcripts during the recovery phase of acute temperature stress is likely associated with the decrease in both Hsp 70 mRNA and protein levels that occurs during that time frame. High expression of Hsp 70 has been shown to consume a large amount of the cell’s amino acid stores and occupy protein synthesis substrates (Krebs and Feder 1997) and can repress the translation of pre-existing messages (Lindquist 1986). Our results suggest that the transcription machinery for a myosin recovery response is only available after the large heat shock response has subsided. (Figure 4.3). Similarly, the decrease in MyHC mRNA transcript levels during chronic heat stress may be the result of the persistence of Hsp 70 transcript and protein levels.

The observation that heat stress had a significant impact on MyHC mRNA and protein levels, and may therefore affect growth, is consistent with our original hypothesis. In several ways, however, the results of the present study were not expected. Our original prediction was that heat stress would likely have a negative impact on growth by reducing MyHC mRNA and protein levels. In both series of experiments, however, MyHC protein levels seem to increase shortly after heat shock. It is possible that increases in MyHC mRNA transcripts were not detected in the time intervals used in this study. This is unlikely given
the 10 h intervals in the acute temperature experiment. It is also possible that the MyHC protein response is post-transcriptional, with higher turnover rates after heat stress. That is, there is an increase in MyHC protein translation from existing transcripts in response to heat stress. Similar increases in myosin protein levels under conditions where MyHC mRNA levels do not change were observed in rainbow trout in response to recombinant bovine growth hormone (rbGH) treatment (Biga et al. 2004). Rainbow trout that were re-fed after 30 d starvation also experienced no increase in MyHC mRNA after re-feeding (Johansen and Overturf 2006). Transcripts of MyHC mRNA may not increase if Tmyogenin and MEF2A expression is not high enough to activate MyHC transcription. Svanberg et al. (2000) suggests that during protein replacement associated with fasting stress in humans, only minimal changes in myosin mRNA levels are necessary to replace protein because myosin protein replacement occurs at the translational level. A rapid protein response would expedite recovery to conditions that would inhibit growth. The increase in protein after thermal stress observed in the present study during acute and chronic thermal stress supports this idea.

In summary, this study shows MyHC mRNA and protein expression in the white muscle of juvenile walleye are significantly influenced by acute and chronic heat stress. These results suggest that heat stress may have significant impacts on growth in walleye. It is also noteworthy, however, that the patterns of these responses are more complex than originally predicted and seem to depend on the nature of the heat stress. Further studies in this area will therefore be required in
order to fully understand the different ways temperature stress may modify levels of myosin, and ultimately how temperature stress affects growth.

Acknowledgments

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Literature cited


**Table 4.1.** Primers used for quantitative real time PCR of Hsp 70 and MyHC genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5' - 3')</th>
</tr>
</thead>
</table>
| Hsp 70 | F: GCAGCCATCGCCTACGG  
           R: ATGGTCAGGATGGACACGT |
| MyHC  | F: GAGGAGGCGGAGGAGCA  
           R: TTTTCCCTTTTCCAGAGTCACG |

F, forward; R, reverse
Figure 4.1. The effects of acute temperature increase on Hsp 70 mRNA content. Hsp 70 mRNA levels were corrected for loading differences using 18S rRNA and were expressed relative to a reference sample loaded during each real time PCR run. Values are mean ± 1 SEM, with statistically different groups indicated by different letters (ANOVA; N = 4; P < 0.05).
Figure 4.2. Temperature profile during the acute temperature increase and recovery (A), and Hsp 70 mRNA (B) and protein (C) levels in response to heat shock. Hsp 70 mRNA levels were corrected for loading differences using 18S rRNA and were expressed relative to a reference sample loaded during each real time PCR run. Protein data was quantified by measuring band density using pixel intensity. Average pixel intensity for each band was normalized to the average for the time zero individuals. Values are mean ± 1 SEM, with statistically different groups indicated by different letters (ANOVA; N = 4; P < 0.05).
Figure 4.3. Temperature profile during the acute temperature increase and recovery (A), MyHC mRNA (B) and protein (C) levels in response to heat shock. MyHC mRNA levels were corrected for loading differences using 18S rRNA and were expressed relative to a reference sample loaded during each real time PCR run. Protein data was quantified by measuring band density using pixel intensity. Average pixel intensity for each band was normalized to the average for the time zero individuals. Values are mean ± 1 SEM, with statistically different groups indicated by different letters (ANOVA; N = 4; P < 0.05).
Figure 4.4. Temperature profile during the chronic temperature exposure (A), and Hsp 70 mRNA (B) and protein (C) levels in response to heat shock. Hsp 70 mRNA levels were corrected for loading differences using 18S rRNA and were expressed relative to a reference sample loaded during each real time PCR run. Protein data were quantified by measuring band density using pixel intensity. Average pixel intensity for each band was normalized to the average for the time zero individuals. Values are mean ± 1 SEM, with statistically different groups indicated by different letters (ANOVA; N = 4; P < 0.05).
Figure 4.5. Temperature profile during the chronic temperature exposure (A), and MyHC mRNA (B) and protein (C) levels in response to heat shock. MyHC mRNA levels were corrected for loading differences using 18S rRNA and were expressed relative to a reference sample loaded during each real time PCR run. Protein data were quantified by measuring band density using pixel intensity. Average pixel intensity for each band was normalized to the average for the time zero individuals. Values are mean ± 1 SEM, with statistically different groups indicated by different letters (ANOVA; N = 4; P < 0.05).
Chapter 5. General Discussion

Myosin heavy chain (MyHC) is the major protein component of myofibrils, and accounts for most of the protein found in muscle (Mommsen 2001). In this regard, it is considered an ideal molecular marker for growth. Fish, in particular, provide an interesting study organism for muscle growth because almost all fish experience indeterminate growth. Fish growth can be divided into hypertrophic (increased muscle fiber size) and hyperplasic (new muscle fiber recruitment) growth. Hyperplasic growth is associated with the persistence of MyHC transcripts throughout all life stages. The MyHC protein itself is a ubiquitous motor protein involved in a number of movement functions including muscle contraction. MyHC is highly conserved, and is therefore very practical for broad applications involving numerous fish species in fields such as aquaculture, where growth equates to economic success.

In this thesis, the primary objective was to investigate MyHC expression in relation to growth and experimental conditions that could impact growth. The experiments were designed to address issues applicable to walleye (Sander vitreus) aquaculture. Declines in natural populations, a rapid growth rate that allow them to reach marketable size within one season of growth, and highly palatable taste make walleye an interesting prospect for culture. While walleye aquaculture has existed for many years, there is a paucity of information about the optimal conditions for growth in this species (Summerfelt 1996). As aquaculture continues its rapid expansion, modifications to create
environmentally sound rearing practices will change conditions for all fish, including those with established rearing protocols such as rainbow trout (*Oncorhynchus mykiss*). It is critical for economic purposes that these modifications occur rapidly to maximize fish growth, and that all variables are optimized for new culture conditions and novel species. In this thesis, I examine the use of MyHC gene expression to rapidly assess growth in walleye.

The first aim of my thesis was to sequence the entire MyHC isoform used in this study. The sequence of this gene was then analyzed in a phylogenetic context to examine this isoform’s relationship with MyHC isoforms from different tissues and species. This isoform was then subjected to two tests to ensure that it was an ideal marker for white muscle growth in juvenile walleye. The marketable tissue of fish is the white muscle, and thus its growth was the focus in this thesis. Analysis of the MyHC isoform content in the cardiac, red, and white muscle of walleye revealed that it was found predominantly in the white muscle tissue. Next, I examined whether higher MyHC mRNA and protein levels were associated with faster growth in this species. Recent literature has revealed conflicting results regarding the response of MyHC expression under various experimental conditions (Overturf and Hardy 2001, Biga et al. 2004, Buckley et al. 2006). However, my study revealed that juvenile walleye exposed to higher nutrient levels, and therefore, faster growth rates, had higher MyHC mRNA content and higher MyHC protein content than those fish grown in ponds with lower nutrient levels. I then proceeded with two subsequent experiments to examine the sensitivity of MyHC mRNA expression during a one-week and a 24 h
fasting period. During one week without food, MyHC mRNA content in the white muscle of juvenile walleye decreased significantly. In the same experiment, however, there were no detectable changes in weight or condition factor. The results of this experiment indicate that a one week fast will have a negative impact on growth in walleye and that MyHC mRNA is a more sensitive indicator of the impact of this fasting period than traditional growth variables such as weight or condition factor. However, MyHC mRNA was not sensitive enough to change significantly over a 24 h fasting period. Taken together, the results of this first series of experiments provide important insight about the expression profile of MyHC, and demonstrate that MyHC may be an accurate marker of growth in fish.

Next, building on the results presented in Chapter 2, an analysis of MyHC mRNA content was applied to a very important dimension to fish aquaculture: feed training. This issue is especially critical in walleye aquaculture as failure to successfully feed train walleye results in starvation and often, cannibalism. Cannibalism can be a major problem during culture, but is relatively common among juvenile walleye likely due to the carnivorous nature of this species and the lack of highly domestic stocks. Prior to these experiments, a successful feed training protocol was developed using a modified commercial pellet diet. The impact of the feed training procedure on growth potential was then measured by quantifying MyHC mRNA content. The ultimate goal of these experiments was to determine how important condition factor and juvenile age were in the feed training process. Walleye are placed in “grow-out” ponds after hatching until they
reach a size where they can ingest commercial pellets. High plankton productivity is critical in these ponds to maximize survivorship and provide the fish with the energetic resources to endure the feed training regimen. The timing of feed training is a logistical issue in which pond capacity typically exceeds the capacity of the tanks used for feed training, creating a temporally staggered feed training system. The results of this study clearly indicate that, despite a typically short training process of 4 d, there are significant decreases in MyHC mRNA content in both the under-nourished fish (FTE2) and the healthier fish trained at the same time (FTE1). Interestingly, the mortality rate and incidence of cannibalism were significantly greater in FTE2, making them unfavorable for feed training. As predicted, the fish trained in late summer (FTE3) were significantly larger and thus less affected by the feed training regimen. Based on the results of these studies, it is recommended that grow-out ponds be designed to maximize the condition factor of the juvenile walleye in order to minimize starvation and cannibalism, and to improve feed training success.

Finally, this thesis examined how heat stress may affect MyHC expression patterns. During the course of my studies, it became apparent that chronically elevated temperatures and acute high temperature events may be relatively common during the rearing process for juvenile walleye. As a result of anthropogenic disturbances such as climate change, it is also likely that many wild walleye populations may face similar problems in the near future. Thus, the potential impact of acute and chronic high temperature events was examined in relation to MyHC expression. For these analyses, Hsp 70 mRNA and protein
content, as well as MyHC mRNA and protein were quantified to determine how MyHC content was affected during a concomitant heat shock response. The data supported the literature with regards to changes in Hsp 70 mRNA and protein expression during acute and chronic thermal stress. In addition, these experiments also supported my prediction that MyHC mRNA and protein would be significantly affected by these disturbances. However, the nature of the changes in MyHC mRNA and protein content were not as initially predicted. After an acute stress of 32 °C, MyHC mRNA content increases during recovery at 22 °C. Protein levels, however, increase immediately after the thermal insult, and then decrease back to baseline levels. During chronic temperature stress, Hsp 70 content again responded as predicted. MyHC mRNA decreased during chronic exposure to 28 °C as initially predicted, but MyHC protein content responded by increasing rapidly and then decreasing as the 1 week experiment neared completion. Together, these results provide interesting insights regarding the response of MyHC during heat stress, and its potential implications for growth. The fact that these responses were more complex than originally predicted also indicates a need for further studies in this area.

Taken together, the studies conducted in this thesis provide valuable information regarding the expression profiles of MyHC mRNA and protein. To the authors’ knowledge, previous studies have not examined MyHC expression profiles over short time scales in fish. Here, we demonstrate the sensitivity of MyHC expression patterns during nutrient manipulation (feed restriction and diet switching) and thermal stress. These studies illustrate the dynamic nature of
muscle tissue and substantiate the efficacy of MyHC as a marker for growth. Sensitive molecular indicators of growth such as this may have important benefits for the aquaculture industry because little is known about the optimal conditions for rearing walleye. Within very short time frames, we were able to assess rearing conditions and provide feedback to our aquaculture partner, Leonard Walleye Culture and Research. Adjustments to temperature and nutrient levels were made within weeks of these analyses rather than the following season after an end-of-year assessment involving traditional growth variables.

Future directions

My research has demonstrated the sensitivity of MyHC as a molecular marker for growth in fish. As a result of this research, however, numerous questions have developed that should be addressed to further understand how expression profiles are manifested in the organism. The discrepancies in the literature and the results presented here may be the result of numerous factors, including lifestage and size of the fish (Gauvry and Fauconneau 1996) and environmental factors such as temperature (Tao et al. 2004). The expression of MyHC isoforms can change as a result of development and temperature. Therefore, to improve descriptions of gene profile and determine functional responses of genes, similar work that takes into account life-stage, size and environmental conditions when measuring MyHC content would be beneficial.

Clearly, increasing the number of markers in time-profile experiments such as those conducted here would also contribute more to our understanding
about how alterations in environmental and rearing conditions can affect the potential for muscle growth. The content levels of genes such as IGF-1 and myostatin have large implications for muscle growth and differentiation, and would be promising targets for future studies. Recent work by Kajimura et al. (2006) has demonstrated the regulatory effects of IGFBP-1 on early muscle growth in zebra fish (*Danio rerio*). Other genes upstream from MyHC, such as Tmyogenin and MEF2A would be interesting to measure in relationship with MyHC expression (Johansen and Overturf 2006).

Concluding remarks

The MyHC gene can provide important information about growth, or the potential for growth, in an organism. My thesis demonstrates how this gene relates to the growth of white muscle, and its relationship with protein production and heat stress. These results will contribute to our understanding of the cellular response of MyHC to nutrient restriction and heat stress in a more detailed manner than that provided in the current literature. In addition, I propose that approaches similar to that used in this thesis that incorporate sensitive molecular markers could be applied in future studies in many different areas to determine how acute and chronic stressors affect growth in fish.

Literature Cited


