Myogenic and Anabolic Gene Expression in Red and White Muscle of Sablefish (*Anoplopoma fimbria*) During Grow Out

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Authorization to Submit Thesis

This thesis of Lisa C. Armbruster, submitted for the degree of Master of Science with a Major in Animal Science and titled "Myogenic and Anabolic Gene Expression in Red and White Muscle of Sablefish (*Anoplopoma fimbria*) During Grow Out," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

We examined muscle growth in aquacultured sablefish (*Anoplopoma fimbria*), a large bodied and fast growing fish from the North Pacific Ocean with economic importance to the United States. A small aquaculture industry, with unrealized growth potential, stands to benefit from knowledge of how different culture environments influence sablefish muscle growth. Species-specific qRT-PCR assays were developed to quantify the transcription of genes with known critical functions in muscle development and nutrient sensing for anabolic signaling in other vertebrates. Expression of these genes was measured in red (oxidative) and white (oxidative, glycolytic, and anaerobic) muscle in males and females over time weighing approximately 0.5 – 2 kg. Differential regulation of these genes by muscle phenotype and at different stages of the growth cycle suggest important and conserved roles in sablefish muscle growth and development. These molecular assay tools will assist in future assessments and design of optimal production measures for sablefish growth efficiency and product quality.

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Dedication

I wish to dedicate this thesis to my husband and family.

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4EBP1	4E-binding protein 1
AD	activation domain
ATP	adenosine triphosphate
ANOVA	analysis of variance
bHLH	basic helix-loop-helix
C	Celsius
cm	centimeter
cDNA	copy deoxyribonucleic acid
ΔCt	delta threshold cycle
DNA	deoxyribonucleic acid
eIF4E	eukaryotic initiation factor 4E
18S	eukaryotic ribosomal RNA subunit with sedimentation rate 18
F1	first filial generation
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FOXO	forkhead box proteins
g	grams
GH	growth hormone
HUFA	highly unsaturated fatty acid
HSD	honest significance difference
C2C12	immortalized mouse myoblast cell line
IGF-1	insulin-like growth factor -1
Kg	kilogram
LSmean	least squares mean
mTOR	mechanistic target of rapamycin
T_m	melting temperature
mRNA	messenger ribonucleic acid
m	meter
µl	microliter
µM	micromolar
mm	millimeter
MAPK	mitogen-activated protein kinase
MAFbx	muscle atrophy F-box/Atrogin-1, muscle specific ubiquitin ligase

MuRF1	muscle ring finger 1, muscle specific ubiquitin ligase
Myod	myogenic determination factor
Myf5	myogenic factor 5
MPCs	myogenic precursor cells
MRFs	myogenic regulatory factors
MRF4	myogenic regulatory factor 4
Myog	myogenin
MHC	myosin heavy chain
ng	nanogram
nm	nanometer
NCBI	National Center for Biotechnology Information
NOAA	National Oceanic and Atmospheric Administration
NRC	National Research Council
ln	natural logarithmic function
NRF	nuclear respiratory factor
Pax7	paired homeobox 7
Pax3	paired homeobox 3
PIT	passive integrated transponder
PGC-1α	peroxisome proliferator activated receptor gamma coactivator-1 alpha
PPARγ	peroxisome proliferator activated receptor gamma
PI3K	phosphoinositide 3-kinase
PUFA	poly-unsaturated fatty acid
K^+	potassium
AKT	protein kinase B (PKB)
G_0	quiescent state
RICTOR	rapamycin insensitive protein binding partner of mTOR
RAPTOR	rapamycin sensitive protein binding partner of mTOR
ROS	reactive oxygen species
RT-PCR	real time – polymerase chain reaction
RNA	ribonucleic acid
SC	satellite cell
SGR	specific growth rate
Na^+	sodium
TORC1/2	target of rapamycin protein complex 1 or 2

Ct	threshold cycle
TCA cycle	tricarboxylic acid cycle
US	United States
USDA-WRAC	United States Department of Agriculture – Western Regional Aquaculture Center

Chapter 1: Literature Review

Introduction to Sablefish

Sablefish (*Anoplopoma fimbria*) are a valuable groundfish of the Northeast Pacific Ocean. The species ranges from Baja California to the north-central Bering Sea, along the Aleutian Islands, with low numbers extending west towards Russia and northern Japan (Allen and Smith, 1988; Jasonowicz et al., 2016; Johnson et al., 2016; Kimura et al., 1998). Sablefish are considered a deep-water species as they are encountered at depths ranging from 0 - 2740 m (Allen and Smith, 1988). Sablefish do not possess a swim bladder, and may maintain partial buoyancy by storage of lipid throughout their muscle (Rummer et al., 2010). Demand for sablefish, better known as "black cod" or "butterfish" (USA), stems from the unique flavor and flakey texture of the lipid rich white flesh, which provides nutritionally beneficial omega-3 fatty acids (Friesen et al., 2013).

Distribution and Natural Life History

The life cycle of sablefish incorporates environmental shifts and depth stratification in tandem with ontogenetic changes (Maloney and Sigler, 2008; Mason et al., 1983). Adults are predominantly found along the continental slope and sea mounts (300 - 1000 m). Natural spawning is poorly understood, but believed to occur below 300 m where eggs are present between January and April (Mason et al., 1983). The pelagic eggs slowly ascend and hatch after a long incubation period, and phototactic yolk sac larvae orient to the surface where the productive photic zone will support their transition to exogenous feeding. Altricial larvae feed on zooplankton over the spring and summer before eventually drifting inshore. By late summer and early fall juveniles measuring 9 cm in length on average (Mason et al., 1983) can be found in near shore bays and inlets (<100 m) where they spend 1-2 years before moving into progressively deeper waters of the continental shelf (100 - 200 m) and subsequently beyond the shelf-slope break (201 - 300 m) (Maloney and Sigler, 2008).

Fisheries studies using mark-recapture techniques starting as early as the 1970s describe sablefish as large-bodied, long-lived and highly mobile fish (Echave et al., 2013). Body length of sablefish in the wild is often between 40 - 80 cm, with the largest individuals reported to grow beyond 100 cm (Hanselman et al., 2014; Head et al., 2014). Most are less than 40 years old, and many are predominantly less than 10 years old (Kimura, 2008). The majority of females surveyed from the west coast region were younger than 60 years old (Beamish and McFarlane, 2000; Head et al., 2014). By age 5 sablefish are large enough to be available to the commercial fishery (Maloney and Sigler, 2008) signifying that for such a long lived-fish, a great proportion of their growth is completed rapidly in the first few years of life (Echave et al., 2012). For a demersal species (living at or near the sea floor)

sablefish are particularly mobile (Hanselman et al., 2014). Adults tagged and released in Alaska traveled an average of 80 nautical miles annually (point to point distance), and females slightly farther than males (Echave et al., 2013). Sablefish are carnivorous, adults typically preying on squid and other smaller forage fish. The longevity and high movement rates are thought to contribute to genetic mixing that maintains a single sablefish population, despite a wide geographic range (Jasonowicz et al., 2016).

Market and Value

Sablefish have become one of the highest value species of the Pacific coast fisheries, second to Pacific halibut by price per pound (NMFS, 2016). Export and ex-vessel prices fluctuate with the Japanese economy, as Japan is the principal market for sablefish where it is cooked in traditional dishes and prepared as sushi (Warpinski et al., 2016). Over the last decade, the popularity for sablefish has risen as a seafood delicacy in smaller markets within the US and abroad (FAO, 2016). With only a handful of small aquaculture industry members in Canada producing sablefish in deep net pens, the cultured product remains a minor proportion of the sablefish trade. Aquacultured sablefish has great potential to be a high-grade, desirable product for the sushi markets due to low incidence of parasites, year-round supply, and consistent quality owing to the controlled conditions during growth and processing.

Commercial Fishery

The US sablefish commercial fishery is modest in terms of mass but lucrative in terms of revenue, earning sablefish a position in the "Top Ten Key Species" of the North Pacific (Alaska) and Pacific (Washington, Oregon and California) regions (NMFS, 2016). Most sablefish are landed with fixed gear (Johnson et al., 2016). In 2014, sablefish commanded the second highest average finfish ex-vessel price per pound in both regions; $ 3.37/pound in the North Pacific and $2.52/pound in the Pacific, making the total ex-vessel revenue for combined landings $110 million dollars for 35 million pounds (NMFS, 2016). Alaska is the largest and most valuable sablefish fishery. Landings from Alaska routinely supply the bulk of world sablefish catch. In 2014, landings from Alaska accounted for 78% of total domestic revenue for this species. An international economic market model suggests that if 2014 landings tripled, prices would drop without a decrease in revenue (Warpinski et al., 2016). Sablefish recruitment is highly variable from year to year (Johnson et al., 2016). Although stock assessments of the west coast region show a spawning biomass decline since the late 1970s, the sablefish population is considered well managed and able to sustain stable catch share quotas that are unlikely to increase in the near future (Johnson et al., 2016).

Growth Variation in Wild Sablefish

Growth variation of wild sablefish is associated with sex, latitude and water depth. Growth and reproduction are intimately connected life history traits. This is especially true for endothermic fish, as growth rates can be drastically altered by temperature and food availability, making body size a better correlate to reproductive maturation than age. Sablefish exhibit a sexual dimorphism favoring greater female growth and maximum body size (Echave et al., 2012). Kimura et al. (1998) reported that females mature at larger sizes and have a greater maximum length than males. Morita et al. (2012) found females were larger and moved greater distances than males, potentially providing a wider forage base to support enhanced growth. Like other fish, sablefish growth slows after reaching maturation (Beamish and McFarlane, 2000). Female sablefish surveyed from the west coast management area in 2010 matured at approximately 50 - 60 cm and 7 years (Head et al., 2014). Maturing females in shallower water (<550 m), and more northerly latitudes were larger. This is corroborated by other work in the fisheries literature documenting variation in sablefish growth and size (length) at maturity between and within management regions that span considerable latitudes (Echave et al., 2012; Hanselman et al., 2014; Head et al., 2014; Kimura et al., 1998). Large fish are more frequently encountered in Alaska than the west coast region, which is consistent with the finding that sablefish length at maturity and maximum length increase for both sexes with increasing latitude (Kimura, 2008; Kimura et al., 1998). Larger adults tend to inhabit deeper waters, but the oldest fish are not the largest individuals (Head et al., 2014). Differences documented in life history traits within a single population of sablefish are suggested to be under the influence of the environment, dispersal, and potentially by undefined seasonal migrations (Head et al., 2014; Jasonowicz et al., 2016).

Candidate Aquaculture Species

Owing to the high value in international markets and fast growth in culture, sablefish have emerged as a candidate cold water marine species with great aquaculture potential in the US (NMFS, 2007; 2015). A modest industry is established in B.C., Canada, and interest groups exist for commercial production in Texas and Washington State. Sablefish have potential as an alternative species for net pen grow out operations in the Pacific Northwest, which predominantly produce salmon. Co-culture with sablefish would diversify production with an endemic species and buffer against fluctuating market demand.

The Case for Domestic Aquaculture

Fish are a source of high-value, lean, nutritious protein. Moreover, fish are efficient converters of feed protein to muscle protein, the conversion of which is enhanced in aquatic environments due to reduced gravity and associated energy expenditure. Human consumption of finfish and invertebrates

has increased steadily over the last several decades. The Food and Agriculture Organization of the United Nations (FAO) is concerned with meeting the food demands of a growing world population, which by 2030 is forecasted to grow by 19% (equivalent to an increase of 1.3 billion people) at which point it will finally surpass the threshold of 9 billion people on the planet (FAO, 2014) under the assumptions that there are no mass deviations to birth or death rates. This population increase is also expected to come with changing demographics and a shift in consumer preferences. Already, the expanding middle class in developing countries is adopting preference toward more healthful and sustainable food options. Collectively, these pressures are expected to be a challenge for our global food security and have elevated the discussion of how aquaculture will contribute to food security (Béné et al., 2015). Given the current level of demand for fish and seafood, the FAO anticipates difficulty fulfilling supply needs soon. The major limitations cited are the exploited state of world fisheries, the amount of available fish protein and oil for use in aquaculture feeds, and the current rate of advancement of technology and development in the aquaculture sector.

Aquaculture is the most rapidly growing agriculture sector worldwide and is positioned to supplement global capture fisheries. Aside from the goal of meeting seafood demand, aquaculture has the potential to reduce pressure on wild populations and indirectly, but perhaps optimistically, promote recovery and stabilization of wild stocks. Aquaculture is the primary manner by which world fish production has grown since the 1960s, despite the plateau of capture fisheries in the mid-1980s (FAO, 2014). Global fish stock assessments estimate that less than 11% of wild fish stocks are underfished, the majority (58%) are fully fished, and nearly a third (31%) are currently fished at biologically unsustainable levels (FAO, 2016). Therefore, increasing wild harvest to meet demand by expanding capture fisheries is limited. In contrast, aquaculture production doubled from 32.4 million tons in 2000 to over 66 million in 2012 (FAO, 2014). In 2014, aquaculture hit a production benchmark and now supplies more than half of fish for human consumption (FAO, 2016).

However, this production increase was not proportional among countries; in 2012 the US contributed less than 1% to total world aquaculture production, which represents a slight decline from US contributions in 2000 (FAO, 2014). An important factor underlying disproportionate growth is more competitive production costs in less developed countries. Considering Americans now eat more seafood annually by weight than ever before, it is not surprising that the US imports of fish and seafood contributed to a trade deficit amounting to more than $10 billion annually (Brooks et al., 2009). Development of the US marine aquaculture industry could help address the seafood trade deficit, provide year-round coastal employment opportunities, and other economic advantages. Domestic aquaculture opportunities should be explored with goals of achieving greater efficiency and socially responsible practices through technology and innovation.

Technology and Development Challenges Relevant to Sablefish Culture

Areas of sablefish aquaculture requiring attention for success and stability of the industry include growth inefficiencies related to sex and age/size, reproductive control, larval culture, feed sustainability, and product quality.

Two biologically driven growth phenomena have been observed during the 24 - 30 month grow out phase in sablefish culture. First, when mixed sex populations are co-cultured and permitted equal access to feed, sablefish growers witness dimorphic growth to favor females (Gores and Prentice, 1984; Luckenbach et al., 2017). This reflects female enhanced growth and larger body size in the fisheries data (Echave et al., 2012; Echave et al., 2013; Mason et al., 1983). Development of a monosex female sablefish population has progressed as a means to capitalize on faster female growth (Luckenbach and Fairgrieve, 2016; Luckenbach et al., 2017). Second, young sablefish are incredibly efficient growers, known to readily receive plant based diets and retain desirable feed efficiency rates higher than 105% (Johnson et al., 2015). However, this efficiency wanes in larger fish before market size is reached (generally by 2 kilos, pre-dressed weight).

Feed sustainability is a major issue for the aquaculture industry because of the finite global supply of forage fish used to produce fish meal protein and fish oil, two staple ingredients in aquaculture feeds (NRC, 2011). The single greatest operational expense to finfish aquaculture is often the cost of feed, and as such will influence profit margins (NRC, 2011). Development of species specific alternative feeds that completely or partially replace fishmeal and/or fish oil with terrestrial plant based ingredients would be more friendly to the environment, sustainable, affordable, and experience less market volatility, but can reduce growth or have other negative consequences (Rust et al., 2011).

Carnivorous marine finfish present special challenges to designing alternative feeds that support health and good growth relative to that achieved with conventional fishmeal based diets (Barrows et al., 2008). Marine fish have a dietary requirement for indispensable omega-3 HUFAs due to their inability to desaturate and elongate the precursor fatty acid (Tocher, 2003), but these omega-3 HUFAs are absent in terrestrial plant oils. Juvenile sablefish respond well to diets that replace anchovy oil with graded amounts of cold pressed flaxseed oil, suggesting substitution up to 75% would not hinder fish growth, proximate composition, or immunity, and still provide consumers with benefits of omega-3 HUFAs (Friesen et al., 2013). Sablefish nutritional requirements are poorly defined and must be assessed for diet formulation of practical plant based feeds that produce efficient, optimal growth. Investigations to date are promising and have already led to improvements in formulated feeds that achieve greater growth. Research shows that sablefish grow better on alternative feeds when supplemented with taurine, an amino sulfonic acid naturally found in animal protein but plants lack

(Johnson et al., 2015). This work contributed to a recent FDA decision to approve taurine as a fish feed additive in the United States, passing a significant hurdle in the ability of alternative feeds to meet amino acid requirements of carnivorous fish. Since little is known about sablefish nutritional requirements at different phases of the growth cycle (Forster et al., 2017), there is potential for future improvements to sablefish growth on formulated alternative feeds.

Sustainability and expansion of the sablefish industry will depend on control of reproduction to close the life cycle, whereby offspring of wild caught adults are produced in the hatchery and maintained in culture (first filial generation, F1) until reproductive maturation, which are then spawned to produce the next generation (F2), and so on. A closed life cycle supports genetic selection programs to achieve domestic strains with desirable production and market characteristics. Due to greater female growth, it has been suggested that the female germline may be especially important to growth rate selection in sablefish (Guzmán et al., 2015). F1 males (2+ years) will mature and produce viable milt in culture conditions, and male gamete availability is further extended by cryopreservation methods (Immerman and Goetz, 2014). In contrast, F1 females have poor reproductive performance and may take a long time (5 - 10 years) to mature in culture, if at all (Guzmán et al., 2015). At least one producer in Canada has closed the sablefish life cycle, while researchers and interest groups in the US continue to rely on annual collections of new sablefish broodstock from the wild for spawning and hatchery production.

Economic feasibility of the sablefish aquaculture industry will hinge on the ability to generate a steady supply of high quality juveniles. The delicate early life stages of altricial marine larvae are notoriously difficult to culture and the hatchery phase of sablefish production represents a bottleneck to juvenile supply. Efforts have advanced aspects of larval culture, including feeding and nutrition, as well as environmental conditions such as temperature (Lee et al., 2017b), tank design (Cook et al., 2015), and water turbidity (Lee et al., 2017a) to achieve higher survival and growth rates for successful weaning and metamorphosis into robust juveniles. Elevated temperature regimes in the hatchery may expedite the expensive live feeding stage but may have negative long term consequences on muscle quality (Lee et al., 2017b).

The quality of flesh produced for market and consumption is likely to become more important to the longevity of the sablefish industry as it gains a stronger foothold in the aquaculture sector. In the sablefish fishery, a “soft-flesh phenomenon” is a reported problem of unknown etiology, with incidence found to increase with depth of capture and in summer landings (Karinen et al., 2010). Compared to firm flesh, soft sablefish flesh had 14% less protein and resulted in much lower (31%) cooked yield (Karinen et al., 2010). Soft flesh has also been reported in other aquacultured species such as Atlantic salmon (Moreno et al., 2012; Morkore et al., 2009), signifying flesh quality problems

are not inherently restricted to wild fish under variable and uncontrolled growth environments. When developing a new species for aquaculture the primary focus is to achieve high survival and growth rates before strategies to enhance product quality characteristics. Accelerating larval sablefish growth through the hatchery stage by increasing temperatures from 12°C to 15°C or 18°C influenced the long term growth trajectory and flesh firmness of sablefish when measured after 9 months of growth in common conditions (Lee et al., 2017b). Based on survival, deformities, and fastest long term growth, the 15°C hatchery temperature was considered optimal, but these fish also had significantly softer flesh. Wild or culture origin, soft flesh can be difficult to process and result in downgrading, which will negatively affect the value. Flesh texture is central to product quality and one of the main sensory attributes influencing consumer willingness to pay for fish. Consumers tend to prefer firmer fish flesh (Hurling et al., 1996; Johnston et al., 2000), and wild Atlantic salmon (*Salmo salar*) and wild sea bass (*Dicentrarchus labrax*) had firmer flesh than farm raised counterparts (Johnston et al., 2006; Periago et al., 2005). Since biotic and abiotic factors directly influence flesh quality by creating patterns of muscle and lipid deposition during growth, it is not surprising that wild and farmed counterparts can have different flesh characteristics. We lack an understanding of the biological mechanisms behind continuous muscle growth in fish even though it is central to developing the commodity and maximizing the quality. Efforts to enhance our knowledge of muscle deposition will lend to improvements in aquaculture methods that practically and reliably support priorities of the producer, such as growth rate and yield, as well as priorities of the processor and consumer, such as quality and fillet firmness. This knowledge has to be species and environmental condition specific so that it translates into improvements in aquaculture through applied research.

The Importance of Understanding Muscle Growth

Teleost skeletal muscle is a specialized tissue designed for the essential function of contraction. This is necessary for fundamental aspects of fish biology including locomotion and ventilation. Depending on species, developmental stage, and influenced by other factors, skeletal muscle can be responsible for 30 - 65% of fish body mass (Whatmore et al., 2013) making it the most abundant tissue type. Because skeletal muscle is a major tissue, increases in whole body weight are closely tied to increases in muscle mass. Muscle is a metabolically active tissue and thus an important driver of whole body energy metabolism that participates in local and systemic signaling to influence overall health. It serves as a dynamic energy reservoir for proteins and lipids (Mommsen, 2001), and plays a role in glycogen storage and glucose metabolism. Attributes of fish muscle have potential to influence survival through swimming performance (Johnston, 2006) as it relates to outcomes in predator – prey interactions, competitive advantage for resources, and disease resistance (Castro et al., 2013). Skeletal muscle condition can have far reaching consequences for the animal, with mass and

composition imparting physical and chemical properties that influence buoyancy and determine survival through periods of scant nutrient availability or feats of migration, timing of maturation, and reproductive fitness (Johnston, 2006).

Fish skeletal muscle has economic importance to both capture fisheries and aquaculture. Fish axial musculature is a source of well-balanced protein for human consumption and often the largest source of the sought-after "heart healthy" long chain omega-3 HUFAs in the human diet. Growth plasticity is influenced by internal and external factors that will translate to meat quality characteristics *ex vivo*, and is associated with responsive elements of the tissue, including nutrient and energy composition, constituent myofiber types, other cell types present, and properties of the collagen matrix. Variation in growth places importance on defining the physiological pathways central to regulation and how they are set by genetics and controlled by different factors to direct muscle development and maturation. Such pathways are highly conserved in vertebrates, with some apparent differences across classes; most commercially important fish species are indeterminate growers, in contrast to terrestrial species in which new fiber formation is restricted to prenatal growth and response to injury (Rowe and Goldspink, 1969). Most fish species retain the capacity to grow via embryonic-like muscle fiber recruitment far into adult stages (Koumans and Akster, 1995; Weatherley et al., 1988). The total effect of inputs on the pathways controlling muscle development and plasticity are not fully understood in mammals, and even less so in fish (Johnston et al., 2011). Physiological systems receive and integrate a complexity of internal and external stimuli, such as cues about the season or nutritional and reproductive status, and must orchestrate muscle growth within dynamic circumstances. Fillet meat is the valuable and marketed derivative of muscle, and as such, product quantity and quality hinge on inherent facets of the living tissue. Cumulative effects of genetics and the growth environment on the many physiological systems governing somatic muscle growth in fish are paramount to establishing desired properties of the final product. Greater knowledge of muscle development and maintenance in fish will enhance our understanding in this area of basic biology and afford greater insight for applications to aquaculture industry, fisheries ecology, and serve as a comparative platform for vertebrate muscle development, aging, and disease.

Fish Skeletal Muscle and Fiber Phenotypes

Bony fish axial skeletal muscle is organized in interconnected muscle segments called myotomes, arranged in a rostral-caudal formation along the length of the body. Myotomes have a complex three dimensional chevron-like shape, fused together by connective tissue sheets called myosepta (Van Leeuwen, 1999). This myotomal architecture creates body flex with sequential muscle contraction in an undulating wave down the length of the body that translates to thrust against the water for propulsion (Altringham and Ellerby, 1999).

White muscle is the predominant muscle type in fish, commonly constituting more than 70% of muscle mass. Fish white muscle is easily distinguished from red muscle by color and anatomical separation (Koumans and Akster, 1995). A lateral strip of red muscle runs superficial to the white muscle, just under the skin and most prevalent near the lateral line (Fernandes et al., 2005). Red muscle extends the length of the body and increases in a caudal direction. White and red muscle are also commonly referred to as fast and slow twitch, respectively. Intermediate fibers (sometimes called pink fibers) can be found at the interface of white and red muscle, and in deep regions most proximal to the medial axis (Scott and Johnston, 2012). These muscle types are specialized for different swim patterns. White muscle is predominantly used in powerful burst swimming events due to the fast twitch contraction speeds and maximum force generation of large fibers, but fatigue quickly. Sustained swimming preferentially recruits slow twitch fibers of red muscle that are small and produce less force, but are resistant to fatigue. The proportion of white and red muscle is influenced by evolutionary adaptation to an ecological niche and associated performance requirements. The major determinant of proportions of muscle types in fish is genetics, suggested by the degree of intra- and inter-specific variation, while environmental factors are secondary in importance and likely affect more reversible characteristics of muscle (Johnston, 2006).

In addition to anatomical location, muscle types are functionally divergent by nature of the constituent fiber types with respect to fiber size (diameter), preferred metabolic type, and contraction properties (van Wessel et al., 2010). Muscle fiber types are classified based on the myosin heavy chain (MHC) isoforms as type I, IIa, IIx, and IIb, which differ in their ATPase hydrolysis rates and thus influence the speed of contraction (Akolkar et al., 2010; Fernandes et al., 2005). Red muscle incorporates a slow MHC isoform (type I) with reduced ATP hydrolysis speed to liberate energy for myosin protein conformation change in each cross-bridge cycle during contraction. MHC isoforms in white muscle (type II) quickly hydrolyze ATP for energy used in the contraction. To support the accelerated ATP demands during such contraction, these fibers generate ATP quickly via glycolytic metabolism. The buildup of lactic acid as a byproduct causes these fibers to fatigue rapidly. White muscle is comprised of type IIa and IIb fibers, which are large diameter fibers (reaching >200 - 400μm in some teleosts) because each contains more myofibrillar contractile proteins for maximal force generation. The glycolytic metabolism of type IIb fibers makes them biochemically suited to fuel short duration anaerobic activities such as sprint swimming. Balancing the function of white muscle, red muscle is an aerobic tissue made up of small diameter, type I oxidative fibers. This tissue is predominantly recruited first for swimming that occurs within an aerobic metabolic range. Myoglobin is a small molecule located in all fiber types that contains an iron-heme group which binds and stores oxygen where it is used. Myoglobin and mitochondria are more abundant in red muscle to help meet

the heavy oxygen demands of the aerobic tissue. The red appearance of slow muscle stems from the iron in myoglobin, an extensive capillary system for enhanced blood supply, and the greater density of mitochondria. Pink muscle can be a mix of fiber types, but may also contain many type IIa fibers. Type IIa fibers are considered "intermediate" because while they are more similar in size and contraction speed to type IIb fibers, their metabolism is shared more with type I fibers, generating more ATP energy via oxidative metabolism.

Differences in White and Red Skeletal Muscle Physiology

The nature of fish muscle organization into discrete zones of fast-white and slow-red muscle facilitates contrast between the predominant fiber type constituents (Duran et al., 2015; Fernandes et al., 2005). As specialized forms of skeletal muscle tissue, white and red muscle maintain many shared bioenzymatic, structural, and contractile apparatus proteins. However, the specialized functions of the fiber phenotypes mean different proportions of these major protein classes are present, and with different rates of turnover. In other words, during fish growth the white and red muscle do not equally emphasize nascent fiber development, hypertrophy, maturation, and maintenance. In fact, a general rule is that structurally white myofibers have a greater number of myofibrils per cell nuclei. In vivo, the sum of these processes is dependent on the developmental stage and condition of the fish, and is reflected in the metabolic rate, preferred energy substrate, nutrient and oxygen availability, and transcriptional profile of the muscle tissue type.

Fast white muscle fibers (glycolytic, type IIa/b fibers) are primarily associated with myofibrillar genesis and sarcomeric assembly to reach large diameters at a mature stage, at which point they are metabolically advantageous in comparison to small diameter fibers (Jimenez et al., 2013). Greater volume also means lower surface area to volume ratio than red muscle, which is demonstrated to translate to energy cost savings to reset the cell membrane action potential with Na^+-K^+-ATPase pumps. Assuming accretionary growth status and non-limiting energy and substrate availability (ex. amino acids), the maintenance costs of abundant white muscle structural and contractile proteins reflect relatively lower energy demands for repair and turnover than red muscle, partly due to low use-related damage. Except following infrequent burst swimming events, the metabolic cost of maintaining large white muscle fibers is therefore lower than red muscle. It is well known that fish reach large sizes due to extensive hyperplasia and hypertrophy in white muscle, suggesting the primary source of metabolic demands reflect some combination of these growth mechanisms. More specifically, energy demands at early developmental stages reflect enhanced rates of myotube production but will eventually shift to fiber maturation and hypertrophy in progressively older, larger individuals (Fernandes et al., 2005).

In contrast, post-larval red muscle fibers (oxidative, primarily type I fibers) have lower rates of hyperplasia, mature at smaller diameters to maintain a high surface area to volume ratio for efficient gas and nutrient diffusion, and require greater mitochondrial density for aerobic energy production. Maturation and growth of red fibers involves extensive mitochondrial biogenesis and energy use is associated with building and maintaining the energy producing infrastructure, such as bioactive enzymes of the TCA cycle and oxidative phosphorylation chain (van Wessel et al., 2010). Elevated oxygen consumption also leads to greater production of reactive oxygen species (ROS; oxygen free radicals). ROS are highly reactive oxidative stressors naturally generated at low levels during ATP production in the mitochondrial oxidative phosphorylation pathway. ROS can be toxic and lead to DNA damage and cell death, but also have cell signaling roles that support diverse physiological processes and adaptation (Barbieri and Sestili, 2012). ROS both increase damage and metabolic costs for protein repair and turnover and activate cell signaling networks for adaptive measures to maintain the oxidative phenotype (i.e., elevate superoxide dismutase availability). Skeletal muscle exercise and contraction produces pulses of ROS that are documented to signal for mitochondrial biogenesis through PGC-1α expression, ultimately creating a more oxidative phenotype and ability to produce more ROS, theoretically. The collective maintenance needs of high oxidative fibers in red muscle lead to greater metabolic load than in low oxidative/glycolytic fibers in white muscle. Consequently, greater transcription and translational demands of oxidative muscle are met by a denser population of myonuclei for smaller myonuclear domains in vertebrates (van Wessel et al., 2010).

Fiber Hyperplasia and Hypertrophy

The type of muscle growth and accretion is the biggest determinant of flesh quality. Fish that grow to large sizes accrete muscle by enlarging existing fibers (hypertrophy) and adding new fibers (hyperplasia). Both mechanisms rely on extensive accretion of nuclei from a population of adult muscle stem cells scattered throughout the muscle. In hypertrophy, existing fibers accrue new nuclei to support increases in myofibrillar protein, length, and size up to a maximum diameter that depends on the species (Priester et al., 2011; Videler, 2011). In hyperplasia, new fibers form when stem cells fuse to each other, creating small diameter fibers alongside existing ones. New fibers must first mature then may be stimulated to grow by hypertrophy. Hyperplasia is a powerful mechanism for lasting muscle growth and is responsible for larger body sizes of female Atlantic halibut (Hagen et al., 2006; Hagen et al., 2008). High rates of hyperplasia are also associated with periods of rapid growth (López-Albors et al., 2008). Indeterminate growth in fish occurs through a combination of increased muscle fiber number and size. Additionally, muscle metabolic requirements will influence fiber size. Plasticity of muscle growth and physiology is why similar sized individuals of the same population and rearing conditions can exhibit different muscle fiber organization (Morkore et al., 2009). Indeterminate growth

and inherent phenotypic differences between metabolic fiber types lend to a mosaic pattern of muscle fibers of variable size, number and density, and potentially orientation, with consequences for muscle firmness.

Meat quality hinges on the muscle attributes upon harvest, and is further modified by handling, processing, and storage conditions. The most important biological factors are muscle fiber organization, nutrient composition, connective tissue cross-linking, and water holding capacity (Listrat et al., 2016). Within and across species, smaller muscle fibers can create a firmer, consumer preferred cooked texture (Hatae et al., 1990; Hurling et al., 1996; Johnston, 2001; Johnston et al., 2004). A sensory assessment of smoked flesh from two strains of Atlantic salmon found increased firmness and higher muscle fiber density in the slower growing strain (Johnston et al., 2000). This is consistent with findings by sensory panels (Hurling et al., 1996) and instrument methods (Hatae et al., 1990; Periago et al., 2005). In sea bass, firmer textured wild fish had greater muscle fiber density and no detectable difference in nutritional composition (Periago et al., 2005). The balance of indeterminate growth mechanisms that develop the fiber sizes and densities is sensitive to both genotype and environment (Listrat et al., 2016; Periago et al., 2005). The idea to control product quality of fish muscle through genetic selection and environmental factors (temperature, nutrition, photoperiod) is not new, but is underutilized in finfish (Johnston, 2006; Johnston, 1999; Johnston et al., 2000; Johnston, 2001; Kause et al., 2011; Videler, 2011). Genetics are known to contribute to fish muscle growth rate, but also growth patterns and muscle texture (Kause et al., 2011). Moderate heritability of muscle fiber number and diameter are suggested quality traits to incorporate into the design of broodstock selection programs in salmonids (Vieira et al., 2007), and Atlantic halibut (Hagen et al., 2008). Sex can influence muscle fiber density and long term hyperplasia in Atlantic halibut (Hagen et al., 2006; Hagen et al., 2008), and normal-sex-ratio diploid Atlantic salmon have more white muscle fibers per myotome than all-female diploids at various stages of the life cycle (Johnston et al., 1999). The quantity, quality, and consistency of nutrition will affect anabolic status and fish muscle hyperplasia and hypertrophy (Overturf et al., 2016; Valente et al., 2016). Complete replacement of fishmeal with plant proteins in Senegalese sole diets had a negative effect on fish growth, fiber cellularity, and myogenic transcription factors (Valente et al., 2016). In this case, the 100% plant protein diet increased the fiber density but this was because it inhibited white muscle fiber hypertrophy, which reduced fish growth and produced softer fillets, ultimately signaling the anabolic condition of the fish was compromised. Appetite and diet consumption is further modulated in fishes by temperature. Temperature will profoundly affect growth by modulating appetite, but can also modulate indeterminate growth in an ongoing fashion as well as through larval imprinting. Larval imprinting at lower hatchery temperatures relative to the fish's natural thermal range appears to set the growth

preference in favor of hyperplasia and has the power to influence growth rate and fiber density in later life (Steinbacher et al., 2011). These genetic and environmental conditions are highly controlled in aquaculture settings. An advantage of aquaculture is the opportunity to better control commodity quality, but an understanding of how flesh is modulated by the culture environment is requisite to develop the desired result.

The Adult Muscle Stem Cell

Myogenesis is the process of building muscle tissue mass. Postembryonic vertebrate myogenesis is sustained by active cell cycling of a heterogeneous pool of pluripotent muscle stem cells called satellite cells (SCs) that originate from the somitic dermomyotome of the embryo (Kuang et al., 2007). SCs are small undifferentiated cells that are transcriptionally repressed and mitotically inactive (Kuang et al., 2007; Mauro, 1961). They are distributed throughout muscle and reside in a niche external to the mature muscle fiber sarcolemma (cell membrane) and just under the basal lamina that envelopes each muscle fiber. Activation of the SC population is responsible and necessary for substantial increases in skeletal muscle mass observed in postnatal growth and repair after injury.

Upon stimulation, SCs exit the mitotically quiescent state (G_0 resting phase) through poorly defined mechanisms and re-enter the cell cycle to proliferate. In the active state, they are referred to as myoblasts (Gabillard et al., 2010), and sometimes as myogenic precursor cells (MPCs). Depending on stimuli, myoblasts can go through numerous divisions to generate new cells for fusion and differentiation into multinucleated muscle fibers (Gabillard et al., 2010; Moss and Leblond, 1971). Each daughter myoblast will make a cell fate decision: continue proliferation, return to quiescence for self-renewal of the SC population (a characteristic of stem cells), or commit to myogenesis and differentiate to become functional contractile muscle (Kawabe et al., 2012; Motohashi and Asakura, 2014; Olguin et al., 2007).

Commitment to the myogenic program directing differentiation begins with primary differentiation of myoblasts to myocytes. Though the mononucleate myocyte has lost capacity as a stem cell it contributes to growth by myonuclear addition to muscle fibers. Myonuclear addition occurs by fusion and terminal differentiation to produce muscle specific proteins for functional contraction. Cell size and the potential for growth via protein accretion is set by upper bounds of the myonuclear domain (the area surrounding and controlled by a single nucleus), which is constrained by metabolic demands and diffusion distances (Priester et al., 2011). When myocytes fuse to existing fibers (hypertrophy) the myonuclear addition increases transcriptional capacity to amass additional protein. Alternatively, when myocytes fuse to other myocytes (hyperplasia), new multinucleated fibers are formed (myotubes) that must further differentiate (generate a contractile apparatus) to yield a functional, syncytial fiber. Myotubes can subsequently grow in length and diameter via hypertrophy.

Hyperplasia “recruits” new fibers into the muscle tissue, while hypertrophy increases the size and strength of existing myofibers.

Fish axial musculature is a mix of muscle, adipose and connective tissues, but the overwhelming majority of the constituent cell types are myofibers. In vivo, other cell types include adipocytes, fibroblasts, neurons, and epithelial cells, all of which are important contributors to the cell signaling network within muscle tissue. These cells contribute chemical signals (cytokines, hormones, growth factors) to the extracellular milieu that directly influence activity of SCs in growth and repair after injury (Motohashi and Asakura, 2014). Mechanical structure of muscle is related to the myofiber and the extracellular collagen matrix, which becomes denser, more cross-linked, and less elastic with age (Hagen 2007 and Valente 2016). This changing SC niche is believed to contribute to the decline in SC regeneration with age in humans and other mammals (Motohashi and Asakura, 2014; Shefer et al., 2006). Deficiency in SC population or regeneration capacity combined with reduced maintenance and protein accretion capacity are the root causes of skeletal muscle pathologies such as muscle loss in aging (sarcopenia), cancer (cachexia), and muscular dystrophies (Motohashi and Asakura, 2014). The dynamic structural and chemical signaling conditions within muscle will influence SC activity and thus tissue deposition, maintenance, and accretionary growth, and extend to texture and flavor attributes in food producing animals and especially sablefish.

Muscle Growth Paradigms

Understanding hypertrophy and hyperplasia is paramount to distinguishing between determinate and indeterminate growth paradigms. Normal skeletal muscle development and growth of higher order vertebrates restricts hyperplasia to two prenatal waves before switching to hypertrophic mechanisms soon after birth or hatch (Rowe and Goldspink, 1969). Since the number of fibers is determined in early life, these species are said to exhibit determinate growth. The only exception to this determinate growth paradigm is in response to severe injury, where hyperplasia is apparent (Rescan et al., 2015). In contrast, many teleosts uniquely retain the ability to recruit new fibers well into adult life stages as a normal growth mechanism, reportedly until approximately 40 - 50% of final body size is reached (Johnston et al., 2011; Weatherley et al., 1988), after which hyperplastic growth may be observed in response to injury as in other vertebrates (Knappe et al., 2015; Montfort et al., 2016; Rescan et al., 2015). This is especially true for species that reach large sizes, such as the fast growing, large bodied species commonly targeted for aquaculture. Teleost indeterminate myogenesis refers to the ability to grow through an undetermined combination of myofiber hypertrophy and hyperplasia, both of which will vary in contribution depending on complex interactions between genetics and environmental factors (Koumans and Akster, 1995). It is important to note that sablefish have a pelagic early life history and are an example of a marine fish with an indeterminate life cycle.

Less is known about the regulation of fiber hyperplasia and indeterminate growth in species with indirect life histories. Indeterminate fish growth requires targeted and species-specific approaches to understand how and when the culture environment modulates muscle development.

Gene Expression Regulating Satellite Cell Specification

Paired Homeobox (Pax) genes are transcription factors important for chordate development of tissue and organs. One of the four subfamilies consists of Pax3 and Pax7, which appear to be the result of a duplication of an ancestral Pax3/7 gene and remain highly conserved in vertebrates (Paixão-Côrtes et al., 2013). Expression of Pax3 and Pax7 specify cell identity to the myogenic population responsible for the initial formation of muscle, and which also give rise to the population of SCs critical for lifelong skeletal muscle maintenance, growth, and repair (Seo et al., 1998). Of a handful of genes used as markers of the SC pool, including those encoding proteins localized to the nucleus and the cell surface (Dumont et al., 2015; Froehlich et al., 2013), Pax3 and Pax7 remain the most widely used and canonical identifiers of SCs due to their key roles establishing and maintaining the SC population.

During development, vertebrate Pax3 is expressed earliest in muscle progenitor cells of the somite that later express Pax7, form embryonic muscle, and give rise to the adult SC compartment (Kacperczyk et al., 2009; Relaix et al., 2005). Murine Pax3 is necessary for normal patterning and mass of trunk muscle and for cell migration required for limb bud formation to develop all appendicular musculature (Buckingham and Relaix, 2015; Relaix et al., 2005). Embryonic Pax7 expression induces neural tissue development and consolidates the signal for SC specification (Seale et al., 2000). Then postnatally, Pax3 expression is restricted to trunk muscles, including the diaphragm and intercostal muscles, and Pax7 specifies SCs in appendicular skeletal muscles. Pax7 expression continues to be critical for maintaining a SC pool (von Maltzahn et al., 2013), at least in part through an anti-apoptotic effect that cannot be substituted for by Pax3 (Relaix et al., 2006).

In comparison, the roles of teleost Pax3/7 are less characterized than in amniotes. Expression of both have been documented during early teleost embryogenesis. In a distant relative of the zebrafish, mitotically active Pax7 expressing cells originating from the dermomyotome of the somite are believed to be key for establishing myogenic precursor cells and teleost fast muscle growth (Marschallinger et al., 2009). The difference in muscle patterning (i.e., no limb development) and sustained hyperplasia of fish skeletal muscle underlie suspicions that expression of both Pax3 and Pax7 may be important for specification and vigorous renewal of the SC pool in teleosts. Research with primary SCs from the indeterminate growing giant danio provides compelling evidence that both Pax3 and Pax7 are important for specification of adult SCs and are differentially regulated during *in*

vitro proliferation and myogenesis (Froehlich et al., 2013). How widely this unique myogenic program may extend to other indeterminate growers is a question yet to be determined.

Mammals have a single Pax3 gene and Pax7 gene, while teleost specific gene duplication event appears to be responsible for two paralogues of Pax3 (Pax3a, Pax3b) and Pax7 (Pax7a, Pax7b) in several fish species. Rainbow trout possess Pax7a and Pax7b paralogues (Chapalamadugu et al., 2015), and the more recent salmonid specific genome duplication event appears to be the cause of a second Pax7a gene, yielding three described Pax7 orthologues in rainbow trout to date (Seiliez et al., 2014). Two Pax3 (Pax3a, Pax3b) and Pax7 paralogues (Pax7a, Pax7b) were recently described with various splice isoforms in Japanese pufferfish *T. rubripes* (Akolkar et al., 2016) and Olive flounder *P.olivaceus* (Jiao et al., 2015a; Jiao et al., 2015b).

The precise dynamics between Pax3/7 or the myogenic regulatory factors (outlined below) in more heavily studied mammalian models is not assumed to transfer exactly across vertebrate phyla, as the roles for these genes in teleosts are under intensifying investigation. Pax7 expression is stably maintained in quiescent SCs, and persists only for a short time in newly activated myoblasts before it is rapidly downregulated and absent in myocytes, making its detection a marker of the SC population (Akolkar et al., 2016; Shefer et al., 2006). Pax3 and Pax7 are considered specifiers of SC identification in the muscle of mammals, birds, amphibians and fish (Chapalamadugu et al., 2009; Peng et al., 2016; Seale et al., 2000).

The satellite cell population declines with age, in young animals constituting over 20% of myonuclei and in adults declining to about 5%, although research in this area for fish is limited. Beginning with very small juveniles, as body size increases the number of myogenic cells per unit tissue weight decreases, which is suggested in reduced recovery of SCs when primary cell lines are extracted from sea bream of increasing size (Vélez et al., 2014). In vertebrates aged SCs have a slower activation process, and in mammals they also have less capacity for proliferation and self-renewal but enhanced commitment to the myogenic lineage (for review see Dumont et al. (2015). As fish body size increases, the SC number per unit area may decrease, but the ability of the SCs to proliferate and self-renew largely remains intact. The developmental stage affects the muscle regeneration process after injury in determinate growing zebrafish, as older larvae had prolonged expression of Myod and myogenin after mechanical injury (Knappe et al., 2015).

Extrapolation of precise gene function across taxa should made with some caution due to the diversity observed across teleost phylogeny, which can be attributed to increased genome complexity following the teleost-specific whole genome duplication event soon after divergence from the common vertebrate ancestor (Glasauer and Neuhauss, 2014). For instance, the zebrafish is a heavily investigated biomedical model species affording many sophisticated tools and conserved sequences

important to muscle growth, but it exhibits determinate muscle growth (Biga and Goetz, 2006), while fish species like sablefish that grow to larger body sizes must rely on some degree of an indeterminate growth paradigm to reach large masses (Mommsen, 2001). Rainbow trout and Atlantic salmon grow to large sizes, and may be two of the most well studied indeterminate growers, however, they fall within a lineage that has experienced an additional salmonid-specific whole genome duplication event resulting in tetraploidy (Macqueen and Johnston, 2014). This polyploidization presents opportunity for retained gene copies to acquire new, different, or sub-functionalization, which can give rise to unique roles of an otherwise conserved suite of genes guiding muscle development and growth (Jiao et al., 2015a; Macqueen and Johnston, 2006). In contrast, sablefish are a diploid teleost (Phillips et al., 2013) and likely have differences in the myogenic program compared to salmonids, at least in part due to differences in the number of gene paralogues present in the genome. The best example of this is noted in the different number of Myogenic determination factor (Myod) gene paralogues. The teleost-specific whole gene duplication created a second gene, Myod2, that has been subsequently lost in some lineages, including zebrafish, giant danio, catfish, and salmonids (*Ostariophysi* and *Protocanthopterygii*; (Andersen et al., 2009). In contrast, additional duplications in salmonids produced several paralogs of Myod (Myod1a, Myod1b, Myod1c) reported in the latter to have diverging roles (Bower and Johnston, 2010; Macqueen and Johnston, 2006).

Gene Expression Regulating the Myogenic Program

A well-documented family of transcription factors called the Myogenic Regulatory Factors (MRFs) are master regulators of vertebrate myogenesis by serving as activators and controllers of SCs and transcription of muscle specific genes. As four copies of a single ancestral MRF gene, Myogenic factor 5 (Myf5), Myogenic determination factor (Myod), Myogenic regulatory factor 4 (MRF4), and Myogenin (Myog) share a conserved basic-helix-loop-helix (bHLH) structure. The MRFs are expressed explicitly in skeletal muscle in a coordinated and specific temporal pattern (reviewed by Bentzinger et al. (2012). The MRFs work in concert to support progression of SCs through the myogenic program and decrease apoptosis: SC activation, myoblast proliferation, determination to muscle lineage, followed by myocyte fusion and terminal differentiation to functional muscle. In vitro work has helped to characteristic timing and hierarchical expression of the different MRFs during myogenesis (Gabillard et al., 2010; Singh and Dilworth, 2013). Myf5 and Myod are expressed by active SCs and myoblasts and are considered necessary to determine cells to the muscle lineage (Rudnicki et al., 1993). Downstream are the differentiation factors, MRF4 and Myog, which permit determined cells to develop, fuse, terminally differentiate, and mature to syncytial tissue. There has been some redundancy in function of these genes and their cognate proteins but all support the myogenic process and myonuclear addition.

As bHLH transcription factors, the MRF proteins exert effects by forming heterodimers with cofactors to locate promotor regions of target genes through recognition of conserved E-box sequences (CANNTG, where N is any nucleotide), while DNA binding is facilitated by the basic domain (Massari and Murre, 2000). By binding the promotors of hundreds of skeletal muscle tissue specific genes located across chromosomes, such as myosin heavy chain, troponin, and tropomyosin, the MRFs recruit *trans*-activating factors and transcriptional machinery to induce target gene expression (Conerly et al., 2016). The potency of MRF expression is demonstrated through induction of the full myogenic program by forced expression, converting non-muscle cells into myoblasts and myotubes (Weintraub et al., 1989).

The accurate spatiotemporal expression of all four transcription factors are critical to proper formation of muscle in the embryo and for adult skeletal muscle stem cell activation for growth, repair and regeneration. MRF expression patterns are distinct to phylogeny and developmental stage. Waves of gene expression are coordinated by mechanisms that are not fully understood, but are known to rely on specific binding of transcription factors, cofactors, and chromosome conformation. Chromatin re-organization facilitates temporal control by creating proximity between genes known for early and late expression during myogenic differentiation that are otherwise located on different chromosomes (Harada et al., 2015).

Gene knockout studies in vitro and in vivo in the mouse have added to the roles assigned to the MRFs. Myod is considered a master regulatory switch in myogenesis whose expression supports activation of SCs, proliferative cycling, and initial differentiation whereby myoblasts are directed into the pathway to become myocytes (Sabourin et al., 1999). Some functional redundancy exists between Myod and Myf5, as each can determine SCs to the myogenic program (Rudnicki et al., 1993). However, crossing Myf5 deficient and Myod deficient mice indicated that at least one of these MRFs is required for myogenesis, as the double mutant offspring lacked skeletal muscle and died shortly after birth (Rudnicki et al., 1993). Myod ^{null} myogenic cells from adult skeletal muscle grown in vitro expressed higher levels of Myf5, continued to proliferate, and produced mainly mononuclear cells in differentiating conditions, while also exhibiting delayed expression of myogenin and MRF4 (Sabourin et al., 1999). This is consistent with other work by Megeney et al. (1996) showing Myod deficient mice had elevated levels of satellite cells that did not efficiently regenerate muscle because Myod expression is necessary for satellite cell progression through the myogenic program. Ectopic expression of Myod in primary cell lines derived from mammals and birds was potent enough to activate expression of muscle specific genes in several differentiated non-muscle cell types and adopt secondary muscle phenotypic characteristics (Weintraub et al., 1989).

Myog is critical to myogenesis and formation of functional skeletal muscle. Studies in determinate growers suggest Myog plays different roles in myogenesis and muscle homeostasis. Homozygous myogenin knockout mice exhibit severe muscle defects and ultimately experience perinatal death (Hasty et al., 1993; Nabeshima et al., 1993). This is caused by a disruption in the transition from myoblast to myofiber because myoblasts fail to fuse into myotubes or differentiate to syncytial fibers. At least one copy of myogenin is sufficient to rescue this pathology, as the heterozygous Myog mutants are indistinguishable from wild type littermates. While redundancies exist in the roles of some MRFs, particularly evident between Myod and Myf5, it appears there is no such compensation for complete lack of Myog during myogenesis. More recent work confirmed this absolute requirement for Myog in prenatal myogenesis and survival, but in contrast, suggested Myog is somewhat dispensable if deleted after birth (Meadows et al., 2008). Several lines of evidence support the notion that Myog expression is connected to oxidative capacity. Myog expression persists in mature muscle fibers (Hughes 1993). Deletion of Myog in mice shortly after birth did not prevent normal development, however, body size at maturity was reduced by 30% concomitant with modified expression of the other MRFs (Meadows et al., 2008). Overexpression of Myog in adult mice shifted fiber enzyme activity away from glycolytic metabolism and enhanced the oxidative phenotype (Hughes et al., 1999). In agreement with the greater oxidative capacity induced by overexpression of Myog, fiber size was also reduced, although fiber type specific myosin heavy chain isoforms were unaltered (Hughes et al., 1999).

Nutrient and Energy Sensing for Metabolic Regulation

Peroxisome Proliferator Activated Receptor gamma (PPARγ) is critical nuclear receptor transcription factor sensitive to lipid availability in order to regulate metabolism and the anabolic state. PPARγ is enriched in adipose tissue and is important for skeletal muscle metabolism. Activated PPARγ is known to control the transcription of genes essential to metabolism that effect adaptation of striated muscle (Baskin et al., 2015). When the nuclear receptor PPARγ binds to a ligand, most importantly a long chain fatty acid or its metabolite, PPARγ can then interact with its coactivator, peroxisome proliferator activated receptor – coactivator 1α (PGC-1α). Transcriptional efficacy of PPARγ is positively mediated PGC-1α, which assists in recruiting and binding factors with strong histone acetylation capabilities that open chromatin for translation (Baskin et al., 2015). This complex efficiently transcribes genes leading to fatty acid uptake, lipid oxidation, and mitochondrial biogenesis. In humans, PPARγ has been targeted to treat and reverse insulin resistance in patients with metabolic syndrome and type 2 diabetes due to its positive influence on fatty acid and glucose metabolism. It also functions to protect from lipotoxicity by inducing lipid uptake and storage in adipose tissue. Mouse models of PPARγ, reviewed by Baskin et al. (2015) indicate the critical nature

of PPARγ; complete deletion proved lethal, while skeletal muscle specific loss increased adiposity, insulin resistance and glucose intolerance. Leaver et al. (2005) found expression patterns of the piscine homolog for PPARγ in two marine teleosts diverged from that of mammals and was expressed in a wide variety of fish tissues, most highly in adipose and intestine. In the cobia, PPARγ expression was consistent with increased adiposity during allometric tissue growth (Tsai et al., 2008). Expression in muscle (dorsal and ventral), liver, and visceral fat depot was often positively and significantly correlated with lipid concentrations in muscle and liver, and with fish age and body weight. The correlation of PPARγ expression with fish adiposity and body size suggests this nutrient sensor could be a useful indicator of nutritional status and pro-growth condition with implications for the lipid and sensory attributes of muscle.

As the name implies, the transcriptional coactivator PGC-1α senses and mediates energy status by functioning as a potent "on" or "off" switch when bound to a variety of transcription factors. It was identified as a stimulatory co-factor to PPARγ to induce brown fat differentiation for neonate mammal thermogenesis (Puigserver et al., 1998; Wu et al., 1999). In adults, it remains highly expressed in adipose and skeletal muscle. PGC-1α has well-established roles in mammalian lipid and fatty acid homeostasis, is known to be an important inducer of mitochondrial biogenesis and angiogenesis to increase tissue capillarity and oxygen delivery for improved oxidative metabolism (Lin et al., 2005). The protein has four functional binding domains that persist across mammalian taxa (the activation domain (AD), nuclear respiratory factor-1 (NRF-1) binding domain, MEF-2c binding domain, and RNA binding domain) (LeMoine et al., 2010). The distinct functional regions add complexity to the molecule's regulation, resulting in an adaptive response to stimuli such as temperature, low oxygen availability, and exercise stress via appropriate muscle phenotype remodeling. For instance, expression of PGC-1α is higher in oxidative than glycolytic fibers, and generally increases in response to endurance exercise to promote transcriptional adaptations supporting higher energy demands, optimal muscle fiber size, and diffusion distances (Hoppeler, 2016; Sandri et al., 2006). PGC-1α is considered a chief coordinator of skeletal muscle metabolic plasticity based on its sensitivity to several converging signaling cascades and its actions must be tightly controlled by transcription and post-transcriptional effects (Hoppeler, 2016). Research in mice suggests it protects from catabolism induced by fasting, denervation, and disuse, and different models of atrophy have measured low expression, suggesting its level of detection may serve as a body condition related nutrient and energy sensor (Sandri et al., 2006).

Despite the central physiological importance of PGC-1α to mammalian metabolism, there is less certainty about its function in teleosts. Orthologous PGC-1 family members are present in teleosts (LeMoine et al., 2010). Investigation of PGC-1α gene sequences from a variety of representative

vertebrate species also revealed the four functional domains to exhibit different rates of evolution in fish compared to mammals (LeMoine et al., 2010). The PPAR activation domain was well conserved across vertebrate taxa, suggesting PGC-1α plays a similar role in lipid metabolism in teleosts. In contrast, the sequences of the NRF-1 and MEF2c binding domains evolved rapidly in bony fishes (Lin et al., 2005). Authors suggest the resulting protein residue insertions in teleost NRF-1 domain of PGC-1α could be enough to prevent interaction with NRF-1, compromising this mechanism of mitochondrial biogenesis to modify oxidative capacity in fish as it does in mammals (Bremer et al., in press; LeMoine et al., 2010).

Insulin like growth factor-1 (IGF-1) is a well-known growth stimulating peptide in vertebrates that signals by endocrine, paracrine, and autocrine fashion to modify metabolism, development and growth of tissues and organs, cumulatively effecting somatic growth. IGF-1 is a downstream effector of the somatotropic axis, a neuroendocrine pathway regarded as one of the most important physiological systems regulating vertebrate growth that is sensitive to internal and external inputs, especially temperature, photoperiod and nutrition (Luckenbach et al., 2007; Picha et al., 2008). Although nutritional regulation of IGF-1 production is well documented in teleosts, it has been difficult to separate the effects of temperature and photoperiod on IGF signaling, especially because of the stimulatory effect increased temperatures have on appetite in fish (Picha et al., 2008). In pro-growth conditions, circulating growth hormone released by the anterior pituitary stimulates primary IGF-1 production by the liver (Duan et al., 2010; Florini et al., 1996). Skeletal muscle also produces IGF-1 in response to cell surface receptor binding of GH and liver IGF-1 to potentiate the growth signal in an autocrine and/or paracrine manner. Increasing IGF-1 stimulates cellular proliferation and anabolic pathways, and serves to complete a classic negative feedback loop to inhibit GH synthesis (Picha et al., 2008). The control of IGF-1 is layered with complexity due to separate regulation of multiple circulating IGF-1 binding proteins and cell surface receptors, whose relative abundance and affinities of IGF-1 can positively or negatively modulate IGF-1 signaling and half-life (Duan et al., 2010; Florini et al., 1996; Fuentes et al., 2013).

Significant attention has been given to the key role IGF-1 plays in metabolism and muscle growth of agriculturally important animal species, including its potential as a growth biomarker in teleosts (Picha et al., 2008). IGF-1 is a potent mitogenic signal for primary trout myoblasts, able to increase proliferation by 2-5 fold depending on culture media (Gabillard et al., 2010). In cultured trout muscle cells IGF-1 induces nutrient uptake, not only stimulating glucose and amino acid (L-arginine) uptake over basal levels, but proving more stimulatory than insulin at equal concentrations (Castillo et al., 2004).

When growth factors like IGF-1 bind to extracellular receptors, intracellular pathways are activated, including the PI3K/AKT and Mapk/ERK pathways, which are mediated by the mechanistic target of rapamycin (mTOR; previously the Mammalian Target of Rapamycin) (Castillo et al., 2004; Fuentes et al., 2015). IGF-1 administered to C2C12 myotubes stimulated hypertrophy by activating the PI3K/Akt/mTOR pathway (Rommel et al., 2001). mTOR is an intracellular node for a variety of stimulatory and repressive growth signals to direct modification of cell size and number. mTOR is sensitive to inputs including nutritional status, stress, hypoxia, and osmotic balance (Fuentes et al., 2015). Activation of mTOR promotes hypertrophic cell growth and differentiation, while inhibition favors self-renewal (Vélez et al., 2014).

mTOR is a conserved serine threonine protein kinase that couples protein synthesis and energy metabolism to regulate cell growth and proliferation (Hay and Sonenberg, 2004). It is sensitive to changes in energy balance (ie. ATP availability) in part because it is a kinase, and because it must be phosphorylated to be active itself (Hay and Sonenberg, 2004). In its phosphorylated state, mTOR can form either of two distinct functional complexes to propagate the intracellular growth signal; TORC1 (with RAPTOR) and TORC2 (with RICTOR) (Hay and Sonenberg, 2004; Laplante and Sabatini, 2012). TORC1 predominantly facilitates protein synthesis, and does so by increasing availability of a rate limiting protein necessary for transcription: eukaryotic initiation factor 4E (eIF4E). This factor is required for 5' mRNA cap binding and ribosomal docking to initiate protein synthesis (Hay and Sonenberg, 2004; Laplante and Sabatini, 2012). TORC1 directly phosphorylates downstream effectors p70S6K and 4E-BP1 to reversibly abolish their growth inhibiting roles. Un-phosphorylated 4EBP1 directly binds and represses eIF4E, but when phosphorylated 4EBP1 can no longer sequester eIF4E, permitting liberated eIF4E to increase rates of mRNA translation. By directly increasing the availability of eIF4E, TORC1 ramps up the rates of protein synthesis in support of cell growth. Increased muscle mass, such as that achieved through strength training, is predominantly regulated by increased translation capacity via TORC1 activation (Hoppeler, 2016). Muscle contraction increases activity of TORC1 where it plays critical roles promoting protein synthesis, mitochondrial biogenesis, and oxidative metabolism, while in adipose it activates PPARγ to promote adipogenesis (see Laplante, Sabatini (2012) for review). As a regulator of cell size, mTOR has also been implicated in preventing muscle atrophy (Bodine et al., 2001). mTOR may indirectly suppress atrophy through AKT activation, which is known to interrupt FOXO transcription factors (Stitt et al., 2004) from expressing the ubiquitin ligases (MuRF1 and MAFbx) responsible for muscle atrophy induced by glucocorticoids and denervation (Sandri et al 2004 Cell). Therefore, under nutrition and energy permitting conditions mTOR resets the potential for muscle accretion by maximizing protein synthesis and minimizing protein degradation.

Hypotheses

Hypothesis 1) Expression of Pax3a/b and Pax7 transcription factors in sablefish are markers of satellite cells in skeletal muscle similar to other vertebrate species, and are related to growth potential.

Hypothesis 2) Expression of myogenic transcription factors Myf5, Myod1/2, MRF4, and Myog in cultured sablefish skeletal muscle across time points will be indicative of skeletal muscle myogenesis, maturation and growth.

Hypothesis 3) Sablefish expression of PPARγ, PGC-1α and IGF-1 genes in skeletal muscle are effective measures to characterize, muscle cell growth, metabolism, maturation and anabolism, that will be useful in terms of future understanding of muscle growth relates to ultimate flesh quality.

Hypothesis 4) Variation in the aforementioned genes will be influenced by environmental conditions, as well as stage of development and sex in cultured sablefish.

Hypothesis 5) Sablefish red (oxidative) skeletal muscle and white (oxidative, glycolytic and anaerobic) skeletal muscle will have varied expression profiles of these genes in accordance with their varied metabolism, stage of growth and physical demands.

Chapter 2: Myogenic and Anabolic Gene Expression in Red and White Muscle of Sablefish (*Anoplopoma fimbria)* During Grow Out

Materials and Methods

Fish and sample origin

Sablefish muscle samples were generously provided by NOAA researchers Luckenbach and Fairgrieve from fish used in a long-term growth experiment conducted at the NOAA Manchester Field Research Station, Port Orchard, WA. Their study was designed to document the overall growth trajectory of this species in culture and characterize sex specific growth of sablefish of known parentage. All muscle samples utilized for gene expression were obtained post-mortem and supplied by NOAA personnel.

A mixed sex sablefish population was spawned in late March of 2013 and larvae were transferred out of the hatchery to first feeding tanks on May 9th, 2013. Juveniles were individually PIT-tagged in late October, 2013, at which point the subset of fish processed herein weighed an average of 151 ± 27 g and were 23.3 ± 1.4 cm in fork length. Stocking for the growth study took place in mid-January, 2014. A total of 676 juveniles were randomly selected from the tagged population to equally represent sex from each of two families, individually measured, and stocked into a 6 m diameter tank. Fish were grown in common garden fashion under natural photoperiod (47.6°N) in this land-based system supplied with single pass, filtered water from Puget Sound at ambient temperature (minimum 8°C, maximum 15°C). Fish were fed commercial salmon diets according to a feed plan developed at our laboratory, and after stocking were fed to apparent excess daily except for sampling days, when feed was withheld. Individual growth history (fork length, weight) was recorded upon PIT-tagging, stocking in January (T0), and every two months for 16 months. From tagging, mortalities totaled 24 fish (3.54% of the starting number). Morphometric data were used to generate specific growth rate (SGR) by length and weight at four time points corresponding to muscle tissue processing for gene expression analysis.

Muscle tissues were collected from randomly selected, lethally sampled fish upon initial stocking (T0, n = 40), and after 6, 12 and 16 months of growth (T6, n = 40; T12, n = 43; T16, n = 40). Muscle was always collected from the fish's right side. Red muscle was collected at the level of the lateral line, just above the vent. White muscle was collected from the dorsal axial musculature at the level of the first dorsal ray. Tissues (~100 mg) were snap frozen in liquid nitrogen and stored at -80°C until processing.

RNA isolation and preparation

Frozen muscle samples were homogenized using a Bullet Blender Blue and RNase free 2.0 mm zirconium oxide beads (Next Advance, Averill Park, NY, USA). Total RNA was extracted from muscle using TRI Reagent (0.5 – 1 ml /100 mg tissue; Molecular Research Center, Inc., Cincinnati, OH, USA) by applying the optional spin step before phase separation by bromochloropropane (Molecular Research Center, Inc., Cincinnati, OH, USA). The RNA pellet was re-suspended in 15 - 17 µl of non-DEPC nuclease free water for quantification on a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA) measured by the absorbance at 260 nm (RNA quantity) and 280 nm (protein quantity). On occasion, initial extractions were poor or missing, which required extraction of a paired sample originally intended for cryo-histological analyses. RNA was treated to eliminate DNA contamination with Turbo DNase Free (Life Technologies, Thermo Fisher Scientific, Waltham MA, USA) in 10 - 20 µl reactions (400 ng / µl). All RNA isolation and sample preparation protocols follow recommendations of the reagent or kit manufacturer, unless noted otherwise.

Reverse transcription

DNase treated RNA (100 ng/µl) was reverse transcribed to first strand cDNA in 20 µl reactions using the High Efficiency cDNA Synthesis Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). DNase treatment and cDNA synthesis were completed with recommended cycling parameters using a MasterCycler with VapoProtect lid (Eppendorf, Hamburg, Germany).

Primer and probe design

TaqMan® MGB probes and primers (Life Technologies) were designed with PrimerExpress3.0 (Applied Biosystems, Foster City, CA, USA) for sablefish (*Anoplopoma fimbria*) genes listed in Tables 2.1. and 2.2. using consensus sequences from other species identified by the NCBI Basic Local Alignment Search Tool (blast; https://blast.ncbi.nlm.nih.gov/Blast.cgi) in conjunction with Clustal W and Clustal Omega alignments (www.ebi.ac.uk/Tools/msa/clustalo/). Teleost sequences used in alignments are annotated in the NCBI Nucleotide database (http://www.ncbi.nlm.nih.gov/) and Ensembl database (http://www.ensembl.org/index.html). Designs always incorporated a published scaffold mRNA sequence or genomic sequence with deduced coding regions. Predicted and unpublished sequences from more closely related teleosts were sometimes added for comparison. Published teleost target gene mRNA sequences were blasted to the *A.fimbria* (NCBI taxonomic ID 229290) transcriptome shotgun and genome assemblies (Rondeau et al., 2013) to identify unannotated species-specific sequences and incorporate into assay designs when available (Tables 2.2. and 2.3.). Amplicons were designed to span exon-exon splice boundaries where possible.

The targeted T_m of primers and probes was 58 - 60°C and 68 - 70°C, respectively. Assay efficiencies were calculated from a 4 - 6 point curve from a 10 or 6 fold dilution series with at least three replicates to produce each point. Efficiencies and sequences of primers and probes can be found in Table 2.3.

TaqMan qRT-PCR

Gene expression analyses were conducted on a Stratagene MX3005P qPCR instrument (Agilent Technologies, Santa Clara, CA, USA) using TaqMan® Fast Advanced Master Mix with uracil-N glycosylase (UNG) and ROX as the passive reference dye (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). For all genes except 18S, 1µl of 1:5 cDNA was amplified in a 14µl total qPCR reaction volume containing 10 µl of 2x Taqman Fast Advanced Master Mix. Final reaction concentrations of forward and reverse primers were each 0.71 µM, and probe was 0.19 µM. For 18S, 1 µl of 1:100 cDNA and 7.5 µl of 2x master mix were amplified in a 14 µl reaction with final primer concentrations of 0.54 µM and probe concentration of 0.15 µM. Reaction conditions began with a 2 minute UNG erase at 50°C, followed by a 20 second enzyme activation step at 95°C, and 40 cycles of amplicon melting for 15 seconds at 92°C, amplification and extension for 30 seconds at 60°C. Reactions were performed in duplicate in 96-well plates for all genes except for eIF4E, which was run in triplicate. Red and white muscle samples from the same fish were run on the same plate for all genes, and plates had a random mix of fish from all sample points. Controls were run without reverse transcriptase for every gene to ensure genomic DNA was not amplified, and controls without template were included on every plate.

Data analysis

SGR for individual fish was calculated for weight and length using (ln kg or cm_{final} – ln kg or $cm_{initial}$ / $Time_{final}$ - $Time_{initial}$) for each growth stanza preceding tissue sampling. We were able to include the T0 growth stanza based on measurements taken at PIT tagging 3 months prior to stocking, while two-month growth stanzas are reflected at T6, T12, and T16. Means and standard deviations are reported for growth metrics (weight, length, SGR).

Genes of interest were normalized to 18S ribosomal RNA subunit or eIF4E using the ΔCt method (Livak and Schmittgen, 2001; Pfaffl, 2001; Souaze et al., 1996). Briefly, the mean threshold cycle (Ct) of a sample's reference gene was subtracted from the mean Ct of the corresponding sample's gene of interest to yield a ΔCt. The ΔCt for all genes and all samples were then converted to relative abundance by $2^{-(\Delta Ct)}$. Gene expression ratios were computed with $2^{-(\Delta Ct)}$ data relative to 18S rRNA. Model adjusted Least Squares means (LSmeans) and percent coefficient of variation are reported for gene expression data. All figures were made in Microsoft Office Excel 2016, 32-bit (Microsoft Corp., Redmond, WA, USA).

Statistical analysis

All statistical analyses were run with SAS 9.4 software (SAS, Cary, NC, USA). Data were evaluated for Gaussian distribution and homoscedasticity. Fork length was log transformed (base 10), while weight and gene expression data were natural Log transformed (Log base *e*). Data points more than 3 standard deviations from the mean of transformed data were omitted from the dataset before statistical comparisons, resulting in few or zero omissions. Differences in body weight, fork length, and SGR by Time (T0, T6, T12, T16) were evaluated with a generalized linear mixed model by invoking Proc Glimmix followed by Tukey's HSD test for multiple comparisons with adjusted p-values. The relationship between gene expression and muscle tissue type (white, red), time point (T0, T6, T12, T16), and sex (male, female) were also evaluated with Proc Glimmix and Tukey's HSD test, listing family cross (1, 2) and sample type (original, cryo-histological) as cofactors in the random statement. P-values for differences by muscle type are reported from this model. Due to significant differences between the tissues we subsequently completed all gene expression analyses per tissue and report these p-values. A p-value ≤ 0.05 was considered statistically significant. Results report the model adjusted means after removing the log transformation.

Results

Sablefish growth

Figure 2.1. Sablefish length and weight significantly increased during the growth period, while SGR by length and weight showed the opposite and significant trend.

Sablefish grew well for 16 months (Fig.2.1.). Relative to initial stocking sizes (T0), fish were 64% longer and 365% heavier by T16 after accruing an average of approximately 110g per month. Mean fish fork length significantly increased at each sample point ($p<0.0001$, Fig.2.1.A; T0 = 33.9 ± 1.5, T6 = 44.7 ± 2.8, T12 = 52.5 ± 3.0, T16 = 55.7 ± 3.6 cm; mean ± SD) with slower rates of growth becoming apparent by T12 - T16. Likewise, mean fish weight significantly increased at each sample point ($p<0.0001$, Fig.2.1.B; T0 = 0.475 ± 0.063, T6 = 1.081 ± 0.206, T12 = 1.840 ± 0.338, T16 = 2.208 ± 0.438 kg; mean ± SD). Because SGR is a factor of the initial size, and since fish lengths and weights substantially increased, significant differences were also found in mean SGR calculated by fork length ($p<0.0001$, Fig.2.1.C) and by weight ($p<0.0001$, Fig.2.1.D). SGRs were significantly lower at each time point for Length SGR (T0 = 0.374 ± 0.026, T6 = 0.122 ± 0.038, T12 = 0.067 ± 0.011, T16 = 0.034 ± 0.014; mean ± SD) and Weight SGR (T0 = 1.131 ± 0.063, T6 = 0.320 ± 0.129, T12 = 0.224 ± 0.059, T16 = 0.058 ± 0.099; mean ± SD).

Muscle gene expression

Figure 2.2. Eukaryotic initiation factor 4E (eIF4E) expression is greater in red muscle and changes in white and red muscle during sablefish grow out.

Muscle type influences expression of eIF4E mRNA (Fig.2.2.). Overall, red muscle expressed more eIF4E relative to 18S ($p<0.0001$; Fig.2.2.A) and when 18S is a covariate ($p<0.0001$; Fig.2.2.B). This suggests red muscle may have higher transcription and translation rates per cell than white muscle. We have used eIF4E as a general marker of metabolic rate.

During sablefish grow out, expression of eIF4E changed in white and red muscle types (Fig.2.2.). In white muscle, expression of eIF4E increased from T0 to its highest point at T6, before declining by T12 and reaching initial levels again by T16 (Fig.2.2.A; $p<0.0001$; Fig.2.2.B, $p<0.0001$). In red muscle, expression elevated after T0 and plateaued at T6 and T12 before declining again by T16 (Fig.2.2.A, $P<0.0001$; Fig.2.2.B, $p<0.0001$). The significant increase in eIF4E expression observed in both white and red muscle between T0 and T6 suggests an increase in metabolic rate and protein synthesis capacity per cell that is consistent with the requirements of myofiber maturation.

Figure 2.3. Paired homeobox 7 (Pax7) and 3 (Pax3b) expression is greater in red muscle and changes during sablefish grow out.

Muscle type influences the relative expression of satellite cell markers, Pax7 and Pax3b. Pax7 expression was greater in red muscle ($p<0.0001$, Fig.2.3.A, B, C).When accounting for 18S, Pax3b expression was also greater in red muscle ($p<0.0001$, Fig.2.3.D, F), but there was no difference relative to eIF4E (Fig.2.3.F). These results suggest that red muscle has more satellite cells than white muscle.

In the red muscle, Pax7 and Pax3b changed as fish grew ($p<0.0001$, Fig.2.3.A-C; $p=0.0032$, Fig.2.3.D; $p<0.0001$, Fig.2.3.E, F) showing similar trends over time. Greatest red muscle Pax7 expression was often observed at T0 and/or T6, before reaching least expression by T12 and/or T16 (Fig.2.3.A-C). Similarly, Pax3b expression in red muscle was greatest at T0, and was lower by T6 where it appeared to plateau (Fig.2.3.D-F). These results are consistent with a larger satellite cell pool in red muscle of smaller fish.

Figure 2.4. Paired homeobox 7 (Pax7) and 3 (Pax3b) expression in white muscle changes during sablefish grow out.

In the white muscle, differences were apparent over time in Pax7 ($p<0.0001$, Fig.2.4.A-C) and Pax3b expression ($p=0.0014$, Fig.2.4.D; $p<0.0001$, Fig.2.4.E, F). White muscle Pax7 expression was typically lower at T0 before stabilizing at greater levels (Fig.2.4.A-C). The pattern of Pax3b expression in white muscle incorporated minimal expression at T6, before increasing again at later

time points (Fig.2.4.D-F). These results suggest a smaller satellite cell pool is present in white muscle of smaller and younger fish.

Figure 2.5. Myogenic determination factor 1 and 2 (Myod1, Myod2) expression is greater in white muscle and changes in each muscle type during sablefish grow out.

Overall, white muscle had greater expression than red muscle of Myod1 ($p<0.0001$, Fig.2.5.A-C) and Myod2 ($p<0.0001$, Fig.2.5.D-F).

In the white muscle, Myod1 appeared to decline over time relative to 18S ($p=0.0309$, Fig.2.5.A) with greater expression at T6 than T16. In contrast, Myod2 relative to 18S had no differences over time ($p=0.37$, Fig.2.5.D). Relative to eIF4E, expression of Myod1 and Myod2 were significantly lower at T6 ($p<0.0001$, Fig.2.5.B, E). The covariate analyses also detected differences over time in Myod1 ($p=0.0006$, Fig.2.5.C) and Myod2 ($p=0.0041$, Fig.2.5.F). Expression of both genes were significantly lower at T16, although Myod2 expression at T16 was not different than T0.

In red muscle, Myod1 normalized to 18S changed over time similarly its covariate analysis ($p<0.0001$, Fig.2.5.A, C) with lowest expression at T0, greater expression at T16, and greatest at T6 and T12. No changes were observed in Myod1 relative to eIF4E in red muscle ($p=0.31$, Fig.2.5.B). Myod2 relative to 18S had significantly lower expression at T0 than T6 which were not different from the intermediate expression at T12 and T16 ($p=0.0331$, Fig.2.5.D). Relative to eIF4E, Myod2 was significantly lower at T6 and T12 ($p<0.0001$, Fig.2.5.E). No changes were detected in Myod2 in red muscle over time by the covariate analysis ($p = 0.3914$, Fig.2.5.F).

Figure 2.6. The ratio of Paired homeobox 7 (Pax7) expression to that of either Myogenic determination factor paralogues, Myod1 or Myod2, or their sum (Total Myod) is greater in red muscle and changes in both muscle types during sablefish grow out.

The ratios of Pax7 to Myod1, Myod2, and Total Myod (all relative to 18S) were always greater in red muscle than white muscle ($p<0.0001$, Fig.2.6.A-C). The expression ratios of Pax7/Myod in the two tissues changed during the growth period in nearly opposite patterns.

In red muscle, the greatest ratio was observed at T0 before significantly lower ratios were detected in at T6 ($p<0.0001$, Fig.2.6.A-C). More significant differences were observed at later stages (T6-T16) in red muscle when ratio calculations included Myod1 (Pax7/Myod1 or Pax7/Total Myod). In red muscle the lowest ratio was observed at T12, which was significantly higher again at T16.

In white muscle the lowest ratios were at T0 and increased significantly by T6 ($p<0.0001$, Fig.2.6.D-F). Gene expression ratios that included Myod1 also appeared to continue to increase, with greatest Pax7/Myod ratios observed in T16 fish. These results indicate a more myogenic proportion of SCs were present in the white muscle than red muscle, and the proportion of determined SCs decreased during growth in white muscle but increased in red muscle. The Pearson correlation

coefficients between Pax7/Myod1 and Pax7/Total Myod in white muscle can be found in Table 2.6, and are significantly and negatively correlated with SGR calculated by weight.

Figure 2.7. Myogenic factor 5 (Myf5) expression is regulated differently by muscle type and during growth, depending on the method of normalization. The ratios of Myf5 to Myod1 or Myod2, or their sum (Total Myod) are greater in red muscle and follow a tissue specific pattern during growth.

Myf5 expression was greater in red muscle relative to 18S and when the analysis included both reference genes as covariates ($p<0.0001$, Fig.2.7.A, C). This situation reversed when Myf5 was expressed relative to eIF4E, indicating white muscle had greater levels Myf5 mRNA relative to protein synthesis capacity ($p<0.0001$, Fig.2.7.B).

Red muscle expression of Myf5 was lower at T6 and T12 relative to eIF4E ($p<0.0001$, Fig.2.7.B) but no changes were observed relative to 18S ($p=0.18$, Fig.2.7.A,) or when reference genes were modeled as covariates ($p=0.51$, Fig.2.7.C).

Myf5 expression in white muscle tended to increase in the large fish at T16. This increase at T16 was significant relative to 18S ($p=0.0003$, Fig.2.7.A) and relative to eIF4E ($p<0.0001$, Fig.2.7.B), although the latter was not greater from expression at T0. No changes were significant by time in white muscle when these reference genes were applied as covariates to the model ($p=0.32$, Fig.2.7.C).

The ratios of Myf5 to Myod1, Myod2, or Total Myod were greater in red muscle ($p<0.0001$, Fig.2.7.D-F). In red muscle the ratio was typically greatest at T0 and T16 ($p <0.0001$, Fig.2.7.D, F; $p=0.0116$, Fig.2.7.E), indicating a strong pro-myogenic signal in small fish (T0) and very large fish (T16). In white muscle the Myf5/Myod ratios always followed a pattern seen in white muscle Myf5 expression relative to 18S: all three ratios were significantly greater at T16 ($p<0.0001$, Fig.2.7.D-F). These results indicate large sablefish (T16) have stronger pro-myogenic signal in white muscle, and an increased likelihood of initiating the myogenic pathway once SCs are activated. Together, these results suggest a greater proportion of myoblasts (from any muscle type) are likely to move onto maturation than be recruited as new myoblasts in the T16 fish with large body sizes and low SGRs.

Figure 2.8. Myogenic regulatory factor 4 (MRF4) and Myogenin (Myog) are greater in red muscle, and both exhibit similar regulation in each tissue during sablefish growth.

More MRF4 and myogenin mRNA was expressed in red muscle as compared to white muscle relative to 18S ($p<0.0001$, Fig.2.8.A, D) and when 18S and eIF4E are included as covariates ($p<0.0001$, Fig.2.8.C, F). When normalized to eIF4E, no differences were significant between muscle types in MRF4 expression ($p=0.98$, Fig.2.8.B) or myogenin expression ($p=0.52$, Fig.2.8.E).

In red muscle, MRF4 and myogenin expression patterns changed similarly over time relative to 18S ($p<0.0001$, Fig.2.8.A, D), significantly increasing at T6, had somewhat intermediate expression

at T12, and lowest expression at T0 and T16. This general pattern was consistent with an increase in expression at T6 when 18S and eIF4E were evaluated as covariates ($p<0.0001$, Fig.2.8.C; $p<0.0002$ Fig.2.8.F), suggesting MRF4 and myogenin expression vary more closely with 18S than eIF4E. Regulation of MRF4 and myogenin expression in red muscle over time demonstrated general agreement ($p<0.0001$; Fig.2.8.B, E), but with a new pattern. Although not entirely identical, peak expression was observed at an earlier stage of growth (T0), before both genes reached lowest expression by T12.

In white muscle, few differences were observed in the regulation of MRF4 and myogenin expression over time. Relative to 18S, MRF4 expression was greater at T6 than at T12 and T16 ($p<0.0002$, Fig.2.8.A) and no change was detected in myogenin ($p=0.3005$, Fig.2.8.D). The covariate model for each gene generally agreed with this, as MRF4 expression was lowest at T16 ($p<0.0001$, Fig.2.8.C) and, even though the covariate model detected differences in myogenin mRNA by time ($p<0.0340$, Fig.2.8.F), differences between time points became insignificant once an adjustment for multiple comparisons was applied to the Tukey HSD post-hoc p-values. Relative to eIF4E, white muscle MRF4 and myogenin expression were influenced by time ($p<0.0001$; Fig.2.8.B, E). The pattern was like our observations in red muscle, as white muscle MRF4 and myogenin were also lowest at T6. This correlates to the same time point we detected an increase in protein synthesis capacity per cell based upon eIF4E mRNA expression. It appears white muscle expression recovered quickly, while red muscle remained low longer before eventually increasing at T16 to reach expression that was not different from T0. MRF4 and myogenin expression patterns over time normalized to eIF4E are specific to the tissue and seem to reflect the inverse of eIF4E expression relative to 18S (Fig.2.8.A). These data suggest MRF4 and myogenin expression in vivo are regulated more closely with 18S during growth in a manner specific to the muscle phenotype and that the red muscle phenotype requires greater expression of both genes.

Figure 2.9. Peroxisome proliferator activated receptor gamma (PPARγ) expression is greater in white muscle and changes during sablefish growth.

White muscle expressed greater PPARγ than red muscle by all gene normalization methods ($p<0.0001$, Fig.2.9.A-C).

White muscle expression of PPARγ changed during growth. PPARγ mRNA relative to 18S changed during growth, with lowest transcription at T0, greatest at T6, and intermediate at T12 and T16 ($p<0.0001$, Fig.2.9.A). A similar trend was observed when 18S and eIF4E are included as covariates, where expression was lowest at T0 and T16 and greatest at T6 and 12 ($p<0.0001$, Fig.2.9.C). In contrast, PPARγ expression relative to eIF4E was least expressed at T0 and appeared to increase over time, reaching significantly greater expression at T12 and T16 ($p<0.0003$, Fig.2.9.B).

Dynamic expression of PPARγ during growth was also measured in red muscle. The expression profile relative to 18S and the covariate model both identified least expression at T0, greater expression at T6 and T12, while lower expression at T16 was not different from T0 and T6 ($p<0.0001$, Fig.2.9.A, C). Relative to eIF4E expression was greater at T0 than T6, while no differences were observed at T12 or T16 ($p=0.0007$, Fig.2.9.B).

Figure 2.10. Peroxisome proliferator activated receptor gamma coactivator - 1α (PGC-1α) expression is greater in red muscle and exhibits different patterns of regulation by muscle type during sablefish growth.

Expression of PGC-1α was always greater in red muscle than white muscle ($p<0.0001$, Fig.2.10.A-C).

In red muscle, PGC-1α expression relative to 18S was lower at T0 than all other time points ($p<0.0001$). The covariate model also found lower expression at T0 than T6, but no differences were significant when compared to intermediate expression levels at T12 and T16 ($p=0.0178$, Fig.2.10.C). Relative to eIF4E, red muscle PGC-1α expression was greater at T0 and T16 than at T6 and T12 ($p<0.0001$, Fig.2.10.B).

White muscle expression of PGC-1α was low and changed over time (Fig.2.10.D-F), and with some consistency in the overall pattern across different normalization methods. PGC-1α relative to 18S appeared to have greater levels of expression at T0 and T12 than T6 and T16, though expression at T0 was not significantly different than the other time points ($p=0.0049$, Fig.2.10.D). Relative to eIF4E, significantly lower expression was observed at T6 ($p<0.0001$, Fig.2.10.E). The covariate model found expression of PGC-1α to be significantly greater at T0 and T12 than at T6 and T16 ($p=0.0005$, Fig.2.10.F).

Figure 2.11. Insulin like growth factor 1 (IGF-1) is expressed higher in red muscle and changes concordantly in white and red muscle during sablefish growth.

IGF-1 expression was expressed less in white muscle than red muscle ($p<0.0001$, Fig.2.11.A-C).

Red muscle transcription of IGF-1 was greatest at T0 and T12, intermediate at T6, and lowest at T16 when expressed relative to 18S and when the reference genes were incorporated into the analysis as covariates ($p<0.0001$, Fig.2.11.A, C). Relative to eIF4E, expression of IGF-1 was greater at T0 than T12, while T12 had significantly more transcript than at T6 and T16 ($p<0.0001$, Fig.2.11.B),

IGF-1 changed in white muscle during growth was greater in the cooler months than the warmer months, similar to red muscle, and had greatest expression at T0. Analysis of IGF-1 expression relative to 18S and with 18S and eIF4E as covariates found significant differences in expression at all time points and followed the same pattern: T0>T12>T6>T16 ($p<0.0001$,

Fig.2.11.A,C). The same profile was observed relative to eIF4E, except there was no difference between T6 and T16, each with the lowest IGF-1 expression ($p<0.0001$, Fig.2.11.B).

Fish sex and muscle gene expression

Sex significantly affected the expression genes in the white muscle (Table 2.5.) and red muscle (Table 2.6.). In white muscle, males had greater expression Myf5 and Myod2 relative to eIF4E, and females had greater ratios of Pax7/Myod2, and greater expression of eIF4E, PPARγ, and IGF-1 only when data was analyzed with reference genes as covariates. In red muscle, males had greater expression of Pax3b and Myod2 when expression was relative to eIF4E. However, the effect of sex was typically present as an interaction with time in both tissues. T-tests conducted at each time point found most sex differences in transcription were present at T16, and were more common in the white muscle.

Discussion

This study evaluates expression of key myogenic and metabolic genes in sablefish fast-white and slow-red muscle. These were characterized in such a manner that they reflect timing and magnitudes of changes in muscle type expression during growth at body sizes equivalent to a substantial portion of the late industry grow out phase. Our assessment is to determine whether sablefish transcribe these genes in accordance with what may be expected given the established roles of the cognate proteins and observed expression in other vertebrates.

Fish growth

Sablefish maintained consistent increases in body mass (Fig.2.1.) but appear to slow their growth by length between T12-T16, after fish reached approximately 52 cm fork length. Declining growth in cultured sablefish of this size agrees with a report by (Luckenbach et al., 2017) who monitored growth of sablefish under similar nutritional and seasonal conditions and found fish slowed their growth near 50 cm fork length. Slowed linear growth at larger sizes in culture coincides with fisheries literature showing declining growth in wild fish as they approach average maximum lengths (specific to sex and location). This may be viewed in von Bertalanffy growth curves, which pairs information on length at age for males and females of the Alaska region (Echave et al., 2012; Hanselman et al., 2014) and females of the West Coast region (Head et al., 2014). Better access to feed in culture permits rapid growth and compression of the growth curve into less time, making the onset of slowed growth in our <2 year old sablefish possible (Luckenbach et al., 2017). During a body length-related transition to slower growth, it is likely that congruent changes take place in the muscle. For instance, hyperplasia is considered important for fast growth in fish (López-Albors et al., 2008). Johnston et al. (2004) found the cessation of fiber recruitment in Arctic char is not related to age but to

body length, and male Atlantic halibut cease myofiber recruitment earlier than females only to be outgrown (Hagen et al., 2008). The positive correlation between fish body mass and mean fiber size implies that at some point hypertrophy overtakes hyperplasia as the primary mechanism for muscle tissue growth in teleosts (Priester et al., 2011). This is interpreted as the increasingly rare occurrence of new myotube formation (diameters <5 μm) in older and larger individuals, such as the tiger pufferfish (Fernandes et al., 2005) and in trout (Rescan et al., 2015). While it is interesting to consider the possibility that slowed growth in sablefish at T12-T16 could point towards more hypertrophic growth, more research is required to establish the relative contribution of hyperplasia and hypertrophy in sablefish at different growth trajectories and stages of development, as well as the environmental and genetic factors that determine this.

mRNA translation capacity

As a rate limiting mRNA cap binding protein, eIF4E availability assists in adjusting the mRNA translation capacity of cells. eIF4E is regulated by activation state (i.e., bound to it's 4E-BP inhibitory binding protein) and by levels of expression. As would be expected based on their high metabolic rates, elevated eIF4E expression is found in a variety of cancers, where the unregulated cell growth incorporates increased cell survival signaling and rates of protein synthesis (Mamane et al., 2004). Greater expression of eIF4E observed in sablefish red muscle (Fig.2.2.) is consistent with elevated metabolic requirements and smaller myonuclear domains relative to white muscle in order to maintain the oxidative phenotype, as described above.

The significant increase in eIF4E detected from T0 to T6 in both muscle types may be interpreted as a transcriptional indicator of fiber maturation. The emphasis to grow and mature red and white muscle types is not expected to occur at the same pace during development. Red muscle is not responsible for large increases in mass like white muscle, does not extensively incorporate as many myotubes via mosaic hyperplasia, but must grow to keep up with gains in body length. Red oxidative fibers remain small in diameter and may be considered a more homogenous and mature muscle type than white muscle, which has a mix of fibers at all stages of development. Accordingly, elevated eIF4E expression appears to be maintained in red muscle while in white muscle expression tends to decline after the peak at T6 (Fig.2.2.). Increased transcription for maturation in muscle includes generating more metabolic enzymes, contractile and structural proteins, and cell components like mitochondria and sarcoplasmic reticulum, to fulfill functions of the respective muscle fiber phenotype with efficiency. Considering the transient nature of gene expression, we examined the timing of suspected fiber maturation relative to changes in the expression of satellite cell population size, activation, and differentiation, which we describe below. Myogenic events indicated by their transcription profile corroborate fiber maturation initiation in red and white muscle by T6.

Paired homeobox transcription factors 3 and 7

Due to the importance of Pax3 during primary muscle development to establish the identity of myogenic precursor cells and of Pax7 in the specification and long term maintenance of the SC population (Buckingham and Relaix, 2015; Seale et al., 2000), the expression patterns of these genes are of interest in teleosts. So far, formal evaluations in a few finfish species have confirmed the existence of Pax3a/b and/or Pax7a/b paralogous genes, including in zebrafish (Seo et al., 1998), olive flounder (Jiao et al., 2015a; Jiao et al., 2015b), *Takifugu rubripes* (Akolkar et al., 2016), and rainbow trout (Chapalamadugu et al., 2015). These works suggest the overall timing of expression of Pax3 and Pax7 is consistent with expression of both genes during embryogenesis of higher order vertebrates. Pax3 is implicated in embryonic myogenesis by functioning as a potent upstream activator of Myf5 and Myod to specify cells to the muscle lineage (Maroto et al., 1997). In olive flounder embryos, Pax3b and not Pax3a co-localized with Myod in the somite (Jiao et al., 2015b). Importantly, we detected low expression of Pax3b in sablefish red and white muscle but never detected Pax3a mRNA. In all cases, it appeared Pax3b was expressed at much lower levels than Pax7 (data not shown). It is possible some of this difference could be due to our Pax7 assay detecting both an ‘a’ and ‘b’ gene, although it remains unconfirmed whether sablefish have a copy of the Pax7 gene. Since Pax3 is expressed during in vitro myoblast proliferation and differentiation of primary cells from the fast muscle of the adult giant danio (Froehlich et al., 2013), it is possible our detection of Pax3b might support a potential role for this homolog in teleost indeterminate myogenesis. However, the low expression of Pax3b compared to Pax7 could indicate that Pax7 is a key regulator of SC specification and renewal for in vivo myogenesis. This would be in accordance with Pax gene expression in *Takifugu rubripes,* where RT-PCR amplified Pax3a/b and Pax7a/b during embryogenesis (days 3-8 and 3-16 post fertilization, respectively), but only Pax7a was detected in adults in both fast-white muscle and slow-red muscle (Akolkar et al., 2016). It appears consistent that in sablefish Pax7 may be a good marker for quiescent satellite cells as has been noted in other animal species.

In vertebrates, larger relative populations of SCs are found in high oxidative fibers compared to low oxidative fibers (van Wessel et al., 2010). This remains true for animals of different postnatal ages (Gibson and Schultz, 1982). We observed transcription of Pax7 and Pax3b to favor greater expression in slow-red muscle in sablefish (Fig.2.4.). As expected, our results indicate oxidative red muscle has a larger population of satellite cells and provide further evidence that Pax7 marks quiescent sablefish SCs.

In white muscle, lower Pax7 and Pax3b expression (at T0 and T0-T6, respectively) can be interpreted as a smaller population of SCs compared to larger fish (at T12 and T16; Fig.2.4.A,D). Initially this may seem counterintuitive, since smaller fish might be expected to have denser SC

populations and thus greater growth potential. However, the smallest sablefish examined were already averaging nearly half a kilogram. A reduced SC pool at this size could result from recruitment of new SCs to the myogenic lineage at rates exceeding SC replenishment. Greater Pax7 and Pax3b in larger and older fish supports the notion that teleosts proficiently repopulate the SC compartment, especially compared to terrestrial vertebrates. Lower Pax7 and Pax3b expression coincide with maximum SGRs, indicating small sablefish may sustain comparable net increases in mass to large sablefish by drawing heavily from the SC population for vigorous myocyte accretion.

Satellite cell determination and activation factors

The MRFs with semi-redundant but essential roles determining SCs to the myogenic pathway Myf5 and Myod (Conerly et al., 2016; Rudnicki et al., 1993). Myf5 can be expressed in both activated SCs and myoblasts, whereas Myod expression marks active and proliferating myoblasts. Expression of both transcription factors are sustained in myocytes and myofibers being anti-apoptotic and myogenic in nature.

Myod activates SCs and determines them to the myogenic pathway (Montarras et al., 2000). Variation across teleosts shows anywhere from one to three distinct copies are retained (Macqueen and Johnston, 2008). Like many other fish species, sablefish appear to have two distinct genes, Myod1 and Myod2. Expression of sablefish Myod1 was greater than Myod2 in each muscle type. BLAST results indicate the partial sablefish Myod1 sequence (Table 2.1.) is more closely related to the single vertebrate Myod gene. Teleost Myod1/2 are known to exhibit differential regulation of the two paralogues (Azizi et al., 2016; García de la serrana et al., 2014). We note the overall tissue specific patterns of Myod1/2 expression are not highly divergent during sablefish growth and muscle development. In the rat, Myod mRNA is preferentially expressed in fast glycolytic fibers and muscles (Hughes et al., 1993). Transcription of sablefish Myod1/2 was also greater in white muscle (Fig.2.5.). Considering sablefish were growing well, elevated expression of Myod1/2 in white muscle would indicate a larger proportion of myoblasts were actively cycling and available for maturation in the muscle tissue responsible for greatest increases in body mass. By T16, the proportion of myoblasts in white muscle was lower (Fig.2.5.A, C, F). This could be because of a dilution effect from greater myonuclei in more mature myofibers of large fish, or because the proportion of quiescent SCs was higher. Greater expression profile in white muscle of sablefish suggests Myod1 and Myod2 appear to mark the population of myoblasts in sablefish as in other species. We should not fail to consider that mRNA expression may not perfectly reflect cognate protein expression, though in general potent transcription factor genes are generally highly translated when mRNA is present.

The ratio of Pax7/Myod expression has been used to infer the fate of SCs in vertebrates (Chapalamadugu et al., 2009; Olguin et al., 2007; Olguín and Pisconti, 2012; Villasante et al., 2016a;

Villasante et al., 2016b). Pax7 expressing SCs must express Myod to activate, but overexpression of Pax7 can prevent Myod expression, leading Olguin, Olwin (2004) to suggest the ratio is a deciding factor for myoblast fate. Higher ratios indicate satellite cell self-renewal, intermediate ratios promote proliferative cycling, and lower ratios support progression to differentiation. In sablefish, the ratio of Pax7 to Myod1, Myod2 or Total Myod is lower in white muscle than red muscle, indicating more myoblast progression to myogenesis in the faster growing tissue (Fig.2.6.). Furthermore, this ratio in white muscle changes in an expected manner with body size and growth rates. Lower Pax7/Myod ratios would be expected during faster growth, and indeed we see the small fish (T0) have the lowest ratios and highest SGRs, indicating greater myogenic progression. In contrast, large fish (T16) have the highest ratios and lowest SGRs, which suggests a shift towards SC renewal. Additionally, the Pearson correlation coefficient (Table 2.4.) shows a significant and negative correlation between the Pax7/Myod1 ratio (-0.627) and Pax7/Total Myod ratio (-0.642), suggesting either of these ratios would be useful to evaluate growth potential in this species. Sablefish fast growth seems to be supported by greater proportions of Myod1/2 expressing myoblasts. Thus, the tissue and growth rate specific regulation of Pax7 to Myod1/2 is in accordance with cell fate regulation to support fast growth in vertebrates, and provides further evidence that sablefish SCs are identified by Pax7 mRNA and myoblasts by Myod1/2 mRNA.

After Seale et al. (2000) demonstrated uniform expression of Pax7 in murine derived SCs, Kuang et al. (2007) found that most quiescent SCs (90%) also express Myf5, and in doing so, are more committed to myogenesis than the minority of SCs that lack Myf5. These investigators also found that the typical $Pax7^{+}/Myf5^{-}$ SC had never expressed Myf5, and instead this population of SCs preferred to repopulate the stem cell compartment. Without initiating myogenesis, Myf5 expression irreversibly shifts the preference of SCs towards efficient differentiation and away from self-renewal. More recent research in mice demonstrates both Myf5 and Myod claim redundant muscle specific DNA binding sites and can promote histone H4 acetylation to open chromatin structure at binding locations, but Myf5 inadequately recruits RNA pol II whereas Myod induced robust transcription (Conerly et al., 2016). These researchers reasoned the preparatory chromatin remodeling function of Myf5 specifies cells to the myogenic lineage and grants ready access for Myod expression to then activate transcription of the myogenic program with less delay. Once activated by Myod, these $Pax7^{+}/Myf5^{+}/Myod^{+}$ myoblasts are more likely to express myogenin, quickly downregulate Pax7, and proceed to differentiation. Myf5 expression can indicate the level of myogenic determination, and the ratio of Myf5/Myod can indicate the success of the pro-myogenic signal in activated myoblasts. Higher ratios indicate a greater proportion of myoblasts progress towards maturity and fewer are recruited as new myoblasts. In sablefish, Myf5 expression was greater in red muscle when normalized

to 18S (Fig.2.7.A, C), implying greater determination in the slower growing, oxidative muscle. This contrasts with other studies, where oxidative and glycolytic muscle types show no difference in expression of Myf5 in mammals and fish (Alves-Costa et al., 2013; Hughes et al., 1993). Myf5 expression was mostly stable in each tissue type during growth, except for when it increased in the white muscle of large T16 fish (Fig.2.7.A, B). Such a change implies greater SC and/or myoblast determination to the myogenic lineage in fast growing muscle of large sablefish. Like Myf5 expression, no changes are observed in the Myf5/Myod ratios in white muscle until T16, when these ratios increase (Fig.2.7.D-F) due to the simultaneous increase in Myf5 and decrease in Myod1 (Fig.2.5.A). Together, these changes suggest a greater proportion of what may also be fewer myoblasts go onto maturity to support growth, possibly spending less time in a proliferative phase. Similarly, elevated ratios of Myf5 to Myod were observed in red muscle at T0, indicating greater commitment to myogenesis. This timeline agrees with the transcription of the other MRFs and suggests the possibility of a wave of myocyte accretion in red muscle at T0 that are maturing at T6, as indicated by higher expression of MRF4, Myog, and eIF4E (described below). As it relates to expression timing and magnitude of the other MRFs, sablefish Myf5 expression appears to support a role consistent with that of other animals as a regulator and identifier of SCs and myoblasts determined to the myogenic lineage.

Muscle differentiation factors

Sablefish dynamically transcribe two MRFs, MRF4 and Myog, which have long been considered important to myogenesis as regulators and markers of myoblast differentiation (Macqueen and Johnston 2011, Garcia de la serrana, et al., 2014). In zebrafish myogenesis, MRF4 expression coincides with myofibril assembly, and expression is stronger in the differentiated slow muscle than in fast white muscle (Hinits et al., 2007). Preferential MRF4 expression in oxidative fibers is noted in other vertebrates, but with some inconsistencies between muscles and across studies (Hughes et al., 1993; Moretti et al., 2016; Walters et al., 2000). Myogenin is more heavily studied than MRF4, is critical to myoblast fusion for myotube formation, and exhibits greater transcription in oxidative fibers (Hasty et al., 1993), (Hughes et al., 1993; Hughes et al., 1999; Meadows et al., 2008; Nabeshima et al., 1993; Rescan et al., 1995). Transcripts of both MRFs persist in differentiated and mature muscle, but MRF4 is the most abundant which suggests it has an important but undetermined function in muscle maintenance (Hughes et al., 1993; Walters et al., 2000). As expected, sablefish red muscle also expresses more Mrf4 and myogenin than white muscle. Sablefish MRF4 and Myog appear to be regulated similarly during growth and especially so in red muscle, further supporting the notion these MRFs have specialized functions at the same stage of myogenesis (Fig.2.8.). Lastly, the timing of transcriptional changes in these MRFs indicate they function as transcription factors of differentiation

and fiber maturation. The increase of MRF4 and Myog in red muscle and increase of MRF4 in white muscle at T6 agree with timing of fiber maturation in both tissues suggested by the elevated expression of eIF4E. It would be expected for greater differentiation and maturation signals to be met with improved mRNA translation capacity to construct of all the necessary cellular organelles, metabolic enzymes, and structural proteins to become a functional part of the syncytial tissue and prepare for further growth by hypertrophy. This time frame also agrees with other transcriptional markers in white muscle, where the increase in MRF4 follows a period of growth by myocyte accretion in white muscle at T0 (suggested by low Pax7 and Pax7/Myod ratio). Altogether, sablefish MRF4 and Myog appear to have roles in muscle differentiation and maturation, and are regulated in accordance with muscle metabolic phenotype as observed in other species.

Nutrient and energy sensors

To gain insight into the nature of accretionary signaling during sablefish growth we evaluated three factors known to be sensitive to nutrient and energy status in other species: PPARγ, PGC-1α, and IGF-1.

PPARγ is a nuclear receptor transcription factor that serves as a nutrient sensor and mediator of anabolism. PPARγ is sensitive to fatty acids, the optimal length of which appears to be species specific. PPARγ expression is further influenced by lipid metabolites and its activity is moderated by the co-factor; PGC-1α as it directly influences binding efficiencies to PPAR response elements in regulated genes. In mammals, it is highly expressed in adipose, but also in muscle where it promotes lipid uptake, storage, and metabolism. Leaver et al. (2005) suggested divergence in teleost PPARγ function based on sequence conservation rates in known functional domains. However, research with cobia strongly suggests PPARγ is the predominant PPAR in connection to lipid metabolism and storage (Tsai et al., 2008). One thing to keep in context is that this research has focused on the growth of muscle but it is also key that we evaluate genes such as PPARs and PGC-1α since these influence the lipid storage and these two factors are ultimately critical for developing sensory attributes of sablefish flesh destined for human consumption. That said, sablefish expressed more PPARγ in white muscle (Fig.2.9.), which is most evident relative to protein translation capacity of the tissue (Fig.2.9.B). This implies a significant degree of lipid uptake in white muscle for metabolism or storage. That PPARγ is expressed more in sablefish white muscle for greater lipid uptake and storage is plausible, given that sablefish is prized for its flavorful, lipid rich meat. When PPARγ is expressed relative to 18S (Fig.2.9.A) transcription peaks in white muscle at T6, coinciding with the timeline of fiber maturation. We recognize both intramuscular adipocytes and myofibers could express PPARγ and thus cannot be certain of the cell type origin of this signal. Nevertheless, the timing of PPARγ upregulation in white muscle supports the maturation of the tissue no matter the cell source and

appears to be a sign of sufficient nutrition and energy availability for anabolism that is an important component of maturation. The increase in PPAR gamma at T6 is likely to correlate with an increase in muscle lipid content. As in salmon (Shearer et al., 1994), sablefish gradually accumulate lipid as they transition from juveniles to adults. Less PGC-1α is expressed in the white muscle (discussed below) to co-activate PPARγ for mitochondrial biogenesis or lipid catabolism, implying lipid accumulation would require PPARγ to bind a different cofactor in white muscle. In contrast, the greater expression of PGC-1α in red muscle suggests greater interaction with PPARγ in oxidative muscle tissue which would appear to support the lipid catabolism and mitochondrial biogenesis known to fuel the oxidative phenotype. Similar to other vertebrates, it appears sablefish PPARγ has retained a role in lipid storage and metabolic homeorrhesis. Its regulation and expression profiles hint at an important role in sablefish muscle as hypothesized.

PGC-1α is a key metabolic regulator and is essential for the establishment and maintenance of the oxidative phenotype, while also being implicated in fiber type switching (Lin et al., 2002). PGC-1α may also be a principal mediator of neuronal signaling in active skeletal muscle (Sandri 1996). It's abundant transcriptional expression in oxidative fibers and muscles transcriptionally activates mitochondrial biogenesis, oxidative metabolism, and prevents muscle atrophy (Sandri et al., 2006). As expected, sablefish preferentially express more PGC-1α in red muscle than white muscle (Fig.2.10.A-C). Coinciding with our proposed timeline of fiber maturation at T6 in both muscle types, PGC-1α was upregulated at T6 in red muscle but not in white muscle (Fig.2.10.A, B) suggesting PGC-1α could be a maturation factor in red muscle. This would be consistent with its ability to drive transcription of components necessary for oxidative metabolism and enhance PPARγ activity. After upregulating PGC-1α at T6, red muscle sustained transcriptional levels, implying it is an important factor in the maintenance of the red muscle in sablefish as has been reported in other vertebrate species. In contrast, expression in white muscle seemed to oscillate with environmental conditions, as we observed greater expression during winter months (T0, T12) than the spring and summer months (T6, T16). These results might suggest improved nutritional and energy status in the white muscle at cooler temperatures, but could also be positively influenced by lower temperatures. The divergence in tissue type and temporal control of PGC-1α transcription in sablefish would strongly suggest a role consistent with directing the metabolic scope of skeletal muscle tissue documented in mammals.

The somatotropic axis and IGF signaling establishes an appropriate growth response based on available energy and the plane of nutrition. Although primary IGF-1 production occurs in the liver, the IGF-1 expression in white muscle of fish has been demonstrated to be more closely tied to nutritional condition than hepatic expression (Fukada et al., 2012) and its autocrine effects are critical for protein accretion. We observed greater levels of IGF-1 transcription in red muscle than white muscle

(Fig.2.11.). It is difficult to interpret this muscle type difference without knowing other confounding factors that modulate the propagation of this growth factor (including circulating IGF-1 and IGF-2, binding proteins, and receptor availability). Since systemic IGF-1 is closely linked to the diurnal rhythm of growth hormone, the IGF-1 transcript abundance just gives us an estimate of the skeletal muscle autocrine and paracrine conditions. Interestingly, both white and red muscle IGF-1 expression was concordantly regulated, though at different magnitudes, under nearly the same pattern. It seems IGF-1 expression tended to decline as fish grew, but possibly with some influence by seasonal conditions (Fig.2.11.). This would be understandable since temperature and photoperiod can influence growth hormone, binding protein and fish appetite and the according nutrient availability. Similar to PGC-1α in white muscle, greater IGF-1 expression was observed in both muscle types during the winter months. Sablefish grown at this facility are believed to have strong appetites in the colder winter months (T0, T12) and in the warm conditions at the growth stage corresponding to T6, but large fish may exhibit appetite suppression to some degree in warm conditions, corresponding to T16. This expression pattern of IGF-1 may still be in line with what would be expected under the changing plane of nutrition, supporting anabolic growth when nutrients are plentiful. More work is necessary to discover the species-specific complexities of the IGF axis and tease apart the confounding effects of fluctuating environmental conditions for the expression of this gene to function as a robust marker of nutritional status.

Fish sex and muscle gene expression

Previously, Luckenbach et al. (2017) found that during culture the phenotypic female sablefish (control XX, 17β-estradiol treated XX and XY) begin to outgrow the male phenotype (control XY, 17α-methyl-testosterone treated XX and XY) before 50 – 55 cm fork length (<2 kg), and prior to the onset of slowed growth and the targeted window for harvest (2 - 2.5 kg). Females in the wild also outgrow males, reaching greater max body lengths in a given management region (Echave et al., 2012), which may be because females travel greater distances (16% farther than males) and thus have a wider forage base (Morita et al., 2012). Furthermore, industry members report sexual divergence during the expensive feeding and grow out phase, making monoculture of the faster growing sex more economical and a priority for research and development (Luckenbach et al., 2017). Altogether, these findings were the impetus for Luckenbach and Fairgrieve to formally document sexual dimorphic growth in sablefish. Using a non-lethal genetic sex marker, their work will characterize the timing of onset and extent of divergence during co-culture, and investigate different physiological systems that could drive divergence. The muscle gene expression reported here originates from a subset of fish used in their research, which has afforded us the opportunity to investigate the effect sex on muscle gene expression.

Given the likelihood our samples spanned the onset of sexually divergent growth, we examined the overall effect of sex on gene expression and tested for an interaction between sex and time (Tables 2.5., 2.6.). Our analysis found few differences by sex alone in either white or red muscle. More commonly, there were significant interactions between sex and time. Many differences were present between sexes in the large fish at T16 in both muscle types. This timing of divergence in the molecular regulation of muscle at this body size (over 50 cm) coincides with the expected timing when faster female growth might be present as greater body length or weight than males. In this context, the expression profiles might support the hypothesis that female sablefish grow faster because of better nutrition. By T16 in the white muscle, females have greater expression of nutritionally sensitive markers PPARγ, PGC-1α, IGF-1, suggesting females are in better anabolic conditions than males. Better anabolic condition in females is further supported by greater transcription of eIF4E, signaling improved mRNA translation. It could be that in grow out conditions with unrestricted access to feed females eat more than males. Future work will have to determine whether this is the case. Our results also provide some indication females have greater Pax7 and Pax3b expression, implying larger SC pool from which to draw in females at T16. Our understanding of the sex regulation of SCs is limited. A study in mice suggests female derived SCs proliferated slower than male SCs, but cycled longer and generated more cells before differentiating (Deasy et al., 2007). This supported more efficient muscle regeneration by female SCs when transplanted into the mouse model of Duchenne muscular dystrophy. The sex differences in sablefish gene expression of nutrient sensors, markers of satellite cells, and myogenic regulatory factors appear to match with sexual divergent growth observed in this species. This research underscores the need to better understand sex based regulation of myogenesis.

Concluding remarks

We developed tools to measure expression of genes in sablefish with known key roles in vertebrate muscle growth and metabolism. Our assessment of these genes in red and white muscle during growth suggests conserved functioning of the SC marker, Pax7, the network of myogenic regulatory factors Myf5, Myod1/2, MRF4, Myog, and the nutrient and energy sensors PPARγ, PGC-1α and IGF-1, in accordance with observations in other species. All genes were differentially regulated by muscle phenotype, in support of the increased maintenance demands of red muscle and greater growth of white muscle. Different temporal expression profiles of the muscle types each indicated maturation of muscle in fish between 40 - 50 cm fork length and weighing approximately 1 kg. The ratio of Pax7/Myod1 and Pax7/Total Myod mRNA correlated to SGR and has potential to be a valuable screening metric to develop new genetic sablefish strains for aquaculture with improved muscle growth and to evaluate growth conditions and diets that support optimal growth. Our results additionally indicate fish sex influences muscle gene expression in this sexually dimorphic species,

which warrants further evaluation in the context of monosex aquaculture production. Taken together, this work contributes to the knowledge base of vertebrate myogenesis by broadening the taxonomic scope of myogenic transcription to a large bodied, fast growing finfish from a unique family, *Anoplopomatidae*. This work advances our understanding of the molecular basis of development of fast-white and slow-red muscle in a commercially important species with an indirect life history. This characterization will assist in the assessment and design of production measures meant to optimize sablefish growth and product quality. This work has effectively and substantively advanced our capacity to strategically improve, and optimize our sablefish aquaculture industry towards efficient production of a high-quality, high-value and healthy flesh for the human consumption market.

Figures

Figure 2.1. Sablefish length and weight significantly increased during growth period, while SGR by length and weight showed the opposite and significant trend.

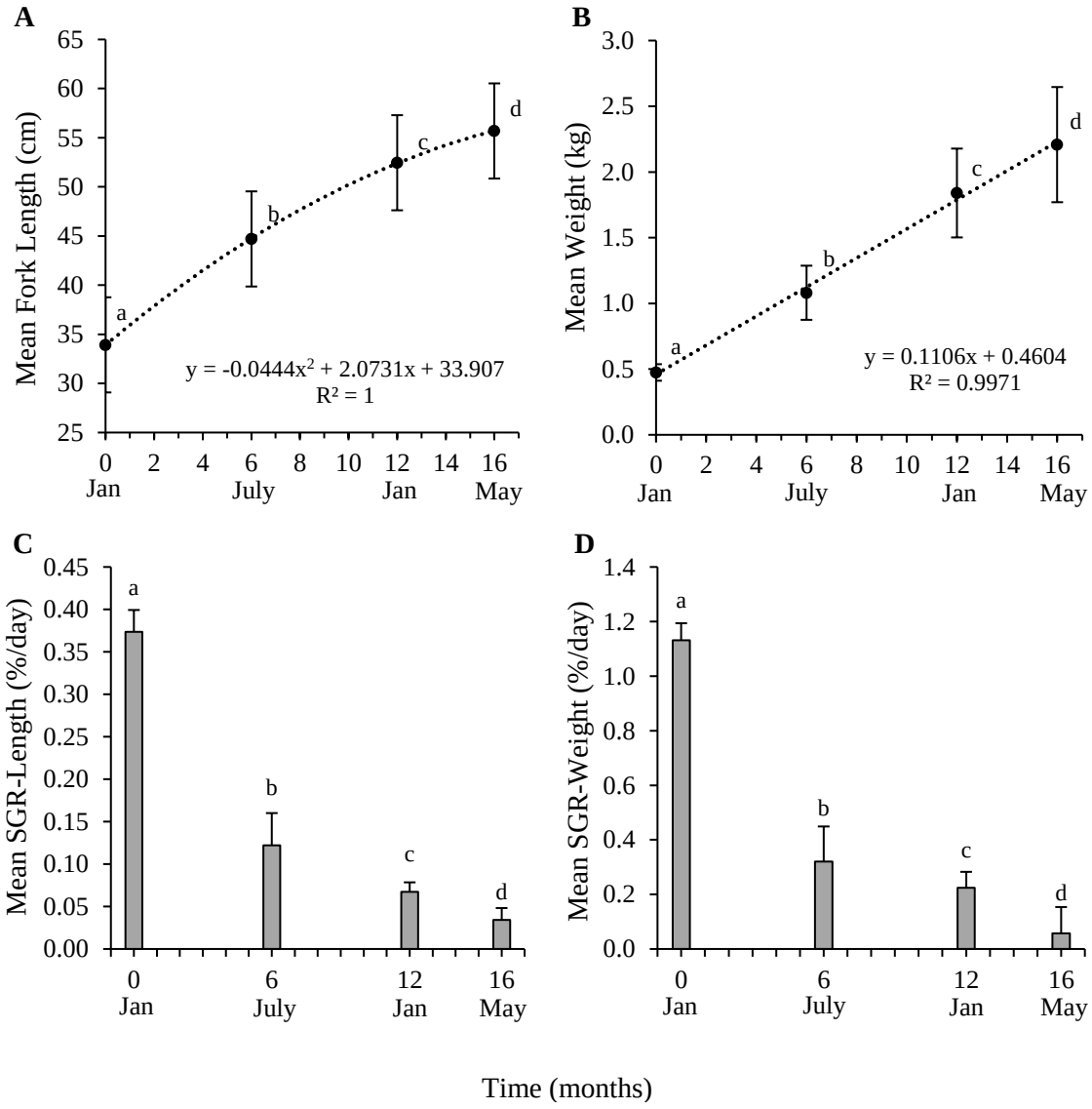

Figure 2.1. Growth of cultured sablefish over 16 months. Fish were reared in common garden fashion under ambient conditions and fed to excess. Mean A) fork length cm and B) weight kg are shown for lethally sampled fish at T0 (n= 40), T6 (n=40), T12 (n=43) and T16 (n=40) months of growth in this study. For the same fish, specific growth rate (SGR) was calculated by C) fork length and D) weight using $(\ln(\text{cm or kg})_{final} - \ln(\text{cm or kg})_{initial} / \text{Time}_{final} - \text{Time}_{initial})*100$. SGRs reflect two months of growth prior to lethal sampling, except for T0, which reflects 3 months of growth following PIT-tagging. Results are reported as mean ± standard deviation. Different letters indicate significant differences at $p \leq 0.05$ (ANOVA, Tukey's HSD test). Dotted lines and equations in (A, B) represent the line of best fit.

Figure 2.2. Eukaryotic initiation factor 4E (eIF4E) expression is greater in red muscle and changes in white and red muscle during sablefish grow out.

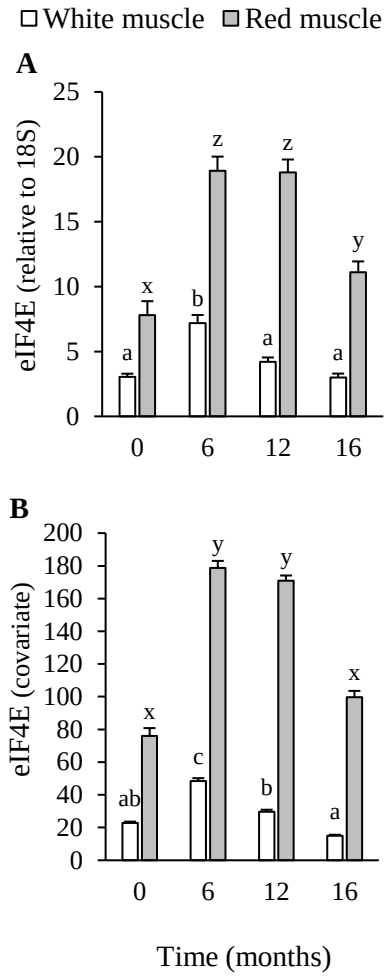

Figure 2.2. Relative mRNA expression of eIF4E in sablefish white and red muscle at four time points spanning 16 months of the grow out period. Mean fish weight at Time 0 (January) was 0.475 ± 0.063 kg (mean ± SD). Fish were reared in common garden fashion under ambient conditions and fed to excess. Tissues were analyzed separately by time after normalizing target gene expression using different methods, A) ΔCt was calculated from 18S endogenous control and B) by incorporating 18S as a covariate (ANOVA) in the statistical model. Error bars of LSmeans represent the numerical coefficient of variation. Time points within a muscle type that differed significantly at $p \leq 0.05$ are represented by different letters (Tukey's HSD test).

Figure 2.3. Paired homeobox 7 (Pax7) and 3 (Pax3b) expression is greater in red muscle and changes during sablefish grow out.

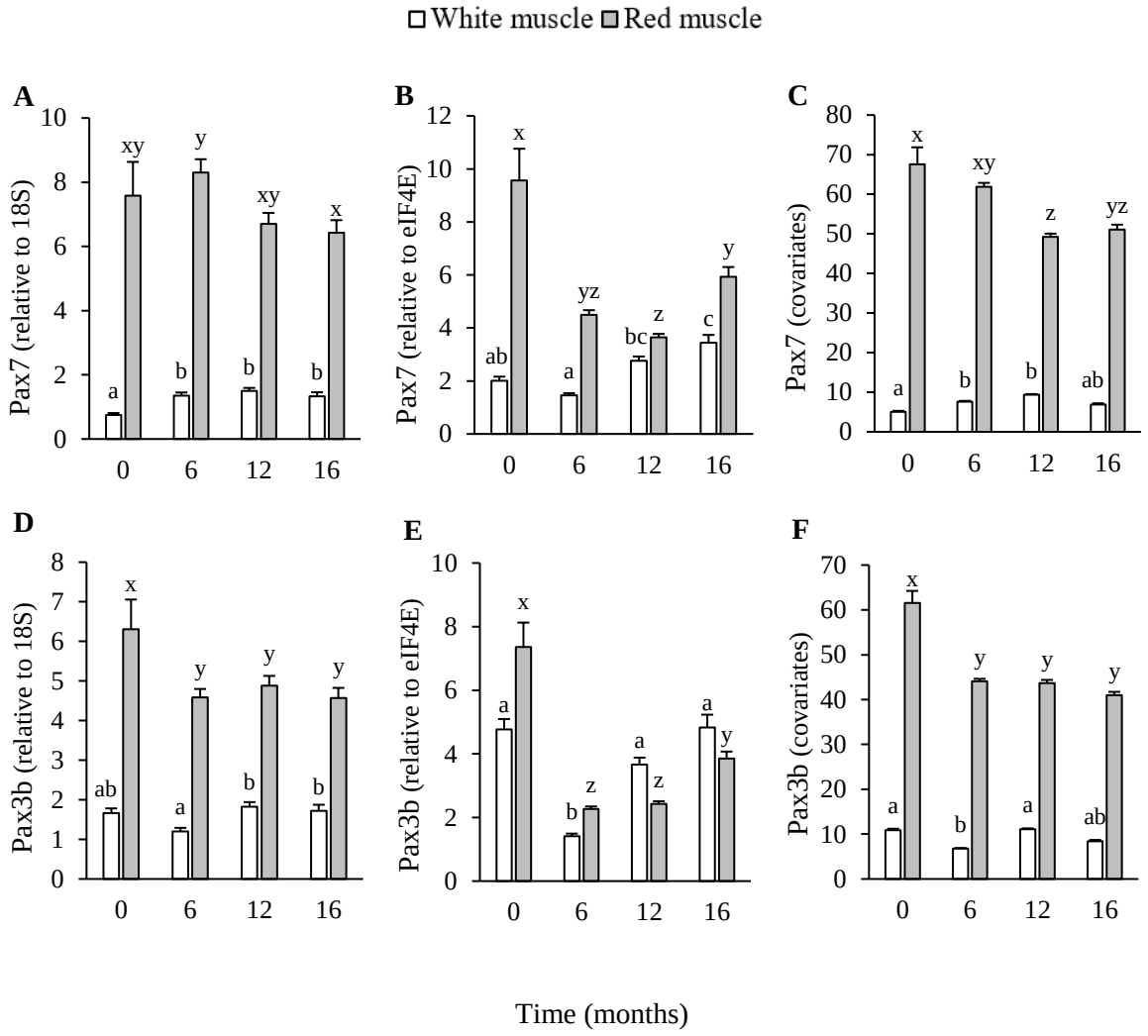

Figure 2.3. Relative mRNA expression of Pax7 (A-C) and Pax3b (D-F) in sablefish white and red muscle at four time points spanning 16 months of the grow out period. Mean fish weight at Time 0 (January) was 0.475 ± 0.063 kg (mean ± SD). Fish were reared in common garden fashion under ambient conditions and fed to excess. Tissues were analyzed separately by time after normalizing target gene expression using different methods and endogenous control genes, A) & D) ΔCt calculated from 18S, B) & E) ΔCt calculated from eIF4E, and C) & F) by incorporating 18S and eIF4E as covariates (ANOVA) in the statistical model. Error bars of LSmeans represent the numerical coefficient of variation. Time points within a muscle type that differed significantly at $p \leq 0.05$ are represented by different letters (Tukey's HSD test). Pax3a expression was not detected.

Figure 2.4. Paired homeobox 7 (Pax7) and 3 (Pax3b) expression in white muscle changes during sablefish grow out.

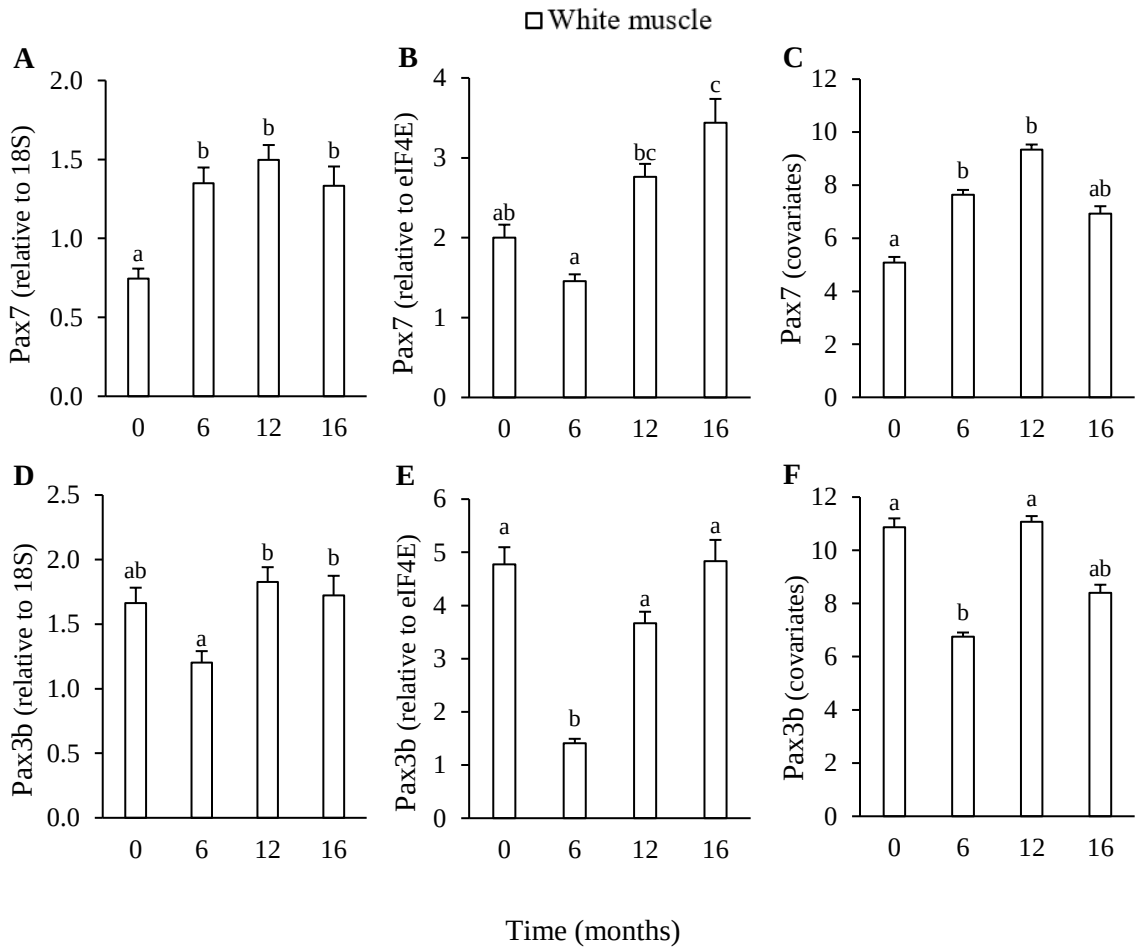

Figure 2.4. Relative mRNA expression of Pax7 (A-C) and Pax3b (D-F) in sablefish white muscle at four time points spanning 16 months of the grow out period. Mean fish weight at Time 0 (January) was 0.475 ± 0.063 kg (mean ± SD). Fish were reared in common garden fashion under ambient conditions and fed to excess. White muscle was analyzed by time after normalizing target gene expression using different methods and endogenous control genes, A) & D) ΔCt calculated from 18S, B) & E) ΔCt calculated from eIF4E, and C) & F) by incorporating 18S and eIF4E as covariates (ANOVA) in the statistical model. Error bars of LSmeans represent the numerical coefficient of variation. Time points within a muscle type that differed significantly at $p \leq 0.05$ are represented by different letters (Tukey's HSD test). Pax3a expression was not detected.

Figure 2.5. Myogenic determination factor 1 and 2 (Myod1, Myod2) expression is greater in white muscle and changes in each muscle type during sablefish grow out.

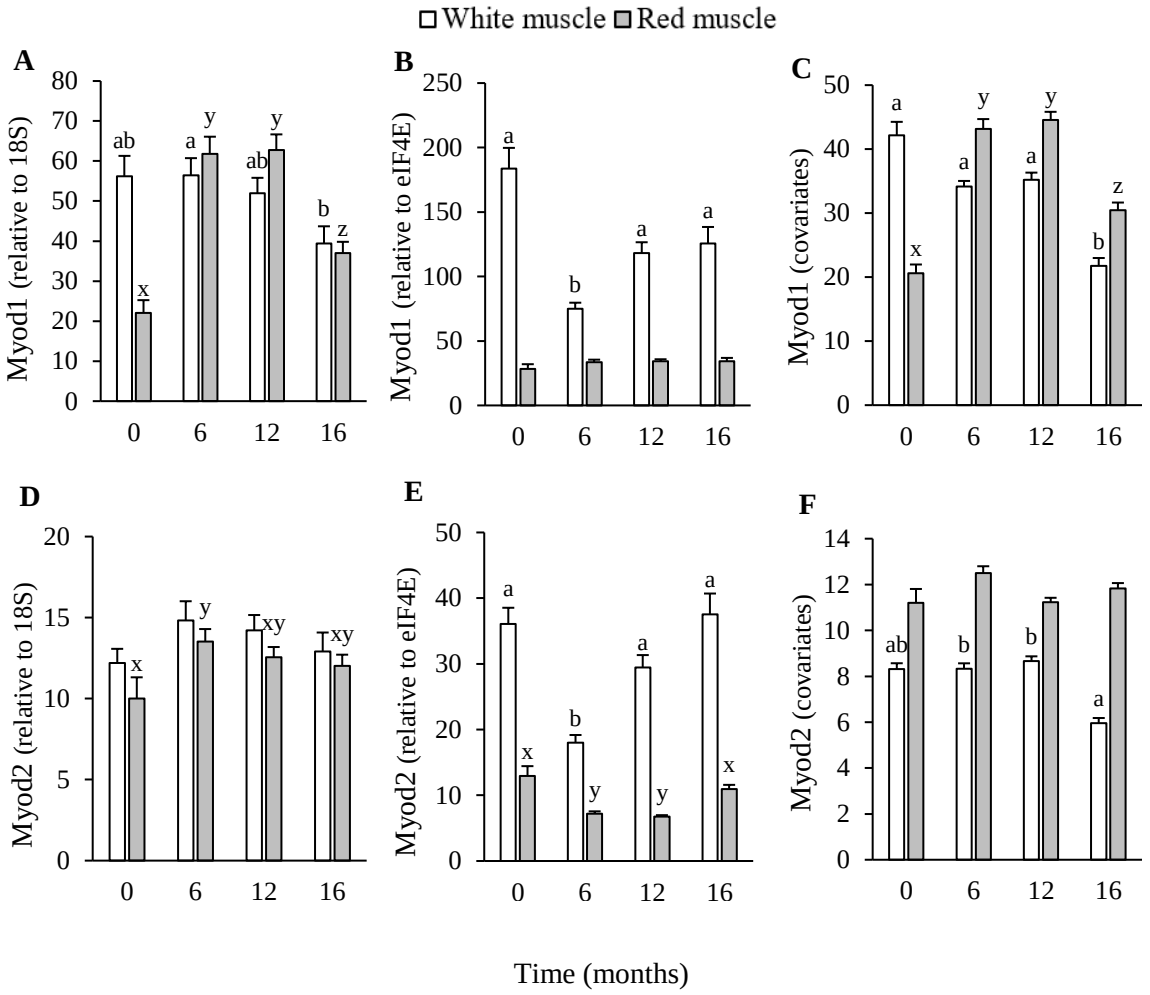

Figure 2.5. Relative mRNA expression of Myod1(A-C) and Myod2 (D-F) in sablefish white and red muscle at four time points spanning 16 months of the grow out period. Mean fish weight at Time 0 (January) was 0.475 ± 0.063 kg (mean ± SD). Fish were reared in common garden fashion under ambient conditions and fed to excess. Tissues were analyzed separately by time after normalizing target gene expression using different methods and endogenous control genes, A) & D) ΔCt calculated from 18S, B) & E) ΔCt calculated from eIF4E, and C) & F) by incorporating 18S and eIF4E as covariates (ANOVA) in the statistical model. Error bars of LSmeans represent the numerical coefficient of variation. Time points within a muscle type that differed significantly at $p \leq 0.05$ are represented by different letters (Tukey's HSD test).

Figure 2.6. The ratio of Paired homeobox 7 (Pax7) expression to that of either Myogenic determination factor paralogues, Myod1 or Myod2, or their sum (Total Myod) is greater in red muscle and changes in both muscle types during sablefish grow out.

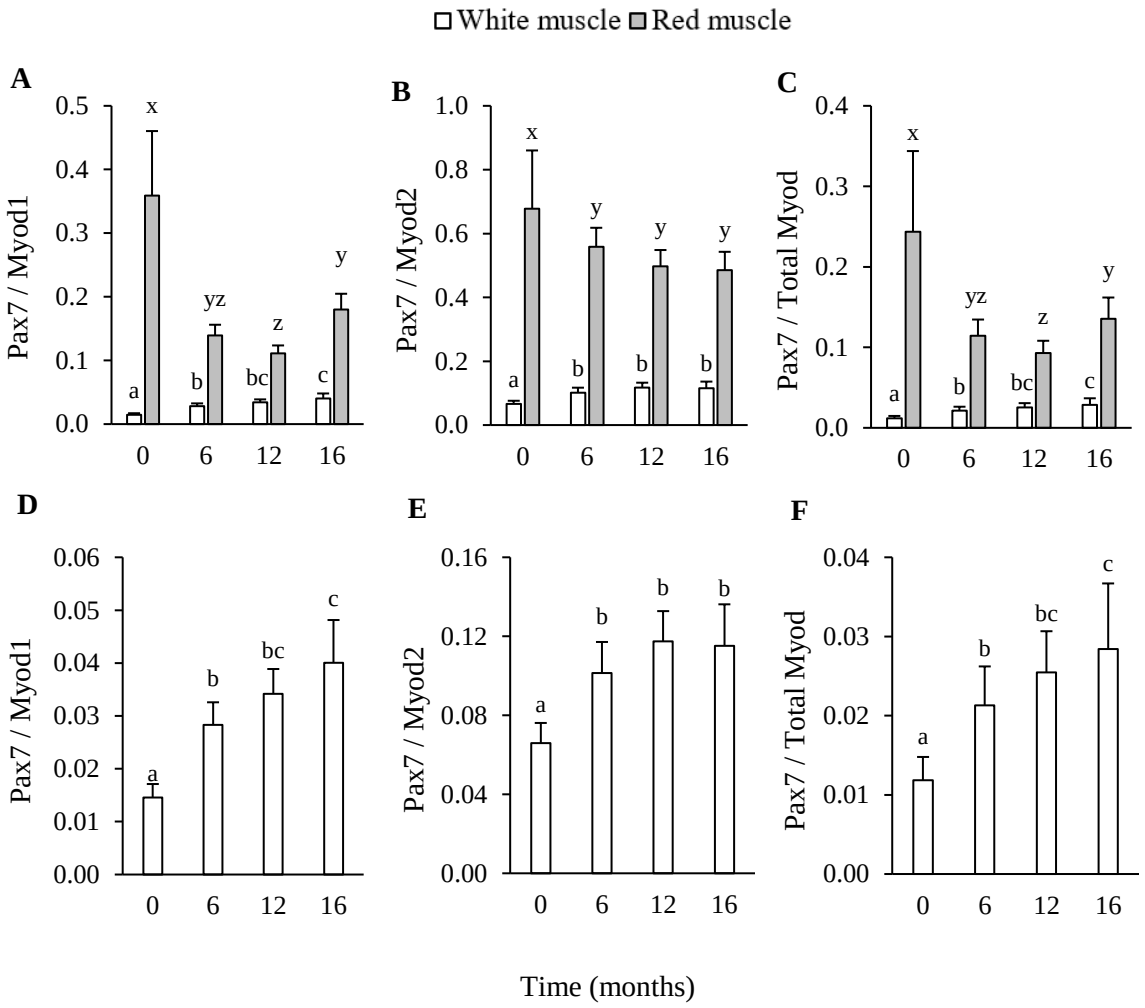

Figure 2.6. The ratio of the relative mRNA expression of Pax7 mRNA to Myod in sablefish white and red muscle (A-C) and in white muscle (D-F) at four time points spanning 16 months of the grow out period. Mean fish weight at Time 0 (January) was 0.475 ± 0.063 kg (mean ± SD). Fish were reared in common garden fashion under ambient conditions and fed to excess. Tissues were analyzed separately by time. Total Myod and gene ratios were calculated using the ΔCt of target gene expression relative to the 18S endogenous control gene. Error bars of LSmeans represent the numerical coefficient of variation. Time points within a muscle type that differed significantly at $p \leq 0.05$ are represented by different letters (Tukey's HSD test).

Figure 2.7. Myogenic factor 5 (Myf5) expression is regulated differently by muscle type and during growth, depending on the method of normalization. The ratios of Myf5 to Myod1 or Myod2, or their sum (Total Myod) are greater in red muscle and follow a tissue specific pattern during growth.

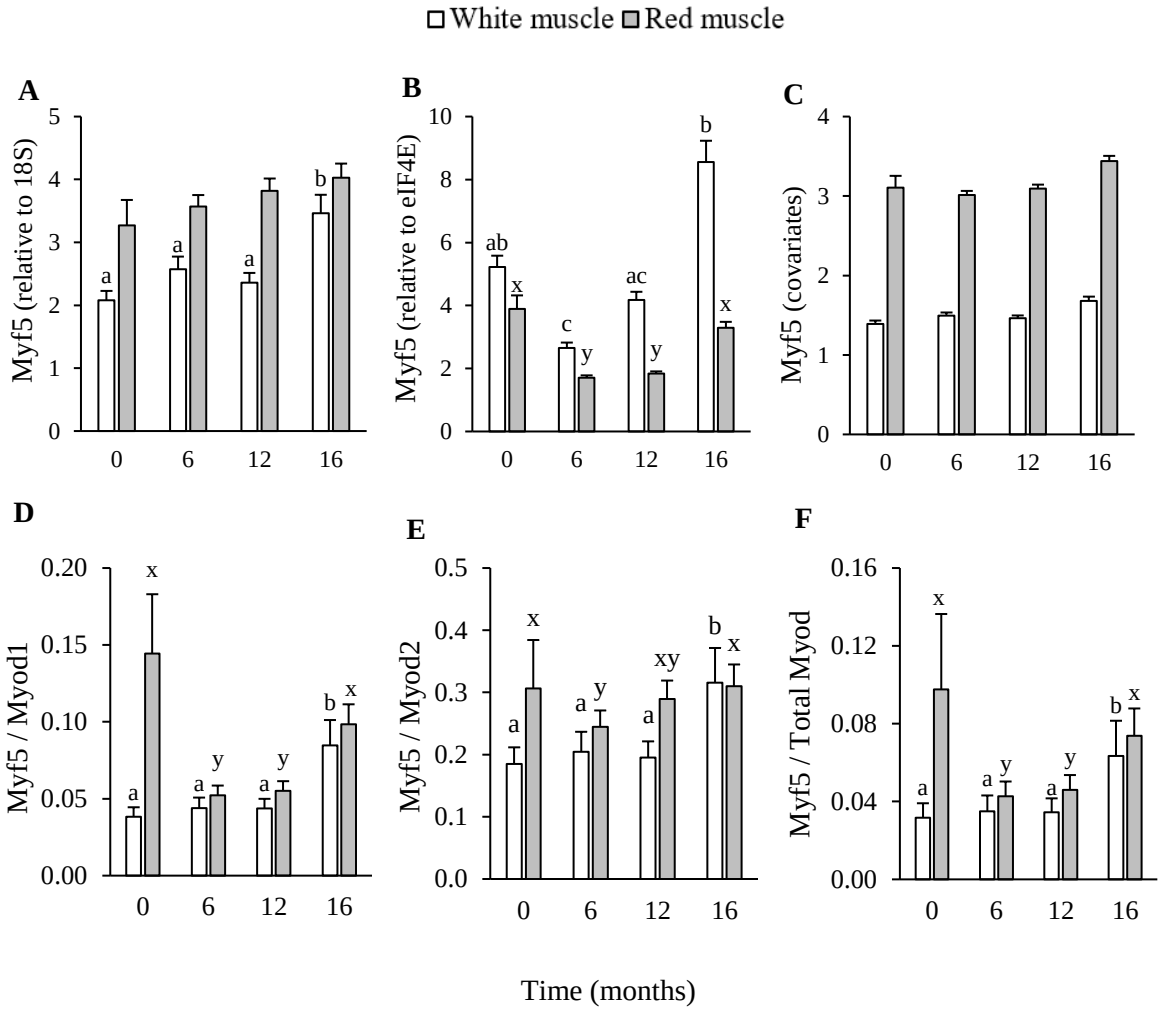

Figure 2.7. Relative mRNA expression of Myf5 (A-C) and the ratio of Myf5 to Myod1(D), Myod2 (E), and Total Myod (F) in white and red muscle at four time points spanning 16 months of the grow out period. Mean fish weight at Time 0 (January) was 0.475 ± 0.063 kg (mean ± SD). Fish were reared in common garden fashion under ambient conditions and fed to excess. Tissues were analyzed separately by time after normalizing target gene expression using different methods and endogenous control genes, A) ΔCt calculated from 18S, B) ΔCt calculated from eIF4E, and C) by incorporating 18S and eIF4E as covariates (ANOVA) in the statistical model. Total Myod and gene ratios (D-F) were calculated after normalizing to 18S by calculating the ΔCt. Error bars of LSmeans represent the numerical coefficient of variation. Time points within a muscle type that differed significantly at $p \leq 0.05$ are represented by different letters (Tukey's HSD test).

Figure 2.8. Myogenic regulatory factor 4 (MRF4) and Myogenin (Myog) are greater in red muscle, and both exhibit similar regulation in each tissue during sablefish growth.

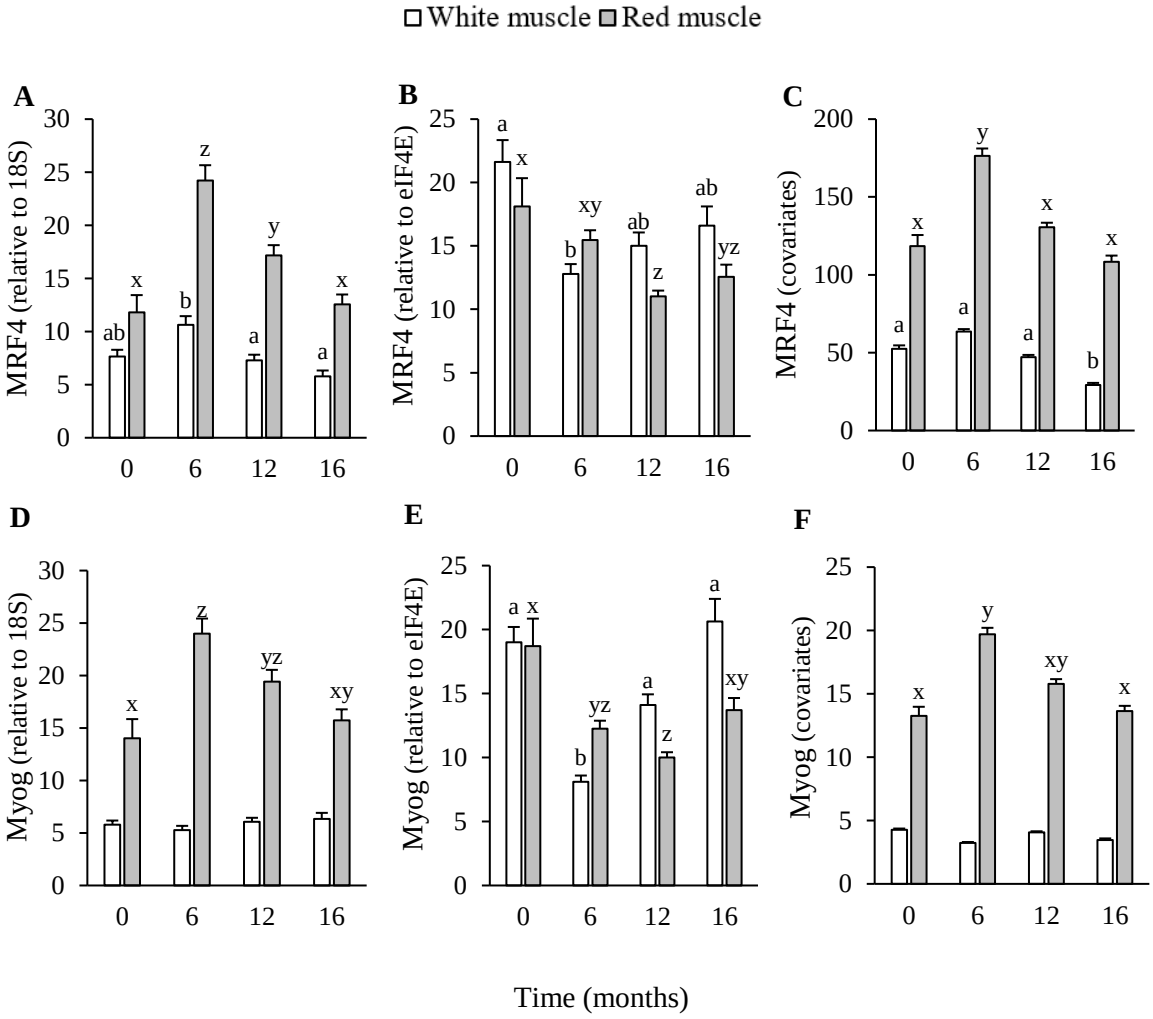

Figure 2.8. Relative mRNA expression of MRF4 (A-C) and Myog (D-F) in sablefish white and red muscle at four time points spanning 16 months of the grow out period. Mean fish weight at Time 0 (January) was 0.475 ± 0.063 kg (mean ± SD). Fish were reared in common garden fashion under ambient conditions and fed to excess. Tissues were analyzed separately by time after normalizing target gene expression using different methods and endogenous control genes, A) & D) ΔCt calculated from 18S, B) & E) ΔCt calculated from eIF4E, and C) & F) by incorporating 18S and eIF4E as covariates (ANOVA) in the statistical model. Error bars of LSmeans represent the numerical coefficient of variation. Time points within a muscle type that differed significantly at $p \leq 0.05$ are represented by different letters (Tukey's HSD test).

Figure 2.9. Peroxisome proliferator activated receptor gamma (PPARγ) expression is greater in white muscle and changes during sablefish growth.

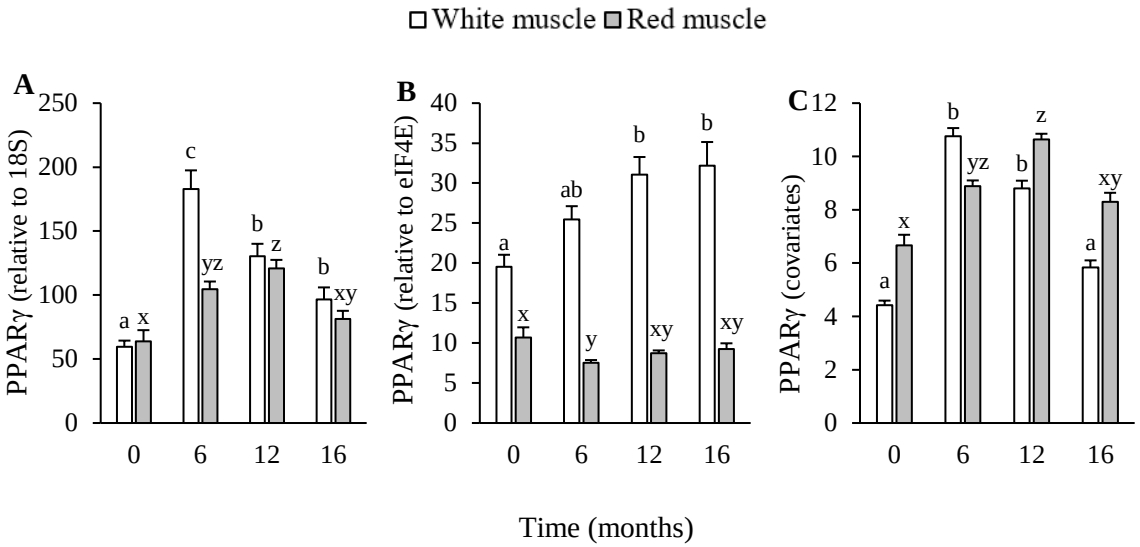

Figure 2.9. Relative mRNA expression of PPARγ (A-C) in sablefish white and red muscle at four time points spanning 16 months of the grow out period. Mean fish weight at Time 0 (January) was 0.475 ± 0.063 kg (mean ± SD). Fish were reared in common garden fashion under ambient conditions and fed to excess. Tissues were analyzed separately by time after normalizing target gene expression using different methods and endogenous control genes, A) ΔCt calculated from 18S, B) ΔCt calculated from eIF4E, and C) by incorporating 18S and eIF4E as covariates (ANOVA) in the statistical model. Error bars of LSmeans represent the numerical coefficient of variation. Time points within a muscle type that differed significantly at $p \leq 0.05$ are represented by different letters (Tukey's HSD test).

Figure 2.10. Peroxisome proliferator activated receptor gamma coactivator - 1α (PGC-1α) expression is greater in red muscle and exhibits different patterns of regulation by muscle type during sablefish growth.

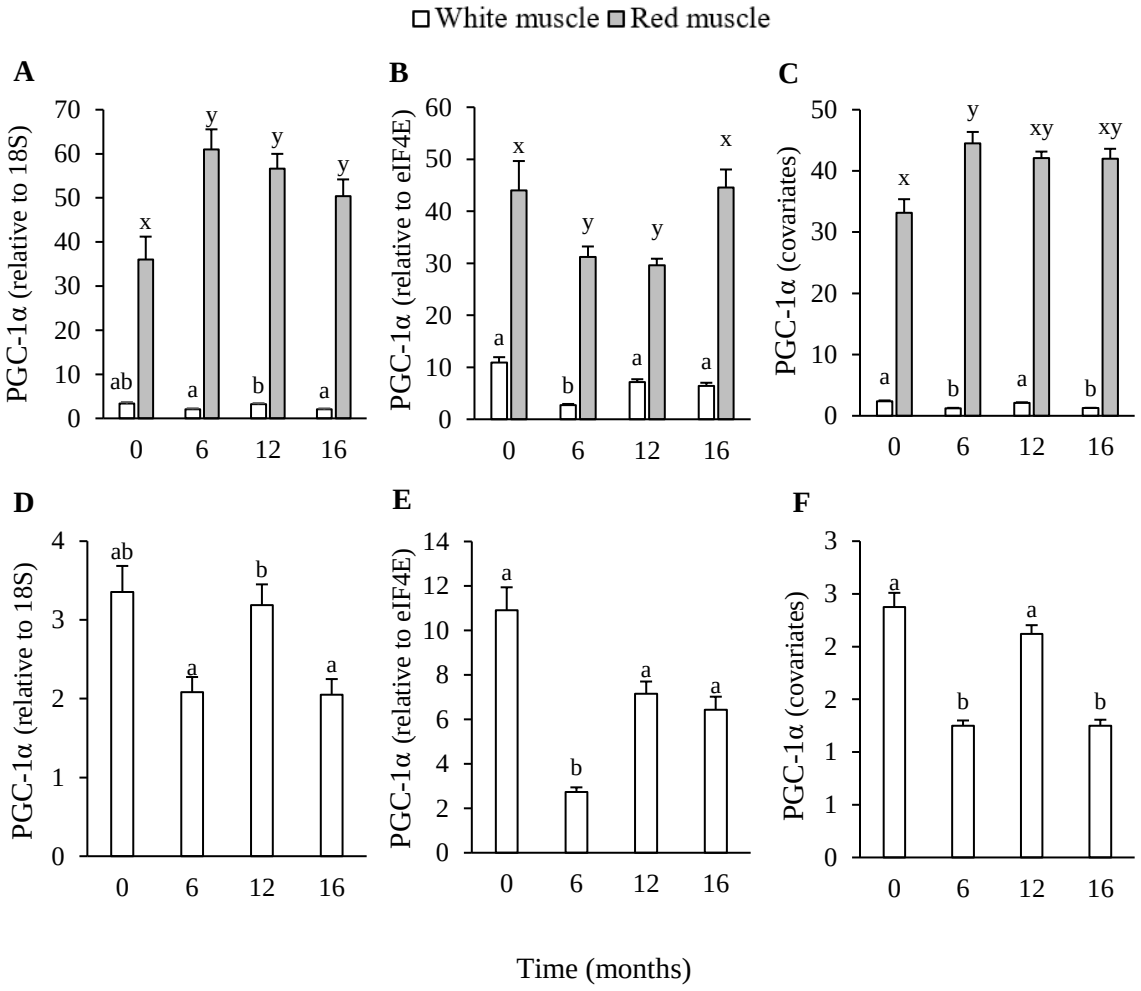

Figure 2.10. Relative mRNA expression of PGC-1α in white and red muscle (A-C) or only white muscle (D-F) of sablefish at four time points spanning 16 months of the grow out period. Mean fish weight at Time 0 (January) was 0.475 ± 0.063 kg (mean ± SD). Fish were reared in common garden fashion under ambient conditions and fed to excess. Tissues were analyzed separately by time after normalizing target gene expression using different methods and endogenous control genes, A) & D) ΔCt calculated from 18S, B) & E) ΔCt calculated from eIF4E, and C) & F) by incorporating 18S and eIF4E as covariates (ANOVA) in the statistical model. Error bars of LSmeans represent the numerical coefficient of variation. Time points within a muscle type that differed significantly at $p \leq 0.05$ are represented by different letters (Tukey's HSD test).

Figure 2.11. Insulin-like growth factor 1 (IGF-1) is expressed higher in red muscle and changes concordantly in white and red muscle during sablefish growth.

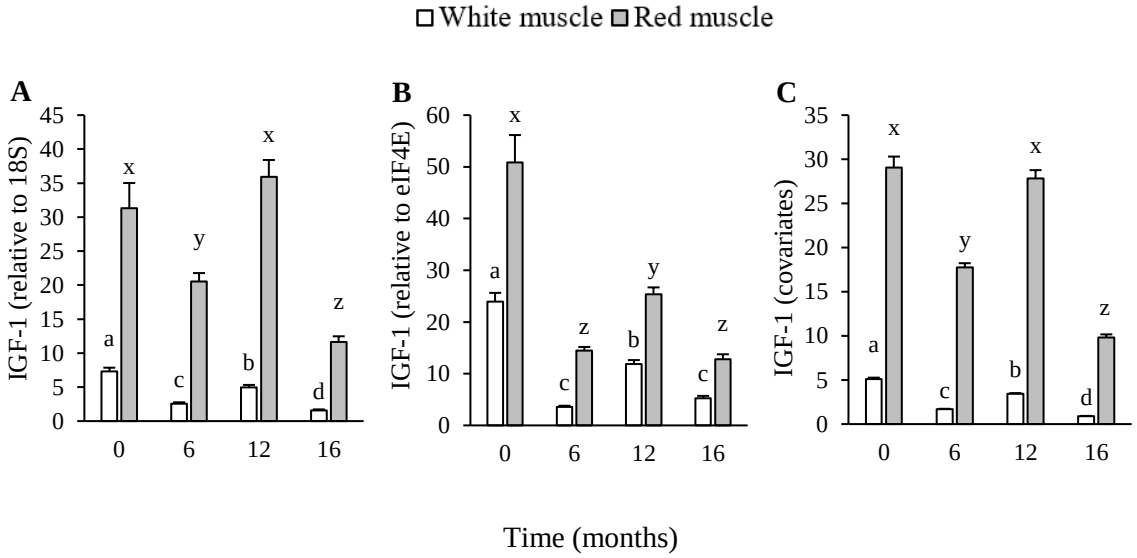

Figure 2.11. Relative mRNA expression of IGF-1 (A-C) in sablefish white and red muscle at four time points spanning 16 months of the grow out period. Mean fish weight at Time 0 (January) was 0.475 ± 0.063 kg (mean ± SD). Fish were reared in common garden fashion under ambient conditions and fed to excess. Tissues were analyzed separately by time after normalizing target gene expression using different methods and endogenous control genes, A) ΔCt calculated from 18S, B) ΔCt calculated from eIF4E, and C) by incorporating 18S and eIF4E as covariates (ANOVA) in the statistical model. Error bars of LSmeans represent the numerical coefficient of variation. Time points within a muscle type that differed significantly at $p \leq 0.05$ are represented by different letters (Tukey's HSD test).

Tables

Table 2.1. Accession numbers of sequences used in sablefish TaqMan assay design of myogenic genes

Gene	Species	Gene	Species
Pax7	*Anoplopoma fimbria* AWGY01143204 *Oryzias latipes* NM_001305635 *Danio rerio* NM_131325 *Sternopygus macrurus* EU624121 *Paralichthys olivaceous* KP323410, KP323416 *Gasterosteus aucelatus* ENSGACT00000017105, ENSGACT00000017120 *Solea senegalensis* JQ001819 *Tetraodon nigridoviridis* AY727905 *Takifugu rubripes* ENSTRUT00000019602, ENSTRUT00000019603 *Sparus aurata* JN034418 *Salvelinus alpinus* AJ634764 *Maylandia sp*. GQ403984	Myod1	*Anoplopoma fimbria* AWGY01039399 *Takifugu rubripes* NM_001032769 *Sparus aurata* AF478568 *Paralichthys olivaceous* DQ184914 *Gasterosteus aculeatus* ENSGACT00000011189 *Hippoglossus hippoglossus* AY999688 *Notothenia coriiceps* XM_010766781 *Danio rerio* Z36945 *Ictalurus punctatus* AY534328 *Ictalurus furcatus* AY562555
Pax3a	*Anoplopoma fimbria* AWGY01186777 *Gasterosteus aculeatus* ENSGACP00000018514 *Fugu rubripes* ENSTRUP00000034277 *Gadus morhua* ENSGMOT00000006726 *Oryzias latipes* ENSORLT00000019948 *Paralichthys olivaceous* KM675813 *Solea senegalensis* HM100237 *Danio rerio* NM_131277, BC076069 *Tropheops sp*. KM272327 *Labeotropheus fuelleborni* KM272328	Myod2	*Anoplopoma fimbria* AWGY01067649 *Hippoglossus hippoglossus* AJ630127 *Takifugu rubripes* NM_001040062 *Larimichthys crocea* NM_001303333 *Epinephelus coioides* HM190250 *Oreochromis aureus* NM_001279721 *Sparus aurata* AF478569 *Gasterosteus aculeatus* ENSGACT00000022964 *Solea senegalensis* FJ009108
Pax3b	*Anoplopoma fimbria* AWGY01097711 *Fugu rubripes* ENSTRUP00000023137 *Oryzias latipes* ENSORLT00000011327 *Paralichthys olivaceous* KM675815, KM675817	MRF4	*Anoplopoma fimbria isotig** *Fugu rubripes* AJ308546 *Sparus aurata* JN034421 *Danio rerio* AY335193 *Trachidermus fasciatus* JQ905628 *Epinephelus coioides* HM190248 *Tetraodon nigroviridis* AY576806
Myf5	*Anoplopoma fimbria* AWGY01133278 *Lateolabrax japonicas* DQ407725 *Marone saxatilis* AF463525 *Fugu rubripes* AJ308546 *Tetraodon nigroviridis* DQ453127 *Sander lucioperca* JF488073 *Notothenia coriiceps* XM010775479 *Epinephelus coioides* HM190249	Myog	*Anoplopoma fimbria* AWGY01002481 *Gasterosteus aculeatus* ENSGACG00000000349 *Hippoglossus hippoglossus* AJ487982 *Marone saxitilis* AF463526 *Sparus aurata* EF462191 *Takifugu rubripes* AY566282 *Tetraodon nigroviridis* AY576805 *Paralichthys olivaceus* EF144128 *Danio rerio* NM_131006 *Trachidermus fasciatus* JQ905626 *Epinephelus coioides* HM190251

* isotig obtained by 454 pyrosequencing, kindly provided by J.A.Luckenbach

Table 2.2. Accession numbers of sequences used in sablefish TaqMan assay design of reference and nutrient sensing genes

Gene	Species
18S	*Eukaryotic rRNA* AF243428
eIF4E	*Anoplopoma fimbria* BT082710 *Danio rerio* AF176317 *Danio rerio* AF176316 *Ictalurus punctatus* JT417680 *Mus musculus* NM007917
PPARγ	*Anoplopoma fimbria* JO674720 *Pachycara brachycephalum* HQ439170 *Takifugu rubripes* NM_001097627 *Epinephelus coioides* KM052849 *Sparus aurata* AY590304 *Paralichthys olivaceus* FJ262993 *Dicentrarchus labrax* AY590303 *Rachycentron canadum* DQ321817
PGC-1α	*Anoplopoma fimbria* JO661126 *Xiphias gladius* FJ710607 *Oncorhynchus mykiss* FJ710605 *Danio rerio* FJ710604 *Carassius auratus* FJ710611 *Acipenser transmontanus* FJ710613 *Amia calva* FJ710614
IGF-1	*Anoplopoma fimbria* AWGY01081561 *Danio rerio* NM_131825 *Hippoglossus hippoglossus* EU682415

Table 2.3. Primer and probe sequences for target gene quantification by qRT-PCR.

Gene	Efficiency (%)	R^2	Amplicon Size (bp)	Primer and Taqman Probe Sequences (5'→3')
18S rRNA	102	0.995	55	FWD: CCACGCGAGATTGAGCAAT REV: GCAGCCCCGGACATCTAA PRB: ACAGGTCTGTGATGCC
eIF4E	100	0.991	61	FWD: TGGAAGACTTTTGGGCATCAT REV: CAGCCAAAGCCAAGTTTGC PRB: CAACCACATACAGCAAC
Pax7	104	0.985	69	FWD: CATCCGACACAAGATAGTGGAGAT REV: CGCAGCTGTCGCGAGATTA PRB: CGGCATCCGACCCT
Pax3a	N/D	N/D	57	FWD: GAAGCAGCGTCGCAGTC REV: CTCTCCAGCTCCTCCAGC PRB: CACCACCTTCACAG
Pax3b	90	0.990	57	FWD: GAAGCAGCGTCGCAGTC REV: CTCTCCAGCTCCTCCAGC PRB: CACCACCTTCACGG
Myf5	99	0.995	56	FWD: TGCCCAAGGTGGAGATCCT REV: CTCCTGCAGGCTCTCGATGT PRB: CGCAACGCCATCC
Myod1	99	0.995	77	FWD: GGAGCTGTCGGATATCTCTTTCC REV: TGTCGCTGGTGTTGAAGCA PRB: CCGACGACTTCTACGACG
Myod2	98	0.998	74	FWD: CCGACCTTTCCTTCCCTCTCT REV: GTTCATGTCGCCCGTGC PRB: CCGATGACCTCTACGATG
Myog	96	0.992	62	FWD: GAAGGTGAACGAGGCCTTTG REV: GCCTCTGGTTCGGGTTCAT PRB: TGAAGAGGAGCACCC
MRF4	97	0.994	60	FWD: CCAGCCTCCTGCGTCTGT REV: TGTTGTTTTTATCGCCATTGGT PRB: CCATCGTGGACAGCAT
PPARγ	99	0.994	65	FWD: ATCTTCACTGTCGCATTCACAAG REV: GCACTTCTGGAAGCGACAGTACT PRB: TCCCGCAACAAATC
PGC-1α	96	0.998	58	FWD: CCTTTGCCGCCCTTGAG REV: TCGAACTGAGGCTCGTTTGA PRB: CGGACACACCTTACG
IGF-1	99	0.996	78	FWD: TGCGATGTGCTGTATCTCCTGTAG REV: CCTGTTGCCGTCGGAGTCA PRB: CACACCCTCTCACTACTG

Forward primer (FWD), reverse primer (REV), and Taqman MGB probe (PRB) sequences designed for sablefish (*Anoplopoma fimbria*). Assay efficiencies, correlation coefficients of the calibration curves, and amplicon lengths are shown. Pax3a was not detected (N/D).

Table 2.4. Pearson correlation coefficients of SGR by weight and the mRNA ratios of Pax7 to Myod1 or Total Myod in sablefish white muscle. Ratios were computed with gene expression normalized to 18S, then Log_e transformed.

	SGR (weight)	p-value
Pax7 / Myod1	- 0.627	< .0001
Pax7 / Total Myod	- 0.642	< .0001

Table 2.5. Indices of white muscle gene expression during sablefish growth

	2-way ANOVA			Sex Differences (2-tailed T-test)			
Gene (normalization method)	time	sex	time*sex	T0	T6	T12	T16
eIF4E (18S)	<.0001	0.0754	0.0143	0.7138	0.3150	0.3946	*0.0006*
eIF4E (18S covariate)	<.0001	*0.0078*	0.0006	0.5118	0.2563	0.2481	*<.0001*
Pax7 (18S)	<.0001	0.3894	-	0.8754	0.4655	0.4989	0.0736
Pax7 (eIF4E)	<.0001	0.1605	0.0203	0.8657	0.1088	0.1631	**0.0144**
Pax7 (18S & eIF4E covariates)	0.0004	0.1210	-	0.6686	0.4046	0.8462	*0.0065*
Pax3b (18S)	0.0014	0.8301	-	0.8572	0.6241	0.3738	0.3867
Pax3b (eIF4E)	<.0001	0.0589	-	0.8596	0.4600	0.1392	**0.0085**
Pax3b (18S & eIF4E covariates)	0.0001	0.5361	-	0.5804	0.7157	0.6711	*0.0350*
Myf5 (18S)	0.0003	0.4277	-	0.3071	0.2077	0.9420	0.1310
Myf5 (eIF4E)	<.0001	**0.0275**	0.0027	0.6774	0.6956	0.3634	**<.0001**
Myf5 (18S & eIF4E covariates)	0.3196	0.3402	-	-	-	-	-
Myod1 (18S)	0.0309	0.3788	-	0.2646	0.5025	0.3037	0.3596
Myod1 (eIF4E)	<.0001	0.1925	-	0.4418	0.2535	0.5523	0.0325
Myod1 (18S & eIF4E covariates)	0.0006	0.1150	-	0.9974	0.7794	0.2910	*0.0137*
Myod2 (18S)	0.3701	0.4189	-	-	-	-	-
Myod2 (eIF4E)	<.0001	**0.0100**	0.0302	0.7024	0.6191	**0.0488**	**0.0027**
Myod2 (18S & eIF4E covariates)	0.0041	0.7311	0.0276	0.5591	0.3856	0.2069	*0.0138*
Myog (18S)	0.3005	0.6011	-	-	-	-	-
Myog (eIF4E)	<.0001	0.2023	0.0052	0.8566	0.1226	0.2562	**0.0021**
Myog (18S & eIF4E covariates)	0.0340	0.1340	-	0.1620	0.3200	0.6407	*0.0103*
MRF4 (18S)	0.0002	0.2597	0.0166	0.6966	0.3455	0.6306	*0.0029*
MRF4 (eIF4E)	0.0157	0.2681	-	0.9681	0.7001	0.2155	0.2343
MRF4 (18S & eIF4E covariates)	<.0001	0.0533	0.0016	0.4881	0.2701	0.9152	*0.0002*
PPARγ (18S)	<.0001	0.0605	0.0180	0.6813	0.3637	0.5592	*0.0003*
PPARγ (eIF4E)	0.0003	0.4556	-	0.9999	0.5129	0.4730	0.1204
PPARγ (18S & eIF4E covariates)	<.0001	*0.0099*	0.0056	0.4743	0.3995	0.3661	*<.0001*
PGC-1α (18S)	0.0049	0.9221	-	0.4406	0.5362	0.6491	0.1069
PGC-1α (eIF4E)	<.0001	0.0859	0.0306	0.2559	0.1514	0.1759	**0.0284**
PGC-1α (18S & eIF4E covariates)	0.0005	0.6724	-	0.6189	0.5171	0.8968	0.0112
Igf-1 (18S)	<.0001	0.0701	-	0.3755	0.2538	0.4564	0.0824
Igf-1(eIF4E)	<.0001	0.7824	-	0.6752	0.1441	0.1666	0.2746
Igf-1 (18S & eIF4E covariates)	<.0001	*0.0176*	-	0.2543	0.2294	0.7820	*0.0056*
Pax7 / Myod1 (18S)	<.0001	0.8803	-	0.5312	0.5972	0.0948	0.8254
Pax7 / Myod2 (18S)	<.0001	*0.0372*	-	0.9093	0.1253	0.2846	0.2007
Pax7 / Total Myod (18S)	<.0001	0.7478	-	0.6347	0.4595	0.3172	0.8376
Myf5 / Myod1 (18S)	<.0001	0.1051	0.0092	0.2142	0.2838	0.5636	**0.0024**
Myf5 / Myod2 (18S)	<.0001	0.9993	0.0175	0.2978	0.9582	0.1472	**0.0069**
Myf5 / Total Myod (18S)	<.0001	0.1600	0.0106	0.2270	0.3518	0.9576	**0.0016**

Significant p-values are italicized if expression is greater in females and in bold if greater in males

Table 2.6. Indices of red muscle gene expression during sablefish growth

	2-way ANOVA			Sex Differences (2-tailed T-test)			
Gene (normalization method)	time	sex	time*sex	T0	T6	T12	T16
eIF4E (18S)	<.0001	0.1887	-	0.7880	0.6760	0.1318	*0.0442*
eIF4E (18S covariate)	<.0001	0.3424	-	0.5563	0.2322	*0.0192*	*0.0222*
Pax7 (18S)	0.0352	0.7331	-	0.1660	0.1704	0.7727	0.1786
Pax7 (eIF4E)	<.0001	0.2709	-	0.8048	0.0913	0.1604	0.1334
Pax7 (18S & eIF4E covariates)	0.0001	0.7252	0.0035	0.2239	0.4226	0.4149	*0.0237*
Pax3b (18S)	0.0032	0.1540	-	0.2837	0.8447	0.6225	0.2557
Pax3b (eIF4E)	<.0001	**0.0091**	-	0.4907	0.5393	**0.0422**	**0.0091**
Pax3b (18S & eIF4E covariates)	<.0001	0.0713	-	0.1819	0.3840	0.7957	0.7798
Myf5 (18S)	0.1770	0.7089	-	-	-	-	-
Myf5 (eIF4E)	<.0001	0.0641	-	0.8623	0.2718	0.1062	**0.0234**
Myf5 (18S & eIF4E covariates)	0.5108	0.8633	-	-	-	-	-
Myod1 (18S)	<.0001	0.8133	0.0142	**0.0241**	0.7975	0.3527	*0.0391*
Myod1 (eIF4E)	0.3079	0.4240	-	-	-	-	-
Myod1 (18S & eIF4E covariates)	<.0001	0.9849	0.0131	0.1137	0.8050	0.1721	*0.0077*
Myod2 (18S)	0.0331	0.6783	-	0.2631	0.5025	0.3037	0.3596
Myod2 (eIF4E)	<.0001	**0.0469**	-	0.4309	0.2023	**0.0107**	0.1550
Myod2 (18S & eIF4E covariates)	0.3914	0.1657	0.0052	**0.0436**	0.9228	0.3343	*0.0115*
Myog (18S)	<.0001	0.0676	-	0.4149	0.0641	0.1512	0.1349
Myog (eIF4E)	<.0001	0.7779	-	0.7889	*0.0262*	0.8486	0.2983
Myog (18S & eIF4E covariates)	0.0002	0.1641	0.0341	0.3591	0.1801	0.0617	*0.0318*
MRF4 (18S)	<.0001	0.9654	-	0.4113	0.3402	0.3736	0.2817
MRF4 (eIF4E)	<.0001	0.1583	-	0.4160	0.5302	0.5138	0.5584
MRF4 (18S & eIF4E covariates)	<.0001	0.4388	-	0.3140	0.0699	0.1363	0.0525
PPARγ (18S)	<.0001	0.9841	0.0430	0.0578	0.9524	0.6736	0.0604
PPARγ (eIF4E)	0.0007	0.1962	-	0.3328	0.5208	0.1122	0.4163
PPARγ (18S & eIF4E covariates)	<.0001	0.4373	0.0237	0.1512	0.5252	0.3919	*0.0342*
PGC-1α (18S)	<.0001	0.8545	0.0175	0.0838	0.9499	0.9932	*0.0072*
PGC-1α (eIF4E)	<.0001	0.1724	-	0.2661	0.5845	0.1583	0.9516
PGC-1α (18S & eIF4E covariates)	0.0178	0.6099	0.0023	0.1504	0.4559	0.7577	*0.0042*
Igf-1 (18S)	<.0001	0.1101	-	0.1346	0.2583	0.3286	0.0553
Igf-1(eIF4E)	<.0001	0.8450	-	0.6007	0.0919	0.8659	0.8471
Igf-1 (18S & eIF4E covariates)	<.0001	0.0840	-	0.7258	0.4803	0.0779	*0.0478*
Pax7 / Myod1 (18S)	<.0001	0.9716	-	**0.0477**	0.5201	0.5027	0.2001
Pax7 / Myod2 (18S)	<.0001	0.3107	-	0.8400	0.5722	0.2471	0.7160
Pax7 / Total Myod (18S)	<.0001	0.8494	-	0.0650	0.5022	0.6528	0.3222
Myf5 / Myod1 (18S)	<.0001	0.3340	-	0.1196	0.8036	0.3917	**0.0139**
Myf5 / Myod2 (18S)	0.0116	0.8312	-	0.4240	0.9431	0.2542	0.0905
Myf5 / Total Myod (18S)	<.0001	0.3624	-	0.1418	0.8264	0.5349	**0.0140**

Significant p-values are italicized if expression is greater in females and in bold if greater in males

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Chapter 3: Future Directions

Sablefish are an emerging species to the US aquaculture industry and research has only begun to investigate the ideal methods of culturing this fish to achieve optimal growth through the entire production cycle. The best growth includes two key areas: the most rapid and efficient growth rates will maintain economic feasibility for the producer, and the type of growth that will support optimal development of the muscle tissue that translates to the highest quality product for processing and that maintains traits related to physical and chemical attributes important to the consumer.

The work described herein provides a baseline for comparison to evaluate myogenic and anabolic gene expression markers in fast-growing sablefish during grow out, and should be evaluated based on genetics, environment, and developmental stage. For completeness, these markers could be evaluated in fish at smaller sizes and earlier life stages. Some of the major changes taking place in aquaculture of sablefish that will influence growth of the fish and the muscle tissue that could be assessed with these molecular tools have to do with establishing genetic strains with the best survival and growth potential, formulation of alternative plant-based feeds for this carnivorous fish, and all-female production populations. Additionally, sablefish fish have typically been grown to harvest size in net pens, but there is interest in evaluating growth performance under alternative methods, including grow out in land-based systems and deep-water, submersible cages. This may better emulate the environment that yields the quality of flesh that the wild sablefish are becoming prized for and the aquaculture industry hopes to retain. In these cases, the tools we have developed could improve our understanding of how these new and different culture techniques modulate muscle growth and development to help answer if stable temperatures and photoperiod in controlled land based systems, or more natural pressures and oxygen concentrations at depth, support rapid growth or produce a better product. We especially consider Pax7/Myod1 or Pax7/Total Myod ratios to have potential value to evaluate any of these intrinsic and extrinsic growth factors due to the correlation with specific growth rate during sablefish grow out.

Due to higher incidence of mortality during the larval life stages, many changes to culture conditions continue to be investigated in the hatchery to immediately improve larval survival and growth, and have potential for long term consequences on muscle development. Our markers of muscle growth provide a means to help industry recognize early on when changes to the environment positively or negatively alter anabolic signaling and activity of the muscle satellite cells. More time and resource efficient decisions could be made by growers when long term consequences are identified earlier by using these molecular methods, helping to eliminate the time and resource demands to grow out the fish to evaluate the phenotype. There are many important factors in the

hatchery to evaluate, including but not limited to temperature, photoperiod, feeding regimes, and the different commercially available enrichments for live feeds.

Moving forward, it is probable that investigations of flesh quality will need to be addressed with paired sensory panel and instrumentation assessments to identify and quantify desirable product characteristics. This knowledge would help evaluate domestic strains and design of the culture environment. A reasonable place to begin would be to compare wild and farmed sablefish, and where differences are found, preferences would help guide strategic modifications to the culture environment. Our markers for muscle growth and anabolism can be linked to the development of the tissue. These could be investigated with respect to genetic and environmental factors that yield preferred growth rate and quality traits. These could potentially be supplemented with gene expression markers that reflect hyperplasia versus hypertrophy, and qualities of the extracellular collagen matrix, as these are known to influence texture, firmness and mouthfeel.

Appendix A. IACUC Approval

University of Idaho

Institutional Animal Care and Use Committee

Annual Protocol Review

Date:	Friday, October 2, 2015				
To:	Gordon Murdoch				
From:	University of Idaho Institutional Animal Care and Use Committee				
Re:	Protocol 2014-1 Determination and practical application of egg quality measures towards reliable culture of high-value marine finfish species				
Original Approval:	1/7/2014	**Annual Expiration:**	11/20/2015	**3 Year Expiration:**	1/7/2017

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. This brief renewal application will provide the basis for an annual review for projects that have not changed or may have only minor modifications from year to year. A new protocol must be submitted for new projects, major changes in existing protocols, and for all protocols every three years.

1) Please indicate the present status of your project by checking one of the statements below:

____ This project is no longer active, please withdraw.

___X_ This project is pending/active and there have been no changes in procedures with respect to animal use or personnel.

____ This project is active and there have been changes in the personnel or experimental procedures. (Describe any such changes below.)

2) Please provide a description of any changes in procedures with respect to animal care and use since your protocol was originally approved. Attach additional pages if necessary.

3) List all changes in personnel involved in your project:

Add	Delete	Name	Department	Email	Training & Experience

Signature: __Gordon K. Murdoch_____ Date: _____10/02/15_____

Send this completed form to iacuc@uidaho.edu or IACU C, Office of Research Assurances, University of Idaho, Moscow, Idaho 83844-3010. **You must immediately cease all live animal activities described under this protocol on or before the renewal date of this protocol, 11/20/2015, unless it is reviewed and approved by the Institutional Animal Care and Use Committee prior to this date.** If you have any questions, please contact the Office of Research Assurances at (208) 885-6162.

University of Idaho

Institutional Animal Care and Use Committee

Annual Protocol Review

Date: Monday, November 03, 2014

To: Gordon Murdoch

From: University of Idaho
Institutional Animal Care and Use Committee

Re: Protocol 2014-1
Determination and practical application of egg quality measures towards reliable culture of high-value marine finfish species

Original Approval:	1/7/2014	**Annual Expiration:**	1/7/2015	**3 Year Expiration:**	1/7/2017

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. This brief renewal application will provide the basis for an annual review for projects that have not changed or may have only minor modifications from year to year. A new protocol must be submitted for new projects, major changes in existing protocols, and for all protocols every three years.

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2) Please provide a description of any changes in procedures with respect to animal care and use since your protocol was originally approved. Attach additional pages if necessary.

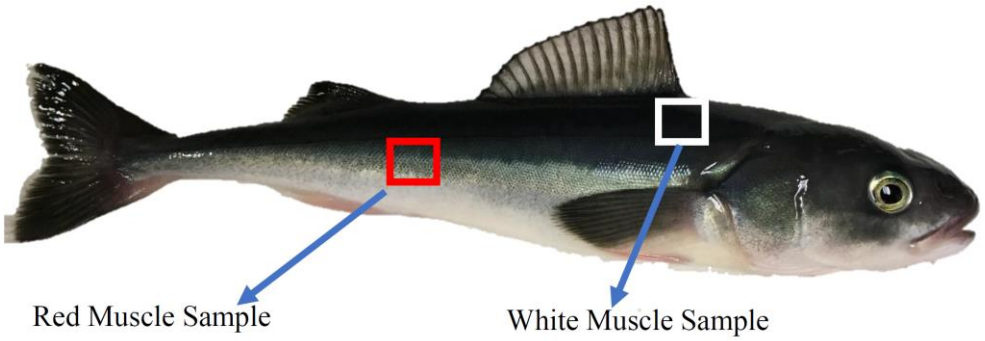
3) List all changes in personnel involved in your project:

Add	Delete	Name	Department	Email	Training & Experience

Signature: ___Gordon Murdoch_____ Date: _Nov 4, 2014_____

Send this completed form to iacuc@uidaho.edu or IACU C, Office of Research Assurances, University of Idaho, Moscow, Idaho 83844-3010. **You must immediately cease all live animal activities described under this protocol on or before the renewal date of this protocol, 1/7/2015, unless it is reviewed and approved by the Institutional Animal Care and Use Committee prior to this date.** If you have any questions, please contact the Office of Research Assurances at (208) 885-6162.

Appendix B. Muscle Sample Origin



Appendix C. Other Products of MS Program: Grant proposals written and submitted during graduate program

1) NOAA Internal Competitive Aquaculture Funding, FY2017. Persistent Effects of Environment on Muscle Growth and Texture in Sablefish (*Anoplopoma fimbria*). Co-PIs: Lee, J., Armbruster, L., Murdoch, G. Submitted 12/14/2016. $ 56,900 - unfunded

2) NOAA Internal Competitive Aquaculture Funding, FY2016. Sablefish Muscle Physiology. Co-PIs: Armbruster, L., Murdoch, G. Submitted 11/25/2015. $ 59,360 - unfunded

3) NOAA Internal Competitive Aquaculture Funding, FY2016. The Effect of Dietary Taurine on Feed Attraction and Physiology of Carnivorous Marine Fish. Co-PIs: Sommers, F., Armbruster, L. Submitted 11/25/2015. $ 40,480 – funded in full

4) NOAA Internal Competitive Aquaculture Funding, FY2015. Sablefish Muscle Physiology. Co-PIs: Johnson, R., Armbruster, L., Murdoch, G. Submitted 5/1/2015. $ 7,798 - unfunded