THESIS

UNDERSTANDING ADAPTIVE REGULATION OF SKELETAL MUSCLE PHYSIOLOGY IN THE WEDDELL SEAL: A PROTEOMICS APPROACH

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY AMBER E. CABLE ENTITLED UNDERSTANDING ADAPTIVE REGULATION OF SKELETAL MUSCLE PHYSIOLOGY IN THE WEDDELL SEAL: A PROTEOMICS APPROACH BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

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Air-breathing, diving vertebrates foster unique adaptations to exercise; namely, these animals are able to exercise for prolonged periods of time while "holding" their breath. Weddell seals (*Leptonychotes weddellii*) routinely undergo progressive hypoxia and ischemia throughout the course of diving activity. In essence, this unique animal has overcome problems that are considered to be otherwise pathological in terrestrial vertebrates. The goal of this project was to verify the use of cross-species analysis and develop a proteomics protocol for use in diving mammals. These steps are necessary in order to ultimately use proteomics to identify age class protein signatures and better understand the molecular regulation of the physiological changes that couple the development of inactive Weddell seal pups into elite diving adults. Proteins from the primary swimming muscle (*M. longissimus dorsi*) of two distinct age classes, pups (3-5 weeks/nondivers) and adults (7+ years/expert divers), were visualized using two dimensional gel electrophoresis (2DE), quantified, and identified. This study validated the use of cross-species analysis, which was of paramount importance due to the fact that the pinniped genome is largely

unidentified, and established a 2DE protocol tailored to suit the unique properties of diving mammal skeletal muscle tissue. To our knowledge, this was the first study in which proteomics was applied to study the proteome of a diving mammal. Understanding thcontrol of these adaptations in the Weddell seal, which *develops* its ability to endure hypoxia associated with breath-hold exercise rather than being born ready to dive, has considerable potential for pharmacological implications for treating various human diseases, specifically those that involve hypoxic conditions such as cardiovascular and pulmonary diseases.

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Chapter 1

Introduction to Diving Physiology

Air-breathing, diving vertebrates represent a model system that has unique adaptations to exercise; namely, these animals are able to exercise while "holding" their breath. As such, research in the area of diving physiology is of particular interest for better understanding exercise in an extremely hypoxic environment. The working skeletal muscles not only maintain function, but actually thrive during breath-hold exercise. Further knowledge of regulatory mechanisms of muscle development in this unique model system is of particular interest due to potential pharmacological implications for treating various human diseases, specifically those that involve hypoxic conditions such as cardiovascular and pulmonary diseases. Weddell seals (*Leptonychotes weddellii*), Antarctic diving specialists, routinely undergo progressive hypoxia and ischemia throughout the course of diving activity. In essence, this unique model has overcome problems that are considered to be otherwise pathological in terrestrial vertebrates. Human stroke victims, for example, experience a wide range of debilitating consequences of brain ischemia, often times leading to death. Understanding adaptations of the Weddell seal, which *develops* its ability to endure hypoxic and ischemic dive conditions, has implications for driving this adaptation in the practice of human medicine.

At a glance, air-breathing diving vertebrates are seemingly an anomaly of exercise physiology in comparison to terrestrial mammals. Under conditions of physical exertion and stress associated with exercise, specifically during diving activity, diving vertebrates undergo a dive response, which, by definition, is essentially the exact opposite of physiological responses experienced by exercising terrestrial vertebrates. Specifically, the dive response entails apnea, bradycardia and

subsequent decreased cardiac output, and vasoconstriction, thus causing the animal to depend solely on a finite supply of oxygen stored internally (Kooyman et al 1981). As this finite oxygen store depletes over the course of the dive, the internal environment of diving vertebrates subsequently undergo progressive asphyxia (Hochachka et al 1986; Hochachka et 1992; Kanatous et al 1999).

This paradoxical exercise response may, therefore, accompany specific adaptations for such a unique exercise environment. Regarding metabolism, there are two apparent strategies for air-breathing animals diving at depth. The first describes the internal environment of birds, which rely on anaerobic metabolism, while the second is found in mammals, which are known to rely on aerobic metabolism during diving activity (Kooyman and Ponganis, 1998). Regarding the latter, sustenance of aerobic metabolism necessitates the presence of oxygen; yet, because these animals enter a state of apnea during dives, oxygen must come from elsewhere. Indeed, diving mammals are known to have increased internal stores of oxygen, specifically as a result of increased expression of oxygen-binding heme proteins, such as hemoglobin and myoglobin (Kooyman et al 1981; Kooyman and Ponganis 1998). These oxygen stores, in addition to an increased aerobic capacity (the maximum amount of oxygen the body can use for a specific period), reliance on fatty acid catabolism (the breakdown of fatty acids into smaller molecules and subsequent release of energy), and decreased dependence on blood-born oxygen and metabolites, allow diving mammals to maintain aerobic metabolism under hypoxic conditions. This aerobic metabolism employed during diving can vary from species to species, depending on diving behavior and activity, thus resulting in varied aerobic dive limits (ADL) (Davis et al 1991; Davis and Kanatous, 1999; Kanatous et al 1999; Kanatous et al 2001; Kanatous et al 2002).

Because oxygen stores are so crucial to a diving animal's ADL, prodigious research efforts have been made to understand the development of diving ability, and specifically the development of these oxygen-binding heme proteins. One study from Burns et al 2005 examined the development of oxygen stores in harbor seals (*Phoca vitulina*) and found that neonatal myoglobin concentrations were low in comparison to adults (1.6±0.2g% vs. 3.8±0.3g% for adults), while blood oxygen stores in the form of hemoglobin were relatively mature (21.7±0.4g% in pups, 23.8±0.3g% in adults). These findings imply that in this particular diving mammal, early diving activity is primarily supported by blood oxygen stores, which develops rapidly, whereas muscular oxygen stores are slowly developed (Burns et al 2005). Another study by Noren et al 2001, which solely focused on the development of myoglobin, compared diving marine endotherms that develop on land (pinnipeds and penguins) versus those that develop at sea (cetaceans). The study determined that regardless of the location of postnatal development, neonatal divers have low myoglobin contents. These myoglobin stores appear to increase throughout development concurrently with increases in physical exertion, thermal demands, and swimming or diving induced bouts of apnea (Noren et al 2001).

Despite a growing understanding of the dive response and the muscle phenotypes corresponding with development, developmental mechanisms describing the ways in which molecular controls manifest themselves into such unique adaptations are poorly understood. As such, knowledge of terrestrial mammal ontogeny must be employed to make sense of the development of diving mammals. Physiological changes in response to ontogeny in terrestrial mammals show nuclear factor activation of T cells (NFAT) response elements as necessary components for muscle-

specific transcription of oxidative fiber and myoglobin. Calcineurin, a Ca^{2+}/calmodulin-dependent serine phosphatase, acts by dephosphorylating NFAT, which in turn allows NFAT to regulate oxidative fiber and myoglobin gene expression (Chin et al 2004, Kanatous et al 2008). Therefore, because this calcineurin pathway described in terrestrial mammals plays an important role in expression of the same phenotypes that are considered key physiological adaptations in diving mammals, it can be hypothesized that Weddell seals may use this type of ontogenic calcineurin pathway.

The goal of this project was to verify the use of cross-species analysis and develop a proteomics protocol for diving mammals. Ultimately, this protocol will identify protein signatures to better understand physiological changes that couple the development of inactive Weddell seal pups into elite diving adults. The primary swimming muscle (*M. longissimus dorsi*) of two distinct age classes, pups (3-5 weeks/nondivers) and adults (7+ years/expert divers), were examined for differences in relevant protein expression using two dimensional gel electrophoresis (2DE). This study validated the use of cross-species analysis (using available sequence data from other species to identify proteins in the seal), which was of paramount importance due to the fact that the pinniped genome is largely unidentified. To our knowledge, is the first study in which proteomics was applied to study the proteome of a diving mammal.

Weddell Seal Background

Weddell seals are elite, Antarctic divers, as seen by their long dive times (approximately two hours) and deep dive depths (approximately 600 meters) in relation to their body size, which averages between 400-600 kilograms in adults. In comparison to their diving mammal

counterparts, Weddell seals rank third in longest dive time at nearly two hours on a single breath, falling only behind the sperm whale and elephant seal, and have an average ADL of 20 minutes (Davis and Kanatous, 1999; Wright and Davis, 2006). Unlike these two diving specialists, however, Weddell seals have a tight link to land and lack terrestrial predators (and subsequent docile behavior), which makes the Weddell seal an ideal candidate for hypoxic exercise research. Weddell seals are known for their reliance on aerobic respiration during dives. An assessment of fiber type in primary swimming muscles showed an abundance of Type I (slow-twitch oxidative) and Type IIA (fast-twitch oxidative) fibers, and a complete absence of Type IIB (fast-twitch glycolytic) fibers (Kanatous et al 2002). Due to the fact that these mitochondria-rich fibers in the Weddell seal rely on oxygen to fuel energy production, this type of fiber distribution in the exercising muscles implies a nearly complete reliance on aerobic respiration during exercise.

A plethora of adaptations allow the working Weddell seal skeletal muscles to maintain aerobic respiration for durations upwards of two hours on a finite supply of oxygen. One of the hallmark adaptations seen in Weddell seals is that they store copious amounts of oxygen internally, thus making oxygen available throughout diving activity despite a cessation of ventilation. These oxygen stores are made possible by having an increase in oxygen-binding heme proteins. Hemoglobin, the blood-born oxygen-binding globin, is approximately 1.5 times higher in Weddell seals in comparison to terrestrial mammals (Wright and Davis 2006). The resultant increase in hematocrite (Hct) causes a substantial increase in blood viscosity, circulatory resistance, and cardiac work; therefore, Weddell seals have a large spleen, which works to sequester RBC, lower Hct, and decrease blood viscosity when they are surfaced, while alternatively RBC and Hct increase during hypoxic dives (Elsner and Meiselman, 1995).

In addition to hemoglobin, Weddell seals also increase their internal oxygen stores through an elevated expression of myoglobin, which represents one-third of the total oxygen stores (Wright and Davis 2006). Unlike hemoglobin, its blood-born counterpart, myoglobin is localized in muscle tissue. Because Weddell seals have oxygen readily available at the muscle, the tissue is less reliant on oxygen delivery from the blood, which allows for aerobic respiration in the muscle in spite of peripheral vasoconstriction (one of the physiological adapatations associated with the dive response). Myoglobin further ameliorates negative repercussions of peripheral vasoconstriction by acting as a scavenger of reactive oxygen species (Garry and Mammen 2008). In the primary swimming muscles, Weddell seals have been shown to have 10 times more myoglobin than their terrestrial counterparts (Snyder 1983; Kanatous et al 2002; Wright and Davis 2006). Weddell seals, therefore, can sustain aerobic metabolism in their skeletal muscles in lieu of blood perfusion and subsequent blood oxygen delivery due to the fact that they have an adaptive increase in myoglobin expression (by an order of magnitude) in these muscles.

Another adaptation in the muscle physiology of the Weddell seal that allows for aerobic respiration is the preferential usage of fat stores as fuel. Enzyme assays revealed that citrate synthase activity, an enzyme marker of carbohydrate aerobic respiration, was significantly lower (47-50%) in the Weddell seal in comparison to its terrestrial counterpart, the dog. Alternatively, ß-hydroxyacyl CoA dehydrogenase, an enzyme marker of fat-fueled aerobic metabolism, was significantly greater (2.3-4.1x) in Weddell seals in comparison to dogs (Kanatous et al 2002). This preferential use of fat stores produces more than 3x the ATP that carbohydrate-fueled

metabolism produces, thus giving the Weddell seal optimal oxygen-fueled energy production in an environment of increasing hypoxic stress for time periods of up to two hours.

Such a unique system of muscle physiology then raises the question, how does this phenotype (increased oxidative metabolism, internal oxygen stores, and fat catabolism) arise? In terms of muscle development, upon the cessation of weaning and commencement of diving, Weddell seals generally experience developmental changes in muscle physiology that parallel changes in activity. Preliminary microarray results for the three age classes of Weddell seals indicate differential expression of RNA transcripts associated with various ontogenic signaling pathways, in addition to differences in transcripts associated with lipid metabolism, between age classes. However, in contrast to terrestrial mammals, where this developmental trend in increasing exercise capacity occurs from birth, skeletal muscle physiology of Weddell seal pups alludes a higher capacity for exercise than juveniles and adults. This is indicated by pups having the highest percentage of type I slow oxidative fibers as well as the highest mitochondrial volume densities of any age class. A relative lack of a simultaneous increase in aerobic enzymes, however, indicates that these results may be due to an adaptation for enhanced nonshivering thermogenesis. Furthermore, juveniles have been shown to have the highest expression of myoglobin (72.4±7 *vs* 55.9±2.5 and 35.5±3 mg g^{-1} wet mass muscle in adults and pups, respectively) (Kanatous et al 2008). These results are consistent with published data linking both hypoxia *and* exercise to increased myoglobin expression, as diving activity is supported by aerobic metabolism in the skeletal muscles and juveniles are the most active divers of the three age classes (Kanatous et al 2009).

Proteomics

Despite a growing library of entire organismal genomic data, these databases of nucleotide sequences present considerable limitations in the understanding of an organism's biology at any given time. An accurate reflection of an organism's environmental and physiological state is generally seen in the expression of a mere fraction of the proteins present in the transcripts of that tissue's genome (Ferguson and Smith 2003). Determining and comparing protein expression is of particular importance due to the ability to detect post-translational protein modifications that otherwise remain undetectable by DNA and RNA studies. The transcription factor hypoxia inducible factor (HIF), for example, exhibits one of these key factors in post-translational modification. This particular protein mediates the effects of hypoxia by upregulating several genes involved in glycolysis and angiogenesis that promote survival in low-oxygen environments. It is always maintained baseline expression in cells; however, upon becoming hypoxic, cellular HIF undergoes a posttranslational modification that stabilizes HIF and allows it to act as a transcription factor. HIF detection, therefore, does not reflect cellular hypoxia; rather, it is the detection of the posttranslational modification that is actually indicative of hypoxia. This and other posttranslational modifications demonstrate why it is necessary to study proteins in studies aiming to understand the environmental and physiological state of skeletal muscles.

The field of proteomics uses mass spectrometry (MS) to establish entire protein signatures of total tissue. This method of studying proteins, which continues to grow in popularity, is considered superior to the formerly popular immunoblot detection due to its ability to identify entire protein complements and modifications (Lin et al 2002; Doran et al 2007).

To determine and compare protein expression, my proteomics study employed 2DE, which involves high-resolution separation of the proteome (Doran et al 2007). This technique uses two applications of electrophoresis to separate proteins first based on their isoelectric points, followed by a separation based on their molecular weights. In the former separation, protein homogenate is applied to a gel strip in which a pH gradient lies, and electric current is applied to the strip, thus allowing proteins to align based on their isoelectric points. In the latter separation, the aforementioned strip is applied to a polyacrylamide gel, which separates proteins in the gel matrix based on their molecular weights upon application of an electric current. Protein representatives of the entire homgenate appear on polyacrylamide gels as spots, and expression levels are determined by the presence, size, and density of the spots. Lastly, identification of protein spots employs a matrix-assisted laser desorption ionization-time-of-flight MS (MALDI-TOF MS) in addition to an online database of protein sequences (Ferguson and Smith 2007; Doran et al 2007) to generate predicted peptide identifications and subsequent predicted protein identifications. Protein expression can therefore be compared between different sample types by comparing expression levels of spots; in turn, for this project, proteomics is capable of providing insight into molecular regulators of ontogeny in the Weddell seal.

Chapter 2 - How do you build a marine carnivore? The potential of microarray and proteomic techniques to identify the molecular regulation of the ontogeny of skeletal muscle adaptations in Weddell seals (*Leptonychotes weddellii*)

(Kanatous SB, LE Pearson and **AE Cable**. How do you build a marine carnivore? The potential of microarray and proteomic techniques to identify the molecular regulation of the ontogeny of skeletal muscle adaptations in Weddell seals (*Leptonychotes weddellii).* 4th CPB Meeting in Africa: Mara 2008. "Molecules to migration: The pressures of life" (Ed S. Morris & A. Vosloo). Medimond Publishing Co, via Maserati June 2008, 40124 Bologna, Italy.)

Air-breathing diving vertebrates, especially species that make deep and long dives, exhibit physiological adaptations in their muscles (and other tissues) that sustain an aerobic, lipid-based metabolism under conditions of hypoxia and ischemia. Our objective was to determine the underlying molecular controls for the ontogenetic changes in the skeletal muscles of Weddell seals that transform a non-diving pup into an elite diving adult. Muscle biopsies were collected from pups (3-5 weeks old), juveniles (1-2 years old) and adults (7+ years old) and previously analyzed for changes in fiber type, mitochondrial density, myoglobin concentrations, and aerobic, lipolytic and anaerobic enzyme activities. Total RNA was isolated from pups, juveniles and adults and compared using subtractive hybridization. In addition RNA was isolated from three adult male samples and compared using Affymetrix microarray technology. Protein was also isolated and analyzed using 2D gel electrophoresis. The objective of this study was to determine the relative success within each technique to utilize cross species hybridization and analysis tools. All three techniques yielded positive results in terms of the cross species hybridizations and analyses. The results from each technique were reproducible and yielded results that were substantiated by the results of our previous studies on the physiological changes within the skeletal muscles of the three age classes.

Introduction

Air-breathing diving vertebrates, especially species that make deep and long dives, exhibit physiological adaptations in their muscles (and other tissues) that sustain an aerobic, lipid-based metabolism under conditions of hypoxia and ischemia (Kanatous et al., 2002; Kanatous et al., 1999; Kanatous et al., 2001; Kanatous et al., 2008). These adaptations increase an animal's aerobic dive limit (ADL) which is the longest dive that an animal can make while relying primarily on oxygen stored in the blood and muscle to sustain aerobic metabolism. Our previous studies of adult Weddell seals (*Leptonychotes weddellii*), harbor seals (*Phoca vitulina*) and Steller sea lions (*Eumatopias jubatus*) have revealed that their muscle adaptations include: 1) an increased aerobic capacity (or one that is matched to routine levels of exertion), 2) a reliance on fatty acid catabolism for aerobic ATP production, 3) enhanced oxygen storage and diffusion capacity, and 4) a reduced dependency on blood-borne oxygen and metabolites (e.g., decreased capillary density) compared to terrestrial mammals (Davis et al., 1991; Davis and Kanatous, 1999; Kanatous et al., 2002; Kanatous et al., 1999; Kanatous et al., 2001; Polasek et al., 2006) Recently numerous studies have started to describe the development of diving capacity and oxygen stores in diving mammals (Clark et al., 2006; Clark et al., 2007; Noren et al., 2006; Noren et al., 2008; Noren et al., 2004; Noren et al., 2005). In addition, we recently detailed the ontogeny of the skeletal muscle adaptation associated with diving in Weddell seals (Kanatous et al., 2008). These studies have found that there are numerous physiological changes that occur during the nursing and weaning periods that are necessary for these animals to initiate independent foraging.

Smaller, subadult animals are less capable divers than adults, in part because of their higher weight-specific metabolic rate and proportionately smaller blood and muscle oxygen stores (Burns, 1996; Burns, 1999; Kooyman, 1983). In addition, the muscle adaptations (as described above) that enhance diving performance may not completely develop until a young animal is several years old. As a result, they can neither dive as long nor as deep as adults. Young Weddell seals are therefore at a disadvantage in their ability to forage on deep-living prey such as Antarctic silverfish (*Pleuragramma antarcticum*), and this appears to influence survival during the initial years of life (Testa, 1987). Therefore the ontogeny of muscle aerobic capacity, lipid metabolism, and oxygen stores in the skeletal muscles is important for diving ability, yet we have only recently begun to describe the development of these physiological variables (Kanatous et al., 2008). To date the underlying molecular regulation of these physiological adaptations have been difficult to discern. Due to the lack of a sequenced genome for seals, researchers must rely on cross species analysis to attempt to decipher their molecular regulation. Thus, the objective of this study was to verify the reproducibilty and utility of using cross species analysis on Weddell seals.

Methods

Animals

Twenty-four newborn Weddell seal pups (age 3-5 weeks, mean mass 75 ± 3 kg), eighteen juvenile (age 1-2 years, mean mass 120 ± 5 kg) and twenty-six adult Weddell seals (age 7+ years, mean mass 385 ± 13 kg) were captured with a purse string net along natural tidal cracks in McMurdo Sound Antarctica over three field seasons. The ages of all the seals were determined from flipper tags with the data provided by personal communication with R. Garrott, J. Rotella, and D Siniff (NSF grant OPP-0225110). The seals were sedated with ketamine (1.5 mg/kg,

(Davis et al., 1983; Davis et al., 1999)), weighed with a hanging digital scale (accuracy ± 0.5 kg), and muscle biopsies taken under local anesthesia. In order to standardize our sampling, all biopsies were taken from the mid-belly of the muscle and at the same location in all age classes (1/3 of the body length from the tail). Pups were returned to their mothers within 30 min after recovery from mild sedation. Adults and juveniles were detained for less than one hour post biopsy and were released near the site of capture after the animals had regained full voluntary locomotion.

Muscle biopsies

Muscle samples of approximately 50 mg each were collected with a 6-mm biopsy cannula (Depuy, Warsaw, Indiana) from the swimming (*M. longissimus dorsi*) muscle. Muscle biopsy samples for transcriptome analyses were placed into RNA later (Ambion, Austin, Texas USA) for 24 hours and then frozen in liquid nitrogen for later RNA analysis. Samples for proteomic analysis were immediately frozen in liquid nitrogen upon collection. Frozen samples were stored at -80 ^{o}C until analysis.

Subtractive hybridization

Total RNA (500 μg) were isolated from three biopsies (one for each age class) of the swimming muscle (*m. Longissimus dorsi*, LD) from pups, subadults and adult Weddell seals. Samples were pooled from each age class to eliminate possible individual variations in gene expression. Poly $(A)^{+}$ RNA (5 μg) was isolated from total RNA using the mRNA Separator Kit (Clontech, Mountainview, California USA) according to the manufacturer's protocol. cDNA transcripts were synthesized from each of the mRNA pooled samples and used to generate

subtractive hybridization libraries using the PCR-Selected cDNA Subtraction Kit (Clontech, Mountainview, California USA) according to the manufacturer's protocol. An example of the subtractive libraries presented was enriched in cDNA transcripts expressed in adults by subtracting cDNA transcripts from LD muscle of newborn pups from cDNA transcripts of adult LD muscle. This yielded cDNA transcripts that were up-regulated in adult muscle as compared to newborn muscle. After generating the subtractive libraries, the cDNA inserts were examined by PCR, sequenced using BigDye Terminator (Applied Biosystems, Foster City, California USA) on a model 377 sequencing machine (Applied Biosystems, Foster City, California USA) and the sequence entered into BLAST (http://www.ncbi.nlm.nih.gov).

Micro-array

Samples intended for micro-array analysis were sorted into Tripure (Gibco, Carlsbad, California USA) to maximize RNA recovery. Total RNA was prepared following standard protocols then treated with Dnase I using the RNeasy column purification system (Qiagen, Valencia, Californa USA). A portion of the total RNA was hybridized to micro-array chips (Affimetrix Human 2.0 gene chip, Affymetrix, Santa Clara, California USA) and analyzed using Affimetrix software.

Proteomic analysis

Individual samples (i.e. samples were *not* pooled) were homogenized in a lysis buffer (9M urea, 4% triton X-100, 50mM DTT, 2% 3/10 ampholytes, cocktail protease inhibitor) and protein concentration was determined using a Coomassie protein assay (Pierce, Rockford, Illinois USA). For first dimension separation, 100µg of protein was diluted in 125µl of rehydration buffer (9M urea, 4% Triton X-100, 50mM DTT, 2% 3/10 Ampholytes, 0.0001% Bromophenol Blue) and

applied to IPG strips (pH 3-10, pH 4-7) for rehydration. Immediately following rehydration, IPG strips ran through isoelectric focusing (IEF) in the Protean IEF Cell (Bio-Rad, Hercules, California, USA), after which they were equilibrated in two different buffers (equilibration 1: 6M Urea, 2% SDS, 0.375M Tris-HCl pH 8.8, 20% glycerol, 130 mM DTT; equilibration 2: 6M Urea, 2% SDS, 0.375M Tris-HCl pH 8.8, 20% glycerol, 135 mM DTT). Then, each strip was placed in a 15% polyacrylamide Next Gel (Amresco, Solon, Ohio, USA) with a layer agarose covering them, and gels were run at 200V for 2 hours in Next Gel (Amresco, Solon, Ohio, USA) running buffer. Protein spots were visualized using Flamingo Fluorescent Gel Stain (Bio-Rad, Hercules, California, USA) and subsequently imaged on a Typhoon scanner. Images were overlayed and analyzed using Decodon 2D software (version 3.5).

Results

In this study we have tested a number of techniques to identify the molecular regulation of the different skeletal muscle adaptations in Weddell seals. While the techniques of subtractive hybridization, microarray and proteomics have been extensively used in model species, their ability to successfully identify transcripts and proteins in such a unique species with a completely undocumented genome remained a large question. Initially we isolated RNA from all of the age classes and transformed it into cDNA for subtractive hybridization analysis. Our initial subtractions between the adult and pups have yielded numerous transcripts that are up-regulated in the adult as compared to the pup (Figure 1). We have identified transcripts for myoglobin, myosin heavy chain IIa, calcineurin, cytochrome c oxidase and NADH dehydrogenase. The subtractive hybridization analysis further corroborated our physiological analysis indicating that the adults had a significantly greater percentage of fast-oxidative fibers as well as myoglobin

concentration. These initial findings also suggest that some of the changes in physiology are regulated at the transcript level further supporting the role of the calcium/calcineurin pathway in regulating the changes in mammalian skeletal muscle even in diving mammals. While this analysis is far from complete, we believe it was an important first step to be able to identify transcripts using the different databases available for other mammalian species such as the rat, mouse and human. In addition, we were encouraged that the transcripts identified were representative of the changes in their skeletal muscle physiology.

The results of the subtractive hybridization were successful but limited, however; based on our initial success with cross species identification of transcripts, we decided to try microarray analysis. We ran three adult male samples (individual samples; *not* pooled together) against the human Affymetrix Human 2.0 gene chip array based on the extensive annotation of the human genome. The results showed 3000 out of a possible 14500 transcripts that were present in all three seal samples (Table 1). This represents approximately 20% coverage of the whole human genome which would have been expected from skeletal muscle. In addition 142 pathway maps were significant, some with near complete representation across the whole pathway (Figure 2 and 3). These pathways included important muscle specific pathways such as NFAT signaling, AKT signaling, G-protein signaling, CREB pathway, WNT signaling, calcium signaling, and lipid metabolism (Figure 3).

Insight into the proteome of an organism can provide invaluable knowledge about its molecular processes, which would complement our transcriptional studies. To our knowledge, this type of 2D proteomics approach has never been applied to the Weddell seal. Because of the

physiological complexity associated with being an elite diving mammal, such as extremely high fat content in biopsies, special consideration in troubleshooting proved necessary. Accordingly, the limited availability and unique nature of the samples required several crucial preparatory steps to produce successful Weddell seal 2-DE gels. Proteins were most effectively extracted from skeletal muscle biopsies by homogenizing tissue with TriPure Isolation Reagent. To further remove impurities that would interfere with the first dimension (e.g. salt, lipids), samples were treated with Bio-Rad's ReadyPrep Clean-Up Kit prior to isoelectric focusing. By using the Delta2D (version 3.5) software, differences in spot size and intensity were interpreted, thus making subsequent protein expression quantifiable. 2-DE gel images analyzed with Delta2D software showed distinct differences between protein expression in the M. Longissimus dorsi of adult verses pup (see Figures 4 and 5). Several spots indicated considerable upregulation in the adult. Specifically, spots labeled A24 1, 2, 3, 4, 5, 6, and 7 were expressed 3.6, 3.1, 1.3, 1.6, 19.1, 25.8, and 30.4 times more in the adult than pup (see Table 2). Conversely, several spots also indicated considerable upregulation in the pup. Spots labeled P221, 2, 3, 4, 5, 6, and 7 were expressed 2.7, 4.9, 9.0, 0, 2.5, 5.3, and 2.3 times more in the pup than the adult (see Table 2). Spot resolution and detection have shown improvement when using 18cm gels rather than 7cm gel. New gels, therefore, are going to use 18cm IPG strips and will include a third age class: the juvenile.

Discussion

The main conclusion of this study was that cross species hybridization and analyses were both reproducible and successful at both the transcriptome and proteomic levels. We initially had limited success identifying unique transcript expressions in the adults as compared to the pups.

This initial success inspired us to attempt to use microarray technology. As a first test, we only used RNA isolated from the swimming muscles of similar aged and sized adult males and ran these samples against a human genome array. On average skeletal muscle accounts for approximately 25-30% of the whole human genome; due to this we were encouraged that we had approximately 20% coverage with seal muscle samples. We were further encouraged to find similarities in transcript expression between our subtraction and microarray. In addition there were numerous muscle specific transcripts that were identified that correlated with our previous physiological results such as myoglobin, myosin heavy chains, calcineurin, nuclear factor of activated T cell, and myocyte enhancement factor. Based on these results we are confident that cross species analyses can be used with Weddell seals to assist in identifying the underlying molecular controls for their adaptation toward diving.

Although proteomic studies are still in progress, preliminary data are promising. Similar to results from subtractive RNA hybridization, initial 2DE gels show marked differences between pup and adult age classes. Furthermore, successful cross species analyses from our transcriptomic studies reassure accuracy in usage of NCBInr BLAST for identifying Weddell seal tryptic peptide sequences. As such, a proteomic avenue demonstrates sufficient promise for assessing molecular controls in skeletal muscle ontogeny of Weddell seals. Accordingly, differentially expressed spots will be digested with trypsin. Following the trypsin digestion, samples will be processed in the MALDI-TOF/TOF (for distinct protein spots) (Ultraflex, Bruker, Madison, Wisconsin, USA) or the LTQ Ion Trap (for spots suspected to have more than one protein) (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Proteins will be identified from mass spectra data with the Mascot Database Search Engine, using calibrated

peptide mass values within NCBInr BLAST (http://www.ncbi.nlm.nih.gov) protein databases for all mammals.

While our initial success is promising, there are still significant limitations to these and future studies. Because the seal genome has not been sequenced we are limited to the use of cross species identification. This fact limits us to only being able to identify transcripts and proteins that are already described in the numerous databases. In other words, we would not be able to identify any transcripts that are unique to the seal without the use of other technologies such as protein purification and sequencing. Therefore, in order to appropriately perpetuate the success of applying new techniques to this unique organism, sequencing of the genome is perhaps a necessary endeavor. Regardless of these limitations, however, transcriptomic and proteomic studies are the new direction in understanding the molecular adaptations to hypoxia and ischemia in this elite diving mammal.

In summary, cross species applications in studying physiological changes in Weddell seals has proven promising in deciphering the molecular regulation of their adaptations. The future utilization of cross-species comparison is still ultimately limited by the lack of a sequenced genome in the Weddell seal. Despite that limitation, cross-species analysis is a very powerful tool to understand differences in the transcriptomic and proteomic expressions within Weddell seals and across different species.

Table 1 Top 50 most highly expressed transcripts across 3 individual adult males

Gene Title	Gene Symbol
tumor protein, translationally-controlled 1	TPT1
tropomyosin 1 (alpha)	TPM1
ribosomal protein L34	LOC342994
ribosomal protein L5	RPL5
tumor protein, translationally-controlled 1	TPT1
myosin, heavy chain 2, skeletal muscle, adult	MYH2
ferritin, heavy polypeptide pseudogene 1	FTHP1
tumor protein, translationally-controlled 1	TPT1
myosin, light chain 2, regulatory, cardiac, slow	MYL2
tropomyosin 1 (alpha)	TPM1
eukaryotic translation initiation factor 4 gamma, 2	EIF4G2
heterogeneous nuclear ribonucleoprotein A2/B1	HNRNPA2B1
tumor protein, translationally-controlled 1	TPT1
ribosomal protein S29	RPS29
titin	TTN
zinc finger, AN1-type domain 5	ZFAND5
tumor protein, translationally-controlled 1	TPT1
ribosomal protein, large, P1	RPLP1
aldolase A, fructose-bisphosphate	ALDOA
ubiquitin C	UBC
protein tyrosine phosphatase type IVA, member 1	PTP4A1
ribosomal protein L15	RPL15
ribosomal protein S12	RPS12
eukaryotic translation elongation factor 1 gamma	EEF1G

ribosomal protein L31	RPL31
Y box binding protein 1	YBX1
high-mobility group box 1	HMGB1
hemoglobin, beta	HBB
ribosomal protein L30	RPL30
eukaryotic translation initiation factor 1	EIF1
eukaryotic translation initiation factor 4A, isoform 2	EIF4A2
ribosomal protein L11	RPL11
eukaryotic translation elongation factor 1 alpha 1	EEF1A1
ribosomal protein S27a	RPS27A
mortality factor 4 like 2	MORF4L2
ferritin, heavy polypeptide 1	FTH1
SMT3 suppressor of mif two 3 homolog 2 (S. cerevisiae)	LOC652489
transducer of ERBB2, 1	TOB1
ribosomal protein L27	RPL27
eukaryotic translation elongation factor 1 gamma	EEF1G
ribosomal protein S4, X-linked	RPS4X
basic transcription factor 3	BTF3
ribosomal protein S15a	LOC646819
ribosomal protein S18	RPS18
eukaryotic translation elongation factor 1 alpha 1	EEF1A1
DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	DDX5
troponin I type 2 (skeletal, fast)	TNNI2
protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform	PPP3CB
calmodulin 2 (phosphorylase kinase, delta)	CALM2
H2A histone family, member Z	H2AFZ

Table 2. Relative quantity of spots (as calculated by Delta2D software, excluding background), where the total quantity of spot volume on a gel is 100%.

Spot ID	%Volume Adult	% Volume Pup	Ratio Adult to Pup	Spot ID	% Volume Pup	%Volume Adult	Ratio Pup to Adult
A24-1	0.7489	0.2095	3.6	**P22-1**	0.2794	0.1048	2.7
A24-2	0.1969	0.0637	3.1	**P22-2**	0.8003	0.1617	4.9
A24-3	1.5745	1.1831	1.3	**P22-3**	0.3034	0.0338	9.0
A24-4	2.7205	1.6828	1.6	**P22-4**	0.3504	0	0
A24-5	4.4237	0.2321	19.1	**P22-5**	0.5353	0.2164	2.5
A24-6	1.7698	0.0687	25.8	**P22-6**	1.8716	0.3546	5.3
A24-7	1.9631	0.0645	30.4	**P22-7**	0.4134	0.1793	2.3

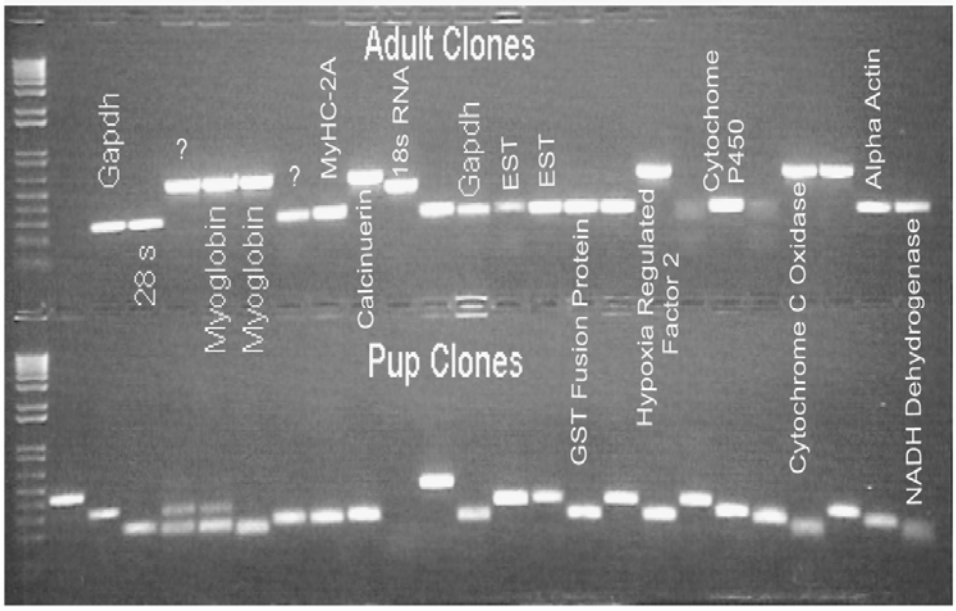


Figure 1. Adult and Pup Subtractive Hybridization Results. Subtractive hybridization analysis between the adults and pups has defined a number of transcripts that were differentially regulated in the adult as compared to the pup. Specifically, we were able to identify transcripts for myoglobin, myosin heavy chain IIa, calcineurin, cytochrome c oxidase and NADH dehydrogenase; analysis further corroborated our physiological analysis indicating that the adults had a significantly greater percentage of fast-oxidative fibers as well as myoglobin concentration

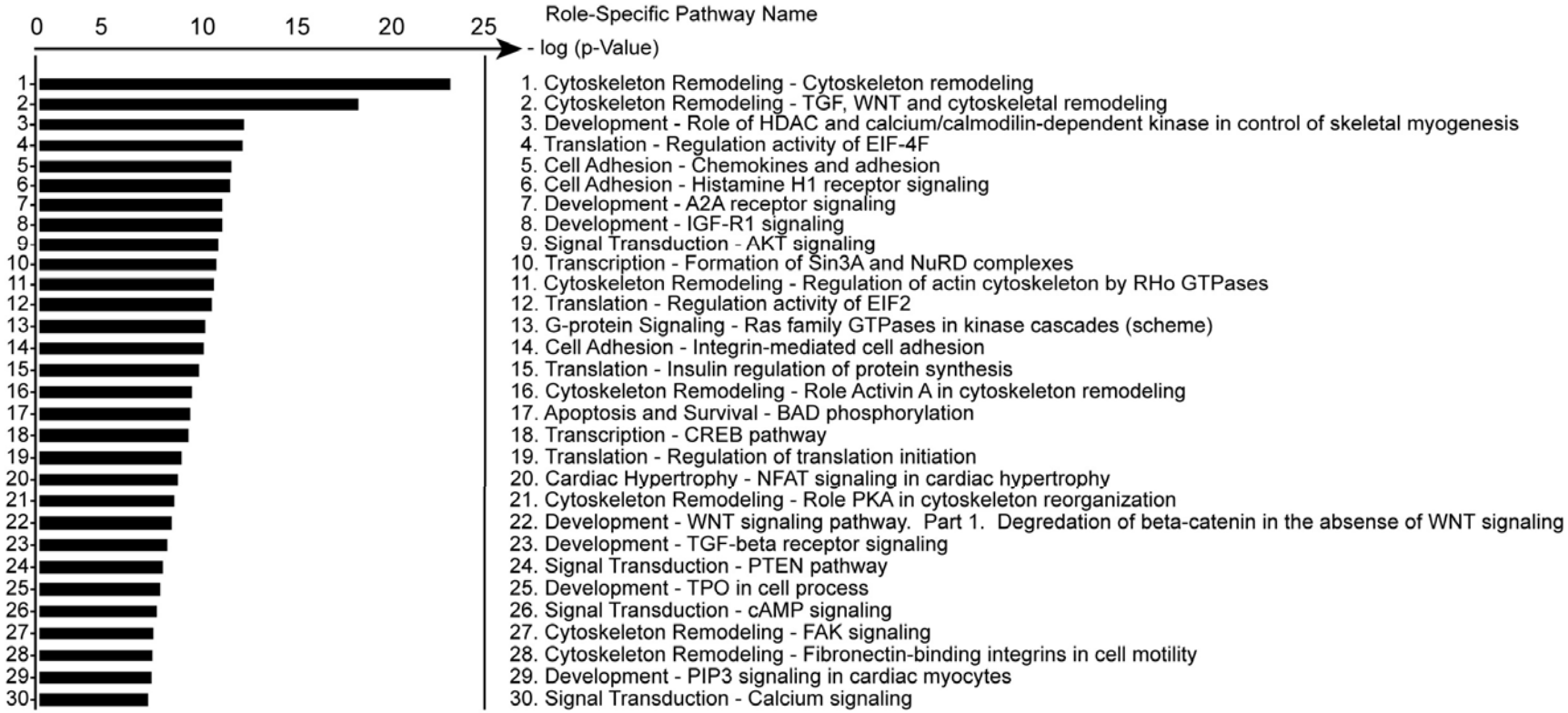
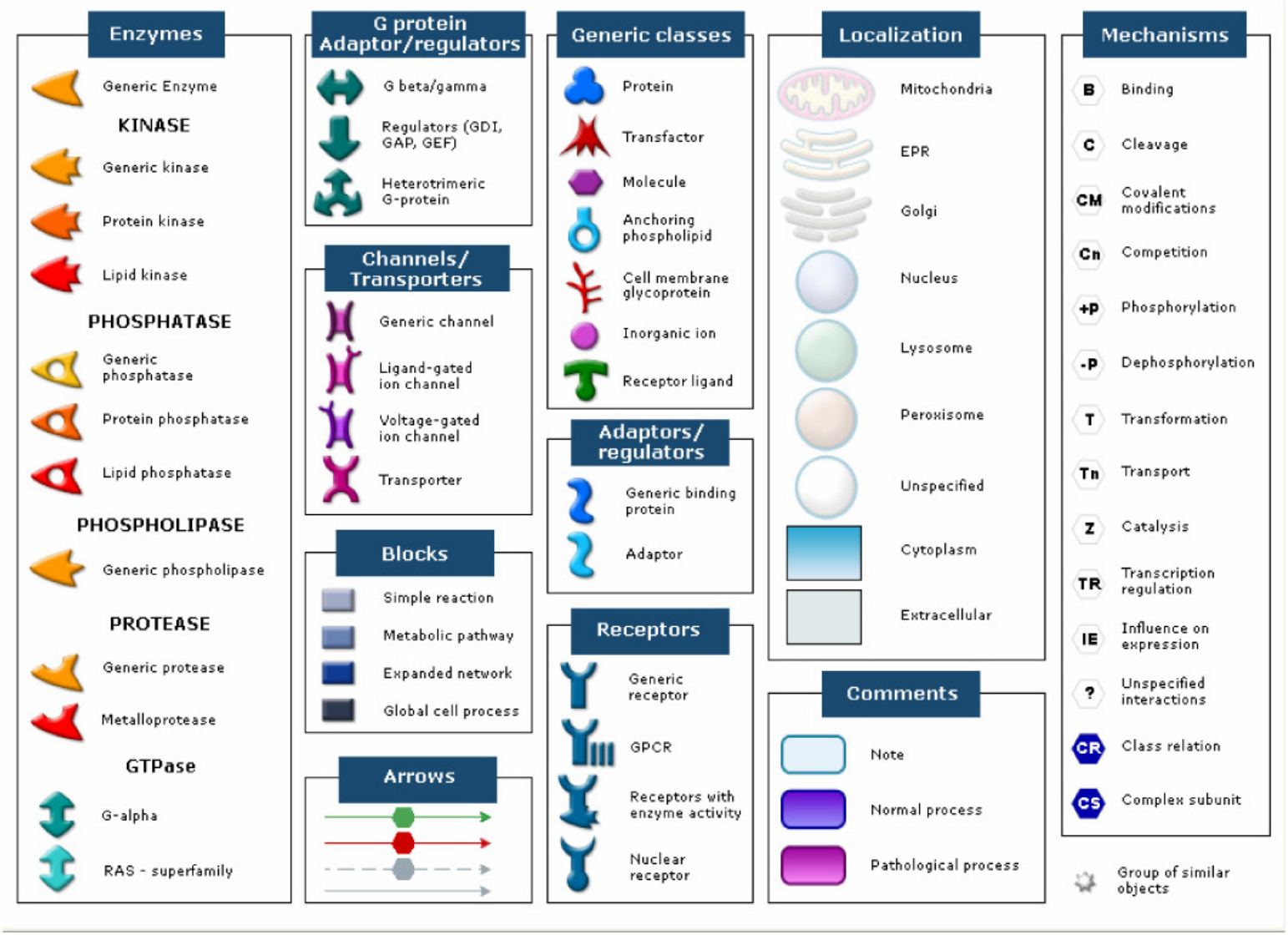
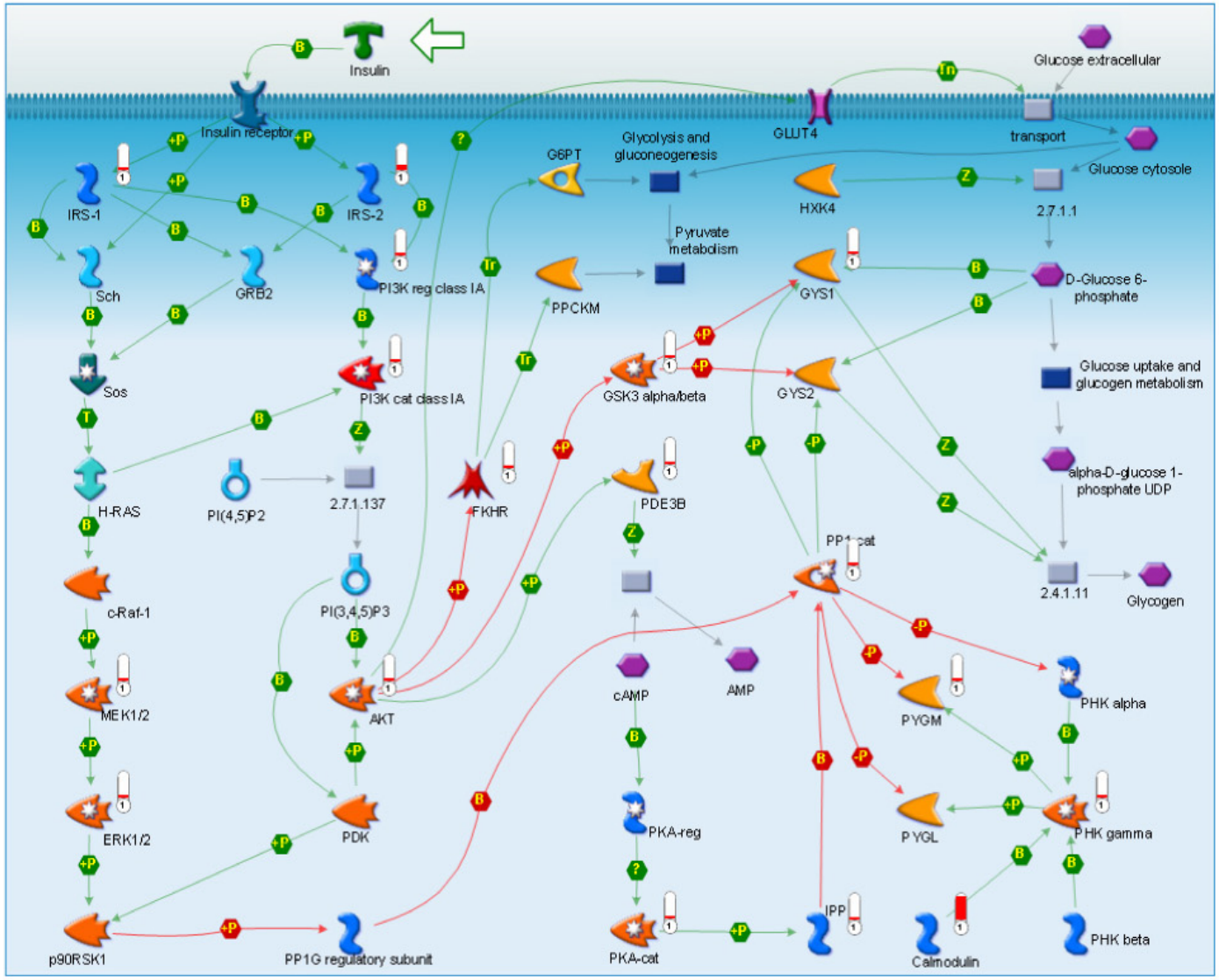


Figure 2. Top 30 Transcript Pathways. Affymetrix pathway analysis identified 148 pathways that were significantly represented by all three seal samples. Shown above are the top 30 pathways.

A.


Enzymes
Generic Enzyme
KINASE
Generic kinase
Protein kinase
Lipid kinase
PHOSPHATASE
Generic phosphatase
Protein phosphatase
Lipid phosphatase
PHOSPHOLIPASE
Generic phospholipase
PROTEASE
Generic protease
Metalloprotease
GTPase
G-alpha
RAS - superfamily
G protein Adaptor/regulators
G beta/gamma
Regulators (GDI, GAP, GEF)
Heterotrimeric G-protein
Channels/ Transporters
Generic channel
Ligand-gated ion channel
Voltage-gated ion channel
Transporter
Blocks
Simple reaction
Metabolic pathway
Expanded network
Global cell process
Arrows
Generic classes
Protein
Transfactor
Molecule
Anchoring phospholipid
Cell membrane glycoprotein
Inorganic ion
Receptor ligand
Adaptors/ regulators
Generic binding protein
Adaptor
Receptors
Generic receptor
GPCR
Receptors with enzyme activity
Nuclear receptor
Localization
Mitochondria
EPR
Golgi
Nucleus
Lysosome
Peroxisome
Unspecified
Cytoplasm
Extracellular
Comments
Note
Normal process
Pathological process
Mechanisms
B Binding
C Cleavage
CM Covalent modifications
Cn Competition
+P Phosphorylation
-P Dephosphorylation
T Transformation
Tn Transport
Z Catalysis
TR Transcription regulation
IE Influence on expression
? Unspecified interactions
CR Class relation
CS Complex subunit
Group of similar objects

B.


Insulin
Insulin receptor
Glucose extracellular
GLUT4
transport
Glucose cytosole
IRS-1
IRS-2
Sch
GRB2
PI3K reg class IA
PI3K cat class IA
Sos
H-RAS
c-Raf-1
MEK1/2
ERK1/2
p90RSK1
PDK
AKT
PI(4,5)P2
2.7.1.137
PI(3,4,5)P3
PP1G regulatory subunit
FKHR
G6PT
Glycolysis and gluconeogenesis
Pyruvate metabolism
PPCKM
GSK3 alpha/beta
PDE3B
cAMP
AMP
PKA-reg
PKA-cat
HXK4
2.7.1.1
D-Glucose 6-phosphate
GYS1
GYS2
Glucose uptake and glucogen metabolism
alpha-D-glucose 1-phosphate UDP
2.4.1.11
Glycogen
PP1-cat
PYGM
PYGL
IPP
Calmodulin
PHK alpha
PHK gamma
PHK beta

C.

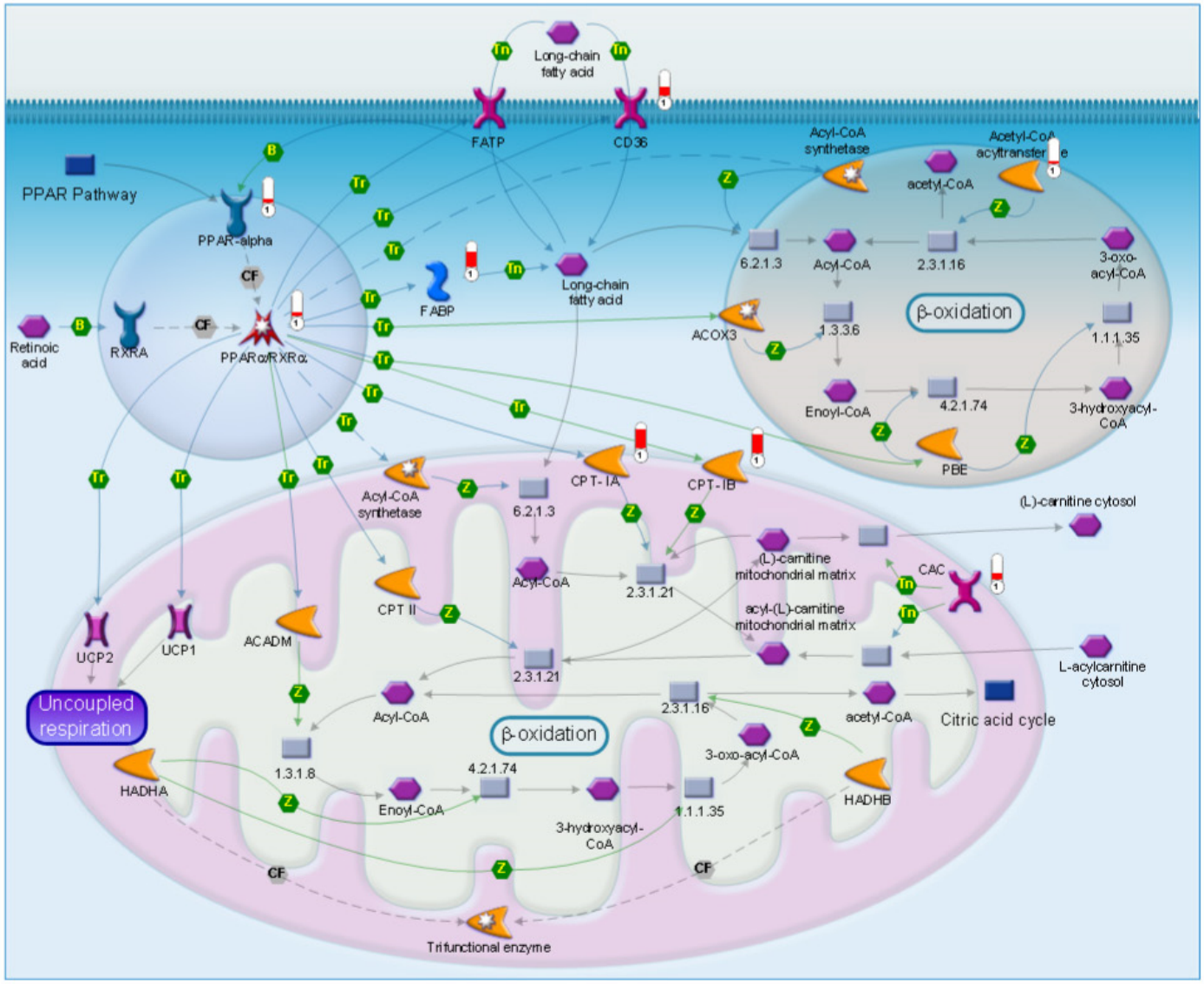

Long-chain fatty acid
FATP
CD36
PPAR Pathway
PPAR-alpha
Retinoic acid
RXRA
PPARα/RXRα
FABP
Long-chain fatty acid
Acyl-CoA synthetase
Acetyl-CoA acyltransfer
acetyl-CoA
6.2.1.3
Acyl-CoA
2.3.1.16
3-oxo-acyl-CoA
β-oxidation
ACOX3
1.3.3.6
1.1.1.35
Enoyl-CoA
4.2.1.74
3-hydroxyacyl-CoA
PBE
CPT-IA
CPT-IB
Acyl-CoA synthetase
6.2.1.3
(L)-carnitine cytosol
(L)-carnitine mitochondrial matrix
CAC
acyl-(L)-carnitine mitochondrial matrix
Acyl-CoA
2.3.1.21
CPT II
UCP2
UCP1
ACADM
L-acylcarnitine cytosol
Uncoupled respiration
Acyl-CoA
2.3.1.16
acetyl-CoA
Citric acid cycle
β-oxidation
3-oxo-acyl-CoA
1.3.1.8
4.2.1.74
HADHA
Enoyl-CoA
3-hydroxyacyl-CoA
1.1.1.35
HADHB
CF
CF
Trifunctional enzyme

Figure 3. Affymetrix-Identified Pathways. Affymetrix pathway maps for the swimming muscle of adult male Weddell seals. A) Affymetrix metacore map legend, B) Regulation of lipid metabolism; insulin regulation of glycogen metabolism map, C) PPAR regulation of lipid metabolism map. In both maps B and C, flags represent the transcripts that had significant expression across all three samples.

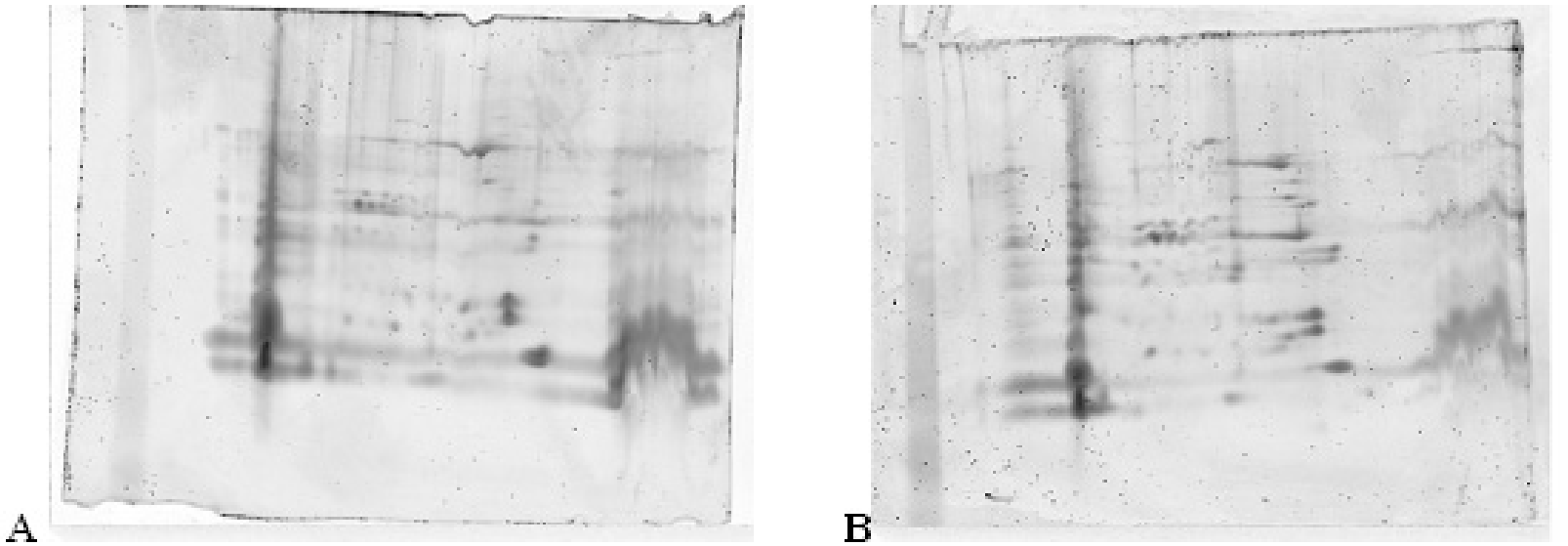


Figure 4. Pup and Adult 2DE Gel Images. 2DE gel images form M. Longissimus dorsi of adult (A) and pup (B) Weddell seal. 100µg of protein was extracted from each sample and separated on a 7cm IPG strip (pH3-10) and a 2-15% SDS-PAGE gel for the second dimension. Gels were stained with Bio-Rad's Flamingo Fluorescent stain.

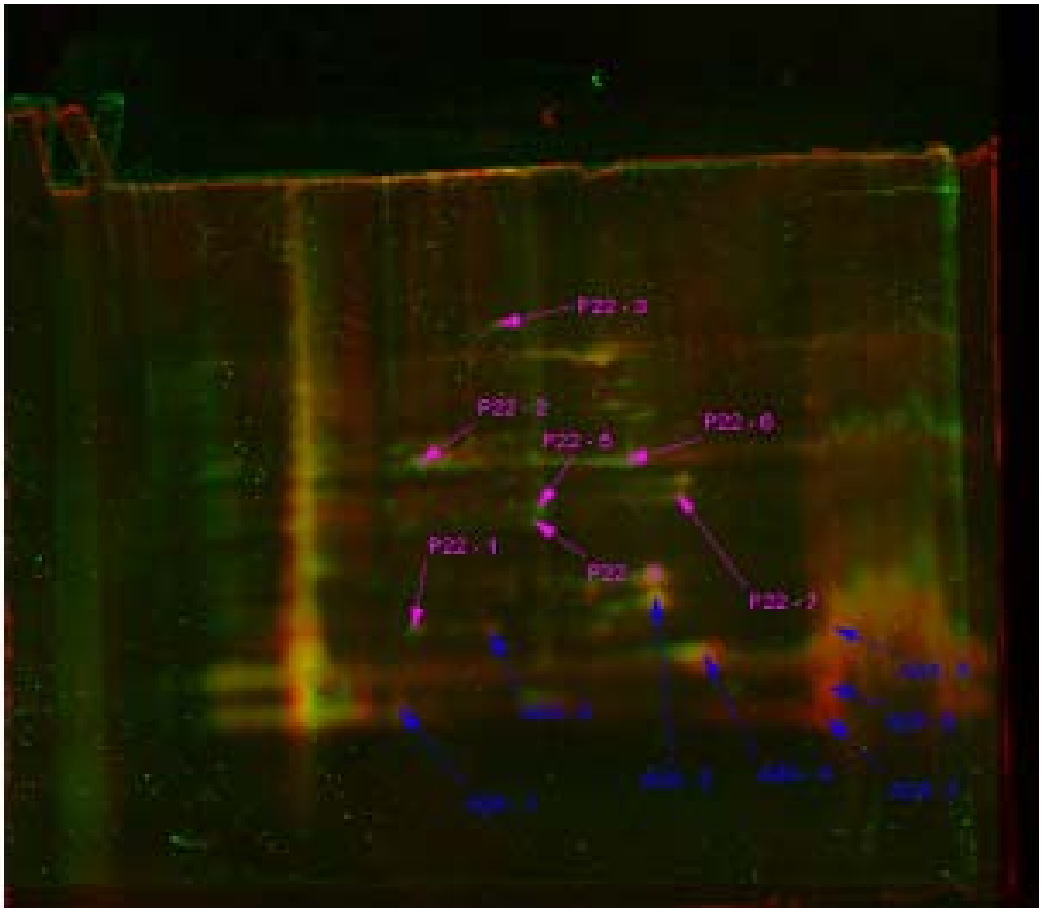


Figure 5. Overlay of Pup and Adult 2DE Gels. Overlay of adult (red spots with blue labels) and pup (green spots with pink labels) 2DE gels using Delta2D (version3.5) software; labeled spots indicate spots that are expressed differently between the two gels.

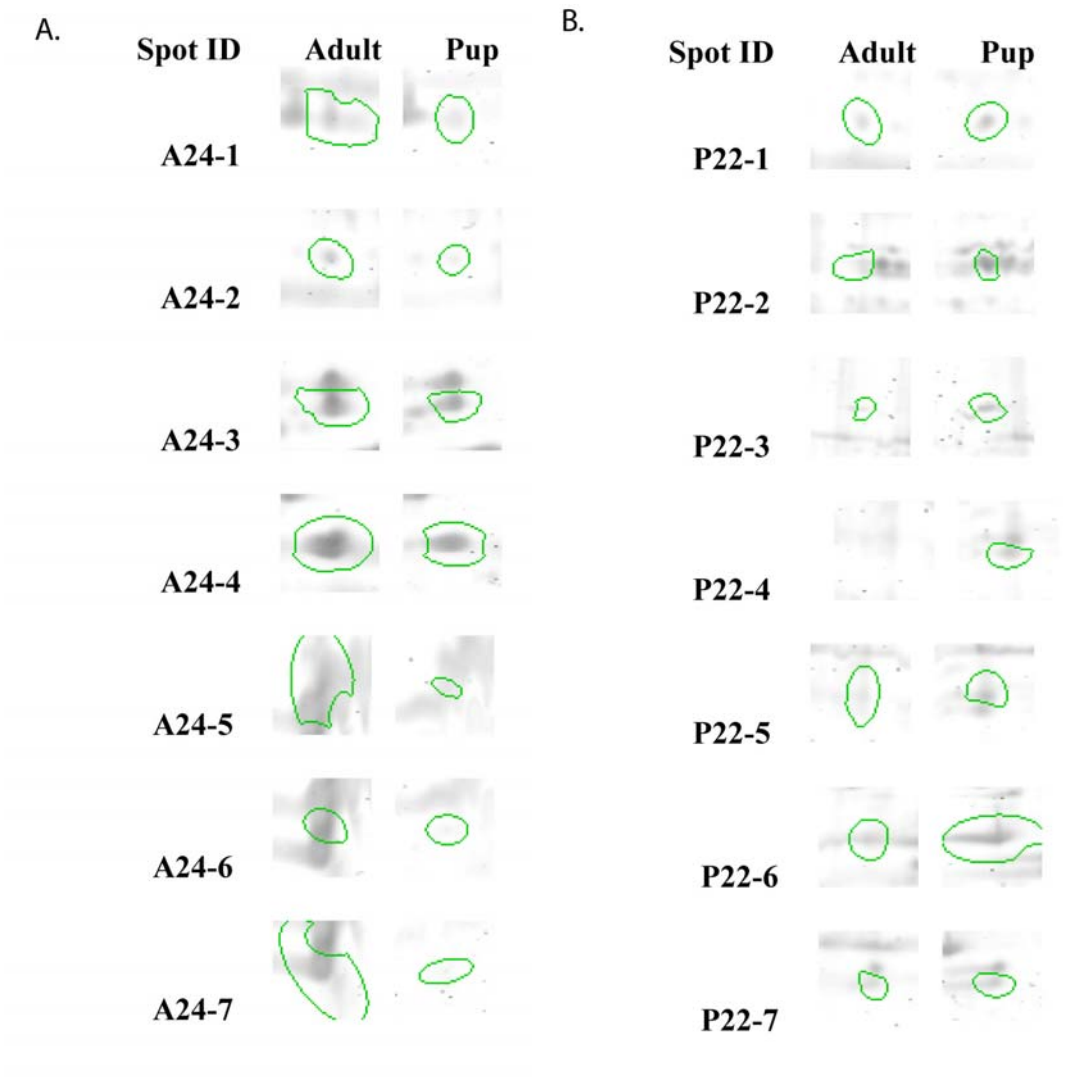


Figure 6. Comparison of 2DE spot expression densities between adult (A) and pup (B) gels. A) Illustrates where indicated spots are expressed higher in the adult gel, while B) illustrates where indicated spots are expressed higher in the pup gel.

Chapter 3 – Preliminary protein signatures for two different age classes of the Weddell seal (*Leptonychotes weddellii*) using cross-species analysis

Introduction

Terrestrial vertebrates respond to exercise by increasing ventilation, increasing heart rate and subsequent cardiac output, and peripheral vasodilation. Interestingly, unlike their terrestrial counterparts, diving vertebrates undergo a dive response upon the commencement of exercise, which entails apnea, bradycardia, and peripheral vasoconstriction. Because diving vertebrates exercise while "holding" their breath and undergo the aforementioned dive response, these animals serve as unique models for studying prolonged exercise under hypoxic conditions.

During prolonged periods of hypoxia associated with breath-hold dives, Weddell seals (*Leptonychotes weddelli*) and other diving mammals are adapted to rely on internal oxygen stores to fuel aerobic energy production in their primary swimming muscles. One of the hallmark adaptations of diving vertebrates is a significant increase in expression of oxygen-binding heme proteins (Kooyman et al 1981; Kooyman and Ponganis 1998; Kanatous et al 1999; Kanatous et al 2002). These proteins allow diving mammals to endure extreme bouts of hypoxia by supplying necessary internal oxygen stores and thus enable the animal to increase its aerobic dive limit (ADL) (Davis et al 1991; Davis and Kanatous, 1999; Wright and Davis 2006).

Because oxygen stores are so crucial to a diving animal's ADL, prodigious research efforts have been made to understand the development of diving, and specifically the development of these oxygen-binding heme proteins. One study from Burns et al (2005)

investigated the development of oxygen stores in harbor seals and determined that neonatal myoglobin concentrations were low in comparison to adults (1.6±0.2g% vs. 3.8±0.3g% for adults), while neonatal blood oxygen stores in the form of hemoglobin were not different from adults (21.7±0.4g% in pups, 23.8±0.3g% in adults). These findings imply that in this particular diving mammal, diving activity in younger, immature animals is primarily supported by blood oxygen stores, which develops rapidly, whereas muscle oxygen stores are slowly developed (Burns et al 2005). Another study, which solely focused on the development of myoglobin, compared diving marine endotherms that develop on land (pinnipeds and penguins) versus those that develop at sea (cetaceans), Noren et al (2001) determined that regardless of the location of postnatal development, neonatal divers have low myoglobin contents. These myoglobin stores appear to increase throughout development concurrently with increases in physical exertion, thermal demands, and swimming or diving induced bouts of apnea (Noren et al 2001). Therefore, the dive response, and particularly the increased expression of myoglobin stores, is has been shown to be a developed adaptation.

Similar to the aforementioned diving vertebrates, Weddell seal pups are not born capable of extreme hypoxic exercise; rather, they *develop* the ability to endure a dive response. Interestingly, nondiving Weddell seal pups have a higher potential for aerobic output than elite diving adults, as seen in the higher mitochondrial volume density and higher percentage of type I slow oxidative fibers in pups. A lack of a coincident increase in aerobic enzyme activity, however, indicates that the pups' high potential for aerobic output does not actually translate into high aerobic capacity. Rather, Weddell seal pups, which lack brown fat, are thought to have this adaptive increase in mitochondria as a

means to meet thermogenic demands in the extreme cold temperatures of Antarctica (Kanatous et al 2008a). Nonetheless, because this developmental trend is opposite that of terrestrial mammals, the process through which this adaptive change occurs is not well understood.

Previous studies from our lab verified the use of proteomics and cross-species analysis to study this unique model system (Kanatous et al 2008b). Specifically, we found that two dimensional gel electrophoresis (2DE) yielded the same age-class associated myoglobin expression trends that were consistent with western blot and myoglobin assay data. The goal of this study is to better understand the differences in skeletal muscle physiology of this unique model system using proteomics to generate protein signatures from the two physiologically distinct age classes: pups (age 3-5 weeks; nondivers) and adults (age 7+ years; expert divers). Building on previous proteomic data, this study found a suite of protein identifications for both age classes that are consistent with skeletal muscle physiology pertaining to hypoxia, ischemia, and aerobic metabolism. Several spots, however, consistently yielded unsuccessful protein matches, suggesting the presence of unique seal proteins. Furthermore, myoglobin was found in multiple gel spots, alluding to the possibility of myoglobin isoforms, which were previously thought to exist only in species of fish. Knowledge of these unique adaptations in skeletal muscle are valuable due to the potential pharmacological implications for treating human disease, specifically those that involve hypoxic conditions such as cardiovascular and pulmonary diseases.

Methods

Muscle biopsies

Muscle samples of approximately 50 mg each were collected with a 6-mm biopsy cannula (Depuy, Warsaw, Indiana) from the swimming (*M. longissimus dorsi*) muscle. Muscle biopsy samples for proteomic analysis were immediately frozen in liquid nitrogen upon collection. Frozen samples were stored at -80 ^{o}C until analysis.

Proteomic analysis

Samples were homogenized in a lysis buffer (9M urea, 4% triton X-100, 50mM DTT, 2% 3/10 ampholytes, cocktail protease inhibitor) and protein concentration was determined using a Coomassie protein assay (Pierce, Rockford, Illinois USA). To test the reproducibility of the technique, 2DE gels were run out on three different samples (i.e. n=3). For first dimension separation, 100µg of protein was diluted in 125µl of rehydration buffer (9M urea, 4% Triton X-100, 50mM DTT, 2% 3/10 Ampholytes, 0.0001% Bromophenol Blue) and applied to IPG strips (pH 3-10, pH 4-7) for rehydration. Immediately following rehydration, IPG strips ran through isoelectric focusing (IEF) in the Protean IEF Cell (Bio-Rad, Hercules, California, USA), after which they were equilibrated in two different buffers (equilibration 1: 6M Urea, 2% SDS, 0.375M Tris-HCl pH 8.8, 20% glycerol, 130 mM DTT; equilibration 2: 6M Urea, 2% SDS, 0.375M Tris-HCl pH 8.8, 20% glycerol, 135 mM DTT). Then, each strip was placed in a 15% polyacrylamide Next Gel (Amresco, Solon, Ohio, USA) with a layer agarose covering them, and gels were run at 200V for 2 hours in Next Gel (Amresco, Solon, Ohio, USA) running buffer. Protein spots were visualized using Flamingo

Fluorescent Gel Stain (Bio-Rad, Hercules, California, USA) and subsequently imaged on a Typhoon scanner. Images were overlayed and analyzed using Delta Decodon 2D software (version 3.5). Differentially expressed spots, as determined by Delta 2D, were excised and digested with trypsin. Following trypsin digestion, peptide fragments were analyzed using the Maldi-TOF/TOF and predicted peptide fragments were matched to predicted proteins using NCIB's Mascot protein database. For this study, an "n" of one was used to determine preliminary protein IDs and expression differences.

Results

Optimizing 2D Methodology for Diving Mammals

Skeletal muscle of diving mammals has an inherent high fat and salt content; as such, many modifications were necessarily made to the standard two dimensional gel electrophoresis (2DE) protocol to optimize the technique for this sample type. Standard homogenization was insufficient in terms of sample prep due to the aforementioned inherent high fat and salt content of our samples. These charged molecules interfere with first dimension isoelectric focusing; therefore, we employed TriPure reagent (Roche, Indianapolis, IN, USA) to separate out all proteins from the RNA, DNA, and lipids. Following the extraction of proteins from skeletal muscle tissue, a 2D ready-prep clean-up kit (Bio-Rad, Hercules, California, USA) was used to further remove salts and any remaining lipids.

Following sample preparation, the protocols for the first and second dimensions of protein separation were modified. For isoelectric focusing, modifications were made to

reduce horizontal streaking/smearing of proteins and increase the amount of clean, definite spots. Specifically, the focusing time and length of the IPG strips were increased. In terms of focusing, conditions were optimized to apply an incremental increase in the voltage gradient, starting from 0-3500V for a total of 23 hours. Alternatively, regarding the length of IPG strips, 7 cm IPG strips were not yielding desired spot resolution, due to the fact that there was not enough physical room for the proteins to separate, so we switched to larger 18 cm strips to increase spot quality and resolution. Lastly, we chose the 3-10 pH range because preliminary gels yielded spot coverage at both acidic and basic ranges of the gel. As for the second dimension of 2DE, the only protocol modifications made were to the equilibration step immediately prior to the second dimension; specifically, the time equilibrating IPG strips was increased to ensure complete solubilization of proteins and thus eliminate any vertical protein streaking.

Regarding the visualization of protein spots, our original Coommassie stain used for imaging the gels was not sensitive enough to detect all protein spots. Furthermore, this particular stain required more protein from our limited samples to be loaded on the gel. Alternatively, silver stain, which is more sensitive, interferes with the MALDI-TOF/TOF spectroscopy; therefore, we found the best spot detection and least spectra interference using a fluorescent stain.

Concerning identification, sensitivity limitations of the Maldi-TOF cannot produce spectra and subsequently identify proteins from lesser abundant spots. This is due to the

fact that there is physically less protein for ionization, which therefore translates into insufficient spectra data. In the future, to solve this problem we will be pooling the same spots from replicate gels to obtain protein IDs for lesser abundant proteins. Moreover, another issue we encountered with spot identification applied to abundantly expressed proteins, which may mask the presence of the lesser abundant proteins. For our initial gels, this was not a problem because we needed myoglobin (an abundantly expressed protein in our samples) to establish the reliability of the technique by confirming previously established myoglobin trends. In the future, however, it may interfere with our being able to visualize smaller, less abundant proteins, such as transcription factors. As a solution to this problem, myoglobin, and myosin, will be precipitated out of the homogenates prior to running out the 2DE gels. Then, 2D gels will be carried out with the pure, extracted myoglobin, and without myoglobin, which will give us better indication of lesser abundant proteins. Another issue with identification lies within peptide spectra that do not get protein matches. This issue is not necessarily a problem, however, because some of these unidentified proteins may actually be proteins unique to seals. The problem lies in the limited data on pinnipeds, which have little known information about their genome/proteome. If, however, we are able to obtain quality spectra on these unidentified peptides, we may be able to use the sequences to establishing a network for pinnipeds within the Mascot database.

Lastly, due to the fact that we are using such a unique model for this study, our samples are very limited in quantity. In the future, we hope to mitigate this problem by collecting more than one muscle biopsy from each animal that will be devoted to proteomics.

As a result of optimizing the technique for the unique properties of diving mammal skeletal muscle, Weddell seal 2DE gels yield reproducible gels (Figure 1).

Preliminary Protein Spot Identifications

Preliminary protein spot identifications included pup and adult gels only, due to the difficult nature of capturing juveniles and subsequent limited sample size. Thus far, thirteen spots have yielded successful identifications, and these are thirteen proteins that have never been identified before in seals using 2DE gels (Figure 2). These proteins correlate with skeletal muscle physiology, which tells us that the technique is working. In addition to the confirmation in this novel technique, there also appear to be expression differences between the two age classes, which implies that we can establish unique protein identifications for each age class. As a point of clarification, the proteins seen more than once are likely isoforms of one another or phosphorylation differences.

Unidentified Proteins

In addition to the proteins that have yielded successful identifications, there are also a few spots that still remain unidentified. Two groups of spots (see Figure 3) consistently show up on every gel and have yet to successfully match to a protein ID. Because these spots do not appear to be of particularly low abundance, it is possible that they may not be matching any proteins in the database because they are unique seal proteins. If these are, in fact, unique seal proteins, we will continue to be unable to identify the proteins until their sequences are deciphered. In future studies, therefore, we plan on doing

further analysis of the peptide spectra sequences to obtain actual sequences of these proteins.

Suite of Age Class-Specific Protein Identifications

Based on spot density quantifications from the Delta 2D software, expression differences were determined in the 13 identified spots (Table 1). As such, we have established unique protein IDs for each age class. Thus far, the pup skeletal muscle appears to have a protein ID indicating an increase in structural proteins, such as actin, and an increase in protein supplying an immediate source of ATP, such as creatine kinase. Conversely, the adult skeletal muscle has increased expression of oxygen-storage and contractile proteins like myoglobin and myosin, respsectively. Now that we have a sense of how these 13 proteins are regulated, we are closer to having a better understanding of the protein signatures of each of these unique age classes.

Discussion

Initial 2DE gels show marked differences between pup and adult age classes. As such, a proteomic avenue demonstrates sufficient promise for assessing protein signatures in skeletal muscle of adult and pup Weddell seals. While our initial success is promising, there are still significant limitations to these and future studies. Because the seal genome has not been sequenced we are limited to the use of cross species identification. This fact limits us to only being able to identify transcripts and proteins that are already described in the numerous databases. In other words, we would not be able to identify any transcripts that are unique to the seal without the use of other technologies such as protein purification and sequencing. Therefore, in order to appropriately perpetuate the success

of applying new techniques to this unique organism, sequencing of the genome is perhaps a necessary endeavor. Regardless of these limitations, however, transcriptomic and proteomic studies are the new direction in understanding the molecular adaptations to hypoxia and ischemia in this elite diving mammal.

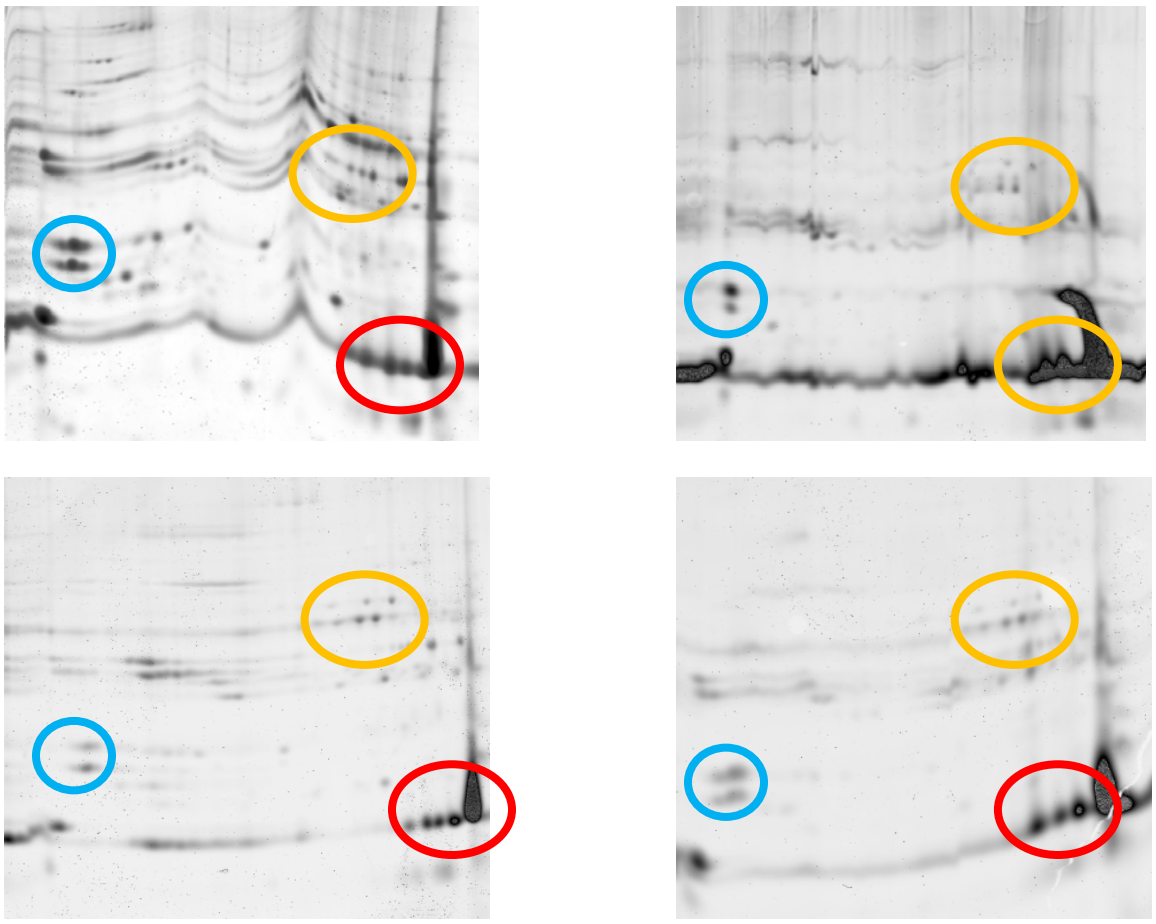
Through the identification of 13 spots from both pup and adult Weddell seal gels, we were able to generate unique protein signatures for both age classes. Of these initial protein identifications, the pup skeletal muscle appears to have a protein signature suitable for building, growth, and their appropriate metabolic demands. Specifically, an increase in structural proteins, such as actin, and an increase in protein supplying an immediate source of ATP, such as creatine kinase, matches the physiology characteristic of the pup's lifestyle. Adult skeletal muscle, conversely, has an increase in expression of myoglobin and myosin, a protein identification that is suitable for supporting a more physically active individual.

Despite pups having a higher aerobic capacity than adults (as seen by increased number of mitochondria), a lack of a simultaneous increase in aerobic enzyme activity alludes to a thermogenic role for these extra mitochondria (Kanatous et al 2008a). Therefore, because these additional mitochondria are not acting as ATP powerhouses as they do in adult Weddell seals, pups must rely primarily on immediate sources of ATP production. Creatine kinase catalyzes the reaction converting creatine to phosphocreatine. The reverse reaction, which is also dependent upon creatine kinase's enzymatic properties, is responsible for rapid generation of ATP. As such, a significant lack of aerobic

production of ATP in the mitochondria warrants the pup to be more reliant on creatine kinase and its hasty generation of ATP.

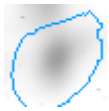
Unlike the nondiver pups, adult Weddell seals live an active lifestyle as elite divers. During these breath-hold dives, energy production is fueled by aerobic respiration via internal oxygen stores bound to myoglobin (Kanatous et al 2002). Expression of myoglobin, however, has recently been shown to rely on both hypoxia *and* exercise (Kanatous et al 2009). Therefore, higher myoglobin expression in adults found in 2DE gels is consistent with activity differences between elite diver adults and nondiver pups. Furthermore, higher expression of myosin, a contractile protein, is also more suitable for the active lifestyle of adult Weddell seals.

In summary, cross species applications in studying physiological changes in Weddell seals has proven promising in deciphering the molecular regulation of their adaptations. The future utilization of cross-species comparison is still ultimately limited by the lack of a sequenced genome in the Weddell seal. Despite that limitation, cross-species analysis is a very powerful tool to understand differences in protein expression within Weddell seals and across different species.



Pup Adult

Figure 1. 2DE gel images from pup and adult samples. Images of 2DE gels from pup (left) and adult (right) samples (n=3). Spots consistently show up in both between age classes and in replicates within each age class.

Protein ID	Pup (P17)	Adult (A23)
Enolase 3		
Creatine Kinase		
Glyceraldehyde Phosphate Dehydrogenase (a)		
Glyceraldehyde Phosphate Dehydrogenase (b)		
Tropomyosin 3		

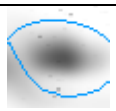
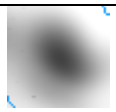
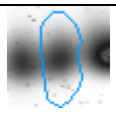
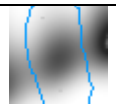
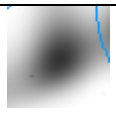
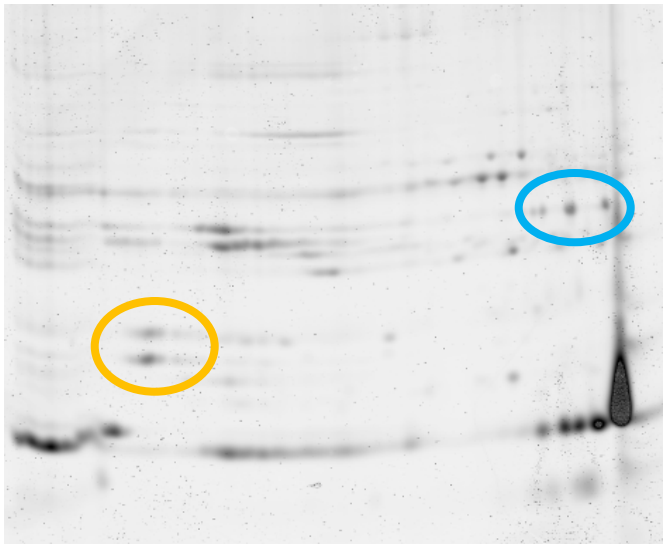
Troponin T		
Myosin Light Chain		
Alpha-Actin		
Actin Chain A		
Peptidylprolyl Isomerase A		
Heat Shock Protein 27		
Myoglobin 1		
Myoglobin 2		

Figure 2. Spot Identifications. Protein identifications for 13 spots from pup and adult gels, demonstrating expression differences between the two age classes.



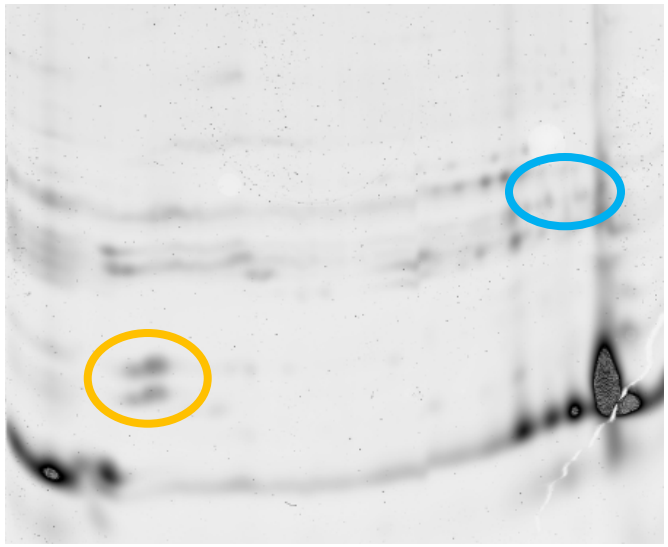


Figure 3. Unidentified Protein Spots. Sets of spots that consistently appear in every gel (pup, left; adult, right), but have yet to yield successful peptide matches in the Mascot database. It is possible that peptide spectra are not matching protein identifications due to the fact that they may be unique seal

Table 1. Protein Identifications and Expression Differences. Thirteen proteins, separated into functional classes, identified in pup and adult samples. Each identification includes the gi accession number for the MASCOT database, the MASCOT statistical score, and the percent volume of total protein compensated for by that particular protein spot.

Functional Class	Protein name	gi number	MASCOT Score	% Volume Pup 17	% Volume Adult 23
Metabolism	Enolase 3	77736349	145	0.608	0.3641
	Creatine Kinase	194018722	270	0.1306	0.0496
	Glyceraldehyde Phosphate Dehydrogenase (a)	1177672	85	0.3392	0.6652
	Glyceraldehyde Phosphate Dehydrogenase (b)	115502204	80	0.1786	0.2227
Structure/Contraction	Tropomyosin 3	58652133	116	1.4682	0.4048
	Troponin T	73947490	119	0.154	0.136
	Myosin Light	57101266	102	2.0656	4.8323

	Chain				
	Alpha Actin	49864	147	0.0833	0.0032
	Actin Chain A	20664362	130	0.231	0.048
	Peptidylprolyl Isomerase A	30102944	72	0.1263	0.362
Stress Response/ Transport	Heat Shock Protein 27	149755998	153	0.2151	0.0211
	Myoglobin 1	62901702	243	2.1128	2.7694
	Myoglobin 2	62901702	210	2.1846	5.0703

Chapter 4 – Conclusion

2DE Protocol for Diving Mammals

Differential protein analysis employing 2DE has, to our knowledge, never been applied to diving mammals, thus making this a pioneer study in the field of diving mammal physiology. Modifications to the protocol, therefore, became a primary focus of this study. Diving mammals, particularly skeletal muscle tissue, have an inherent elevation in fat and salt content. These charged molecules interfere with isoelectric focusing, making careful removal essential for proper spot resolution. Applying extra steps in sample preparation, specifically the use of TriPure (Roche, Indianapolis, IN, USA) to isolate protein and a 2D Ready Prep Clean Up Kit (Bio-Rad, Hercules, CA, USA), is therefore necessary to optimize focusing. Further additional steps, such as increasing IPG strip size to clean up isoelectric streaking and pooling together small spots from different gels to yield accurate peptide peaks and subsequent protein identifications, have also proved necessary for this unique sample type.

Cross-Species Analysis

Adding yet another layer of complication to this study, Weddell seals and pinnipeds as a whole do not have a sequenced genome; therefore, identifying proteins necessarily relied on the information available from other species. As such, another crucial main objective was to verify the reproducibility of using cross-species analysis on the Weddell seal.

To achieve this verification, protein expression results from the current study were compared to previously published data from the Kanatous laboratory. Specifically, trends in myoglobin expression from 2DE gels were found to match that of myoglobin assays, with juveniles showing the highest expression, followed by adults with the second highest myoglobin expression and pups with the lowest (Kanatous et al 2008a).

Further adding to the verification of cross-species analysis, identified proteins were not unrelated to skeletal muscle; meaning, the thirteen identified proteins in both adult and pup 2DE gels (Chapter 3, Figure 1) consistently correlated with the physiology of skeletal muscle tissue. Identified Weddell seal proteins, of which included metabolic, structural, stress response, and transport protein classes, were relevant to the skeletal muscle functionality.

Until the pinniped genome is sequenced, proteomics studies in pinnipeds (and other diving mammals, such as cetaceans) are contingent upon cross-species analysis. This study's verification of cross-species analysis has demonstrated that this is a reliable technique that can be employed to study skeletal muscle physiology of diving mammals.

Preliminary Suite of Protein Signatures

Initial protein identifications and quantifications in pup and adult Weddell seal 2DE gels demonstrate both the presence of proteins consistent with skeletal muscle physiology and differential expression of the identified proteins between the two age classes, thus creating unique protein signatures for both the pup and adult. Of these initial protein

identifications, the pup skeletal muscle appears to have a protein signature suitable for building, growth, and their appropriate metabolic demands. Specifically, an increase in structural proteins, such as actin, and an increase in protein supplying an immediate source of ATP, such as creatine kinase, matches the physiology characteristic of the pup's lifestyle. Adult skeletal muscle, conversely, has an increase in expression of myoglobin and myosin, a protein identification that is suitable for supporting a more physically active individual.

Despite pups having a higher aerobic capacity than adults (as seen by increased mitochondrial volume), a lack of a simultaneous increase in aerobic enzyme activity alludes to a thermogenic role for these extra mitochondria (Kanatous et al 2008a). Therefore, because these additional mitochondria are not acting as ATP powerhouses as they do in adult Weddell seals, pups must rely primarily on immediate sources of ATP production. Creatine kinase catalyzes the reaction converting creatine to phosphocreatine. The reverse reaction, which is also dependent upon creatine kinase's enzymatic properties, is responsible for rapid generation of ATP. As such, a significant lack of aerobic production of ATP in the mitochondria warrants the pup to be more reliant on creatine kinase and its hasty generation of ATP.

Unlike the nondiver pups, adult Weddell seals live an active lifestyle as elite divers. During these breath-hold dives, energy production is fueled by aerobic respiration via internal oxygen stores bound to myoglobin (Kanatous et al 2002). Expression of myoglobin, however, has recently been shown to rely on both hypoxia *and* exercise

(Kanatous et al 2009). Therefore, higher myoglobin expression in adults found in 2DE gels is consistent with activity differences between elite diver adults and nondiver pups. Furthermore, higher expression of myosin, a contractile protein, is also more suitable for the active lifestyle of adult Weddell seals.

Future Directions

Development of a 2DE protocol tailored to the uniqueness of diving mammal skeletal muscle tissue in conjunction with verification of cross-species analysis allows for continual use of this novel technique in diving mammal research. Future research will aim to include more Weddell seal RNA (microarray) work. Such studies will prove beneficial by comparing transcript expression to protein expression, which will provide insight as to when protein modification actually occurs (i.e. transcriptionally or post-translationally).

In addition to the inclusion of more RNA analysis, another avenue of interest in our protein studies includes immunoprecipitation. Using antibodies to separate out proteins has promise for better understanding of functional proteomics due to its ability to separate out individual protein complexes while still maintaining their inherent function (Monti et al 2009). Furthermore, precipitating out proteins of large abundance within the sample (e.g. myoglobin and myosin) will allow us to focus on lesser abundant proteins, such as transcription factors, which are important molecular regulators.

Immunoprecipitation of myoglobin will also allow us to focus on the potential myoglobin isoforms detected in this study by running native protein gels including only myoglobin. Alternatively, if these are not isoforms, the series of multiple myoglobin spots in the gel may be due to phosphorylation differences. To determine whether or not myoglobin is being phosphorylated, phosphoproteins will be isolated from the entire sample for 2DE analysis (Lee and Mykles 2006).

Finally, future studies will amalgamate the results from protein and RNA studies aforementioned. Specifically, transcripts and proteins related to regulation of muscle ontogeny that appear to be differentially expressed between adult and pup samples will be transfected into a C2C12 line of cells. These cells will then be cultured against Weddell seal primary cells in attempt to drive the seal phenotype. This research is of particular importance because if the unique exercise phenotypes of seals can be driven in a control cell line, then there is potential for this phenotype to be pharmacologically driven in people in efforts to offset deadly the deadly repercussions associated with hypoxic diseases, such as cardiovascular and pulmonary diseases.

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