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EXPRESSION OF ALTERNATIVE OXIDASE IN THE COPEPOD *T. CALIFORNICUS* WHEN EXPOSED TO ENVIRONMENTAL STRESSORS

by

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Abstract

In addition to the typical electron transport system in animal mitochondria responsible for oxidative phosphorylation, some species possess an alternative oxidase (AOX) pathway, which causes electrons to bypass proton pumping complexes. Although AOX appears to be energetically wasteful, studies have revealed its wide taxonomic distribution, and indicate it plays a role in environmental stress tolerance. AOX discovery in animals is recent, and further research into its expression, regulation, and physiological role has been impeded by the lack of an experimental model organism. DNA database searches using bioinformatics revealed an AOX sequence present in the arthropod *Tigriopus californicus*. Multiple sequence alignments compared known AOX proteins to that of *T. californicus* and examined the conservation of amino acid residues involved in AOX catalytic function and post-translational regulation. The physiological function of a native AOX has never been identified in an animal that produces it, but I hypothesize that AOX protein levels will change in response to environmental stress in *T. californicus*. In order to test this hypothesis, copepods were exposed to five different temperatures (6, 10, 15, 22 and 28°C), and extended periods of light/dark. Samples were taken after 24 hours (acute) and 1 week (chronic) of incubation at each stress treatment. In conclusion, *T. californicus* possesses the necessary residues required for AOX function. Furthermore, Western blots demonstrate that there are fluctuations in AOX expression when exposing *T. californicus* to temperature stress. In contrast, during light stress AOX is constitutively expressed when animals were subjected to changes in their circadian rhythms. AOX has been most thoroughly characterized in a number of plants; however, the physiological function of a native AOX has never been identified in an animal that produces it. This is the second study to confirm AOX protein expression in an animal and is the first study to look at a native AOX protein in an

animal and its response to biotic stress. By understanding why *T. californicus* possesses AOX, we can better understand why some other organisms, including humans, do not express or have lost the AOX gene. More thorough investigation of AOX in copepods may aid in the development of a drug that can be added to fish aquaculture to exterminate parasitic copepods and prevent the loss of economically valuable fish.

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CHAPTER 1

Introduction

1. Introduction

1.1 Cellular Respiration and the Alternative Oxidase Pathway

Living cells need to make energy, in the form adenosine triphosphate (ATP), in order to sustain life. This is accomplished through a process called cellular respiration, which is a biochemical pathway that releases energy from chemical bonds, stores the energy as ATP, and uses it for essential life processes (Hirsch *et al*., 2002). ATP is biosynthesized through the controlled breakdown of organic molecules. In most animals, organic molecules go through 4 main stages in order to synthesize ATP: 1) glycolysis, 2) pyruvate oxidation, 3) the citric acid cycle, 4) oxidative phosphorylation (Morris, 2013). The first three stages produce a small quantity of ATP directly through substrate level phosphorylation, and also reduce nicotinamide adenine dinucleotide (NAD^+) to NADH and flavin adenine dinucleotide (FAD) to $FADH_2$. The role of NADH and $FADH_2$ is to shuttle electrons that are released from the breakdown of organic substances to the electron transport system (Morris, 2013). The electron transport system (ETS) is embedded in the inner membrane of the mitochondria and is comprised of protein complexes. The four complexes, numbered I, II, III, and IV, shuttle electrons and at the same time create a proton gradient across the inner mitochondrial membrane (Lodish *et al*., 2008). The ETS accepts the electrons from NADH and $FADH_2$. As electrons move down the ETS, energy is released and used to pump protons out of the matrix of the mitochondria and into the intermembrane space, thereby creating a gradient. ATP synthase allows the protons to return to the matrix, from the intermembrane space, through the use of the proton-motive force, which transduces energy into ATP (Lodish *et al*., 2008). ATP is used as an energy source in a multitude of intracellular processes.

The alternative oxidase (AOX) pathway is a distinctive pathway of electron withdrawal, which causes the electrons to bypass complexes III and IV, of the ETS, and results in cyanide-resistant respiration (Rogov *et al*., 2016) (Figure 1). AOX catalyzes the oxidation of reduced ubiquinol (i.e. it is a terminal quinol oxidase that reduces oxygen to water) and is situated in the ETS, in the mitochondria of certain organisms (McDonald, 2008; Rogov *et al*., 2016). The pathway's ability to reduce oxygen to water requires a transition metal, such as iron (Moore *et al*., 2013). This pathway introduces a branch point at ubiquinone, bypassing two sites of proton translocation within the ETS and as a result yields less ATP per oxygen consumed (McDonald and Vanlerberghe, 2004). AOX was previously considered to only be possessed by fungi, plants, and protists, but recently it was discovered in certain animals (McDonald and Vanlerberghe, 2004; McDonald 2008).

1.2 Physiological Role(s) of AOX

AOX is widely distributed in nature, but its physiological function(s) remains ambiguous. It is energetically wasteful for an organism to use AOX in terms of ATP biosynthesis, the operation of AOX dissipates the energy of the electrons that it uses as heat. Researchers have hypothesized numerous advantages conferred by AOX in order to explain its broad taxonomic distribution. One confirmed benefit AOX provides to some plants is thermogenesis, which is defined as the production of heat by an organism (Angioy *et al*., 2004). The heat generated through thermogenesis facilitates the emission of an odour that aids in pollination by attracting insect pollinators (Angioy *et al*., 2004). Despite this finding, it is unknown why AOX is present in non-thermogenic plants, a group making up the bulk of plant species (McDonald, 2008).

Previous studies indicate that AOX likely plays a role in the ability of organisms to tolerate various environmental stresses. Research has shown that there is an increase in AOX

mRNA expression, when plants are exposed to cold stress, which contributes to the tolerance of plants to cold (Li *et al*., 2011). Furthermore, it has been demonstrated that when the AOX pathway is up-regulated during cold stress, it leads to a 20% reduction in plant growth in *Arabidopsis* plants (Fiorani *et al*., 2005). When plants face conditions of cold stress, the AOX pathway may serve an essential role in controlling the balance between antioxidant defenses and metabolism (Li *et al*., 2011).

Another hypothesized advantage that AOX confers to cells is controlling reactive oxygen species (ROS) production (Møller, 2001). ROS are chemically reactive molecules of oxygen that damage macromolecules, and AOX may limit their generation by acting as an overflow pathway in the ETS (Møller, 2001). ROS such as superoxide, the hydroxyl radical, and hydrogen peroxide are formed in organisms through normal metabolic processes (Maxwell *et al*., 1999). In eukaryotic cells, the majority of ROS comes from the mitochondria (Maxwell *et al*., 1999). A study conducted by Maxwell *et al.* in 1999 demonstrated that AOX serves to keep mitochondrial ROS formation low in plant cells. It was proposed that this is done through a second oxidase (namely AOX) downstream of the ubiquinone pool, in the ETS, which maintains upstream electron transport components in a more oxidized state, leading to a lower generation of ROS by preventing the over reduction of electron carriers (Maxwell *et al*., 1999).

The fungal phytopathogen *Ustilago maydis* has also been shown to possess alternative oxidase (Juárez *et al*., 2006). A study conducted by Juárez *et al*. (2006) found that one of the metabolic roles of AOX in *U. maydis* is the prevention of ROS production. It has been extensively reported that AOX makes a significant contribution to the prevention of ROS production (Czarna and Jarmuszkiewicz, 2005; Maxwell *et al*., 1999; Robson and Vanlerberghe, 2002). Juárez *et al*. (2006) also discovered that AOX increases the metabolic plasticity of the

cell, and enables it to avoid metabolic collapse when placed in conditions that impairs the cytochrome pathway. Similar to other cosmopolitan organisms, *U. maydis* is subjected to numerous changes in environmental parameters. During *U. maydis* cell culturing, when assay temperatures were increased to 28°C, there was a 3.5-4.3× increase in AOX function (Juárez *et al*., 2006). Juárez *et al*. (2006) concluded that AOX allows the mitochondrial metabolism to be active when subjected to biotic and abiotic stressors that can limit the activity of the cytochrome pathway. These hypotheses propose that organisms that contain cells which express AOX may be able to respond effectively to a wide range of abiotic environmental stressors.

1.3 AOX Protein Structure and Regulation

AOX is a mitochondrial, membrane bound protein that catalyzes the oxidation of ubiquinol while reducing oxygen to water (Pennisi *et al*., 2016). AOX is not inhibited by cyanides, which are frequently used to inhibit cytochrome c oxidase (McDonald and Gospodaryov, 2018). This inability to be inhibited by cyanide is due to AOX not possessing heme or copper; instead it contains a di-iron centre (Moore *et al*., 2013). AOX is instead inhibited by salicylhydroxamic acid and alkylated gallates (Rogov *et al*., 2016). AOX is a homodimeric protein which is categorized within the group of di-iron carboxylate proteins (Berthold *et al*., 2002). Each monomer is composed of six long helices and four short helices, which are arranged in an antiparallel fashion (Figure 2) (Moore *et al*., 2013). Being composed of a di-iron centre and four helices (Figure 3), alternative oxidase is associated with the inner mitochondrial membrane (McDonald, 2008). Located in the four helices are highly conserved amino acids that are confirmed to play a role in the enzymatic function and regulation of the AOX protein. There are several glutamate (Glu, E) and histidine (His, H) residues required for the activity of AOX, as they are responsible for coordinating the di-iron centre (Figure 3)

(Siedow & Umbach, 1995). This finding has been confirmed in multiple studies using AOXs found in plants such as *Sauromatum guttatum* (Albury *et al*., 2002) and *Arabidopsis thaliana* (Berthold *et al*., 2002) and the protist *Trypanosoma brucei* (Kido *et al*., 2010).

1.4 AOX in Animals

Previously, AOX was deemed limited to such organisms as bacteria, plants, fungi and protists, but not long ago it was discovered in some animals (McDonald and Vanlerberghe, 2004; McDonald 2008). The presence of an AOX gene was discovered just over a decade ago (McDonald & Vanlerberghe, 2004). Over the past couple of decades extensive sequencing and analysis of animal genomes has uncovered a non-conventional mitochondrial respiratory system enzyme (McDonald & Gospodaryov, 2018). AOX sequences have been identified in a multitude of animal phyla, including *Placozoa, Porifera, Cridaria, Annelida, Echinodermata, Mollusca, Nematoda, Hemichordata and Chordata* (McDonald & Gospodaryov, 2018). The expression of AOX mRNA was originally confirmed in several tissues in the Pacific oyster *Crassostrea gigas* (McDonald & Vanlerberghe, 2004). AOX is present in the simplest multicellular animal, *Trichoplax adhaerens,* and several members of the phylum *Chordata* (McDonald *et al*., 2009). One research paper identified the alternative enzymes in mitochondria isolated from *Artemia franciscana* nauplii (Rodriguez-Armenta *et al*., 2018). Subjection of *A. franciscana* to cyanide and octyl-gallate, which causes inhibition of the organism's mitochondrial oxygen consumption, suggests that alternative oxidase is present (Rodriguez-Armenta *et al*., 2018). Work on animal AOX has been limited by the lack of an animal model in which to conduct experiments. Recently, AOX was identified in members of the phylum Arthropoda, in several species of copepods.

1.5 Copepods

With over 12,000 species, copepods are one of the most numerous multicellular organisms on Earth (Lee *et al*., 2005). The success of these crustaceans is dependent on their high reproductive rates and fast development times. Similar to other crustaceans, copepods have separate sexes, with males and females that differ in their sexually dimorphic characteristics, which develop during the copepodid stage. Typically, females are larger than males and live-longer than males. Conversely, males are smaller, short-lived, and their fifth legs are highly modified, possessing antennules that are used during mating (Lee *et al*., 2005). The species *Tigriopus japonicas* is reproductively active after 21 days of development under laboratory conditions (Raisuddin *et al*., 2007). In aquatic ecosystems, the male copepod typically locates their female counterpart, by using chemoreceptors present in the body (Sehgal, 1983). The male swims after the female and catches her with his modified antennule (Fraser, 1936). The period of copulation lasts anywhere between a few minutes to a couple of days (Sehgal, 1983). The fertilization of the female's eggs does not occur until after the male and female separate and the eggs leave the female's oviducts. The number of eggs that can be carried by a single female varies with seasonal characteristics (temperature, food availability, etc.) (Sehgal, 1983). Normally, females are highly reproductive and carry multiple broods of eggs, which develop following a single mating interaction (Koga, 1970). The brood size can very between 30-50 nauplii, depending on the species of copepod (Raisuddin *et al*., 2007). The fertilized eggs are carried in either one sac (calanoids) or two sacs (cyclopoids). When females lay their eggs, they either deposit their eggs freely into the surrounding environment or gravid females carry them affixed to their genital segment in egg masses until the nauplii are hatched.

1.6 Copepods and Their Role in the Ecosystem

Numerous marine fisheries yield small pelagic eggs. The larvae that hatch from these small eggs require a source of live food shortly after the commencement of exogenous feeding (Lee *et al*., 2005). Copepods play an essential role in the aquatic food chain. They constitute an intermediate trophic level between bacteria, algae and protozoans on the one hand and are prey to small and large plankton eaters, which consists of mainly fish (Sehgal, 1983). Several research studies suggest that harpacticoid copepods are a better food source in marine aquaculture compared to other zooplankton such as rotifers or brine shrimp (Lee *et al*., 2005). Specifically, harpacticoids have a higher concentration of unsaturated fatty acids compared with other live feeds used in mariculture (Lee *et al*., 2005).

Harpacticoid copepods promote the rapid growth and/or high reproductive rates in fishes and invertebrates (Cutts, 2002). One study done by Volk *et al*. (1984) demonstrated that food conversion efficiency was higher in juvenile *Oncorhynchus keta* fed the harcapticoid copepod *Tigriopus californicus* in comparison to when they were fed calanoid copepods or amphipods. They ascribed this difference to the higher caloric content of *T. californicus* compared with amphipods and a lack of escape response compared with calanoid copepods. Indeed, harpacticoid copepods serve as a vital food resource for many species of marine fish (Coull, 1990). Throughout a fish's lifetime they undergo an ontogenetic shift from a diet composed largely of harpacticoid copepods to larger-bodied prey (McCall & Fleeger, 1995). On the other hand, for some fish, harpacticoid copepods may serve as prey for the entire lifetime of certain marine fishes (Tipton & Bell, 1988).

1.7 Copepods and Environmental Stress

Copepods face a wide variety of environmental stresses that are both abiotic (e.g.

fluctuating in salinity levels, temperature levels, and day/night cycles) and biotic (i.e. competition and predation) (Burton & Lee, 1994). Within the life cycle of many copepods there is a dormancy or suppressed development feature, which is utilized when faced with environmental stress (Seebens *et al*., 2009). Depending on the species, this suppressed development might occur at the embryonic, naupliar, copepodid or adult phase of their life cycle (Seebens *et al*., 2009). Dormancy encompasses a range of suppressed development, whether it is quiescence or diapausing (Ortells *et al*., 2005). Quiescence is defined as a prompt response to adverse environmental conditions (Zhou *et al*., 2016). In general, copepods experiencing quiescence resume development as soon as the immediate environmental condition is alleviated (Zhou *et al*., 2016). For example, when a warm water species of copepod is subjected to cold temperature, its developmental rate begins to slow down, but once placed back into its naturally occurring temperature its developmental rate begins to speed up again. Conversely, diapause refers to an organism undergoing a biochemical, physiological, and/or endocrinal adaptation and females who are pregnant begin to produce diapausing eggs (Lee *et al*., 2005). Diapausing eggs, also known as resting eggs, are encysted embryos in an arrested state of development (Montero-Pau *et al*., 2008). In order for aquatic invertebrates to cope with the unpredictability of their environments, they produce resting eggs. These eggs face a wide range of environmental extremes and have developed mechanisms to survive these conditions (Carlisle, 1968). Diapausing will only halt following the completion of a refractory phase that could last days to months on end. During the above mentioned refractory phase, copepods will not resume development even if conditions become favourable. These eggs can face a wide range of environmental extremes and have developed mechanisms to survive these conditions (Carlisle, 1968). This stage will allow the encysted embryos to withstand their harsh environment and they

will remain in this dormancy stage (Caceres, 1998). There is one very important distinction between quiescence and diapausing, diapausing eggs of copepods are able to survive long-term (several months) even when exposed to toxic chemicals such as hydrogen sulfide, whereas copepods undergoing quiescence are not capable of surviving long periods of time especially when exposed to toxic chemicals (Lee *et al*., 2005).

1.8 *Tigriopus* as a Model Organism

Harpacticoid copepods, which belong to the genus *Tigriopus*, are a subclass of Copepoda and belong to the phylum Arthropoda. Copepoda are the second largest Crustacean taxa and over 12,000 species of copepods exist (Raisuddin *et al*., 2007). Furthermore, copepods are of high ecological importance as they are one of the most dominant taxa in aquatic zooplankton communities, making up 70% of the ocean's biomass (Wells, 1984). Copepods tend to be more abundant in still bodies of water, such as pools and ponds (Sehgal, 1983). *Tigriopus* encompasses four well studied model species (*T. brevicornis, T. californicus, T. fulvus* and *T. japonicus*) and numerous other less studied species.

Over the past couple of decades, there has been an increasing interest in the copepod genus *Tigriopus*, with a substantial number of publications having focused on these organisms (Raisuddin *et al*., 2007). There are multiple characteristics that make *Tigriopus* a favorable model organism for environmental studies. The majority of the species of *Tigriopus* spp. are small in size (i.e. an adult's length is approximately 1.0 mm) and they possess a discernible brown orange colour, but their colour is highly depend on their diet (Harris, 1973). Similar to all copepods, *Tigriopus* spp. goes through 12 post-embryonic stages of development: 6 naupliar stages, 5 copepodid stages and an adult stage (Figure 4) (Fraser, 1936).

T. californicus is an intertidal species of copepod that inhabits rock pools on the Pacific coast of North America (Burton and Lee, 1994). Due to *T. californicus'* intertidal habitat, it is constantly being exposed to ever changing environmental stressors including: temperature, salinity, predation, and oxygen levels (Burton and Lee, 1994). Copepods reproduce sexually and fertilized eggs are held in a sac and against the urosome of the females (Marini and Sapp, 2003). This sac appears dark brown in colour, in comparison to the rest of the organism's body. The brood size of a gravid *T. californicus* female is 17±4.2 eggs, when they are acclimated to temperatures between 10-15°C (Powlik *et al*., 1997). These copepods emerge from eggs as a nauplius, which is composed of six stages, N1-N6 (Kvile, 2015). During the nauplius stages this organism experiences periods of growth and changes to their overall body shape (Kvile, 2015). The nauplius stages are followed by six copepodid stages, which are characterized by growth to a maximum size of approximately 1 mm in length (Marini and Sapp, 2003). Furthermore, the animals appear segmented, with prominent antennae and five sets of legs (Kvile, 2015). Between each of the six developmental stages the copepods continue to grow and shed their exoskeletons (Marini and Sapp, 2003). It is not until the 5th copepodid stage that the gender of the copepod can be identified (Marini and Sapp, 2003). When *T. californicus* copepods are exposed to environments possessing higher temperatures and salinities their average lifespan is 21 days (Powlik *et al*., 1997). Conversely, when they reside in habitats that possess lower temperature and salinity levels they can survive approximately 30 days (Powlik *et al*., 1997).

T. californicus is characterized by a short generation time, small space requirements, and many genetically divergent populations which can be cross-bred in the laboratory (Burton and Feldman, 1981). These attributes make *T. californicus* an emerging model organism in biology for the study of environmental stress responses in animals.

1.9 Heat Shock Proteins: Structure and Function

Heat shock proteins (HSPs) are highly conserved and present in all prokaryotic and eukaryotic organisms (Li and Srivastava, 2004). They are characterized as stress-inducible molecular chaperones and are proteins which range in size from 12 to 43 kDa (Seo *et al*., 2006). Molecular chaperones are proteins that aid in the folding, unfolding, and assembly of other molecular structures (Seo *et al*., 2005). Furthermore, HSPs have a molecular mass of 200-800 kDa when organisms are subjected to stressful conditions. This is caused by the interaction between small HSP subunits, which results in the formation of a multimer, which is crucial for chaperone activity (MacRae, 2000). The products of these genes are responsible for protecting cellular proteins and repairing DNA damage (Kim and Hagiwara, 2011). When animals experience thermal stress, heat shock proteins are induced to help the organism survive (Dutton and Hofmann, 2009). The role of HSPs are to assist in the refolding of stress denatured proteins, thereby preventing them from aggregating in the cell and permitting the cell to cope with the environmental stress (Wang *et al*., 2013). HSPs constitute a large family of proteins that are classified based on their molecular weight (e.g. HSP20, HSP70, HSP90, etc.).

1.10 Housekeeping Genes

A housekeeping or reference gene or genes are those that are expressed ubiquitously and constitutively by different cell types and are utilized when normalizing data, whether it be for protein expression or reverse transcription quantitative polymerase chain reaction (qRT-PCR) (Reboucas *et al*., 2013). Housekeeping genes act as internal standards that allow for the normalization of signals and enable different samples to be compared to one another by eliminating variations arising due to technical reasons, such as differences in the amount of sample loaded and transfer efficiency (Ferguson *et al*., 2005). One of the best-known

housekeeping genes in literature is tubulin, along with glyceraldehyde-3-phosphate desidrogenase (GAPDH), β-actin, and several ribosomal proteins (RPL) (Reboucas *et al*., 2013, Ferguson *et al*., 2005).

1.11 Gaps in the Literature

In addition to the typical electron transport system in animal mitochondria responsible for oxidative phosphorylation, in some species there exists an alternative oxidase (AOX) pathway, which permits an alternate root of electron exit. The discovery of AOX in animals is recent and further research into its expression, regulation, and physiological role has been impeded by the lack of a versatile experimental model organism. The physiological function of a native AOX has never been identified in an organism that produces it. DNA database searches using bioinformatics revealed an AOX sequence present in the organism *Tigriopus californicus*. *T. californicus* is a marine invertebrate copepod which inhabits rock pools located along the west coast of North America and is subjected to daily fluctuation in environmental stressors. This makes *T. californicus* an excellent organism for the investigation of animal AOX in order to gain a deeper understanding of its physiological function and its role in temperature regulation and light stress. It has been previously confirmed that *T. californicus* possesses an alternative oxidase (AOX) gene, transcribes AOX mRNA, and translates AOX protein (Tward *et al*., 2019). Unfortunately, the function of AOX and T. californicus is unknown. Based on previous research on plants and fungi, it is thought that the AOX pathway provides metabolic flexibility and gives the organism the ability to survive under a multitude of environmental stressors.

2. Purpose

The first purpose of this thesis is to analyze the primary sequence of the AOX protein in *T. californicus* by comparing it to AOX protein sequences from other organisms. This will allow

us to determine if it possesses several conserved residues that are required in order for enzyme function. In addition, this analysis may reveal protein characteristics that are unique to animal AOXs and/or the AOXs of arthropods and copepods. The second purpose of this research project is to determine if the AOX protein is expressed in the copepod *T. californicus* and whether AOX protein levels change in response to temperature and light stress. *T. californicus* will be exposed to varying environmental temperatures (6, 10, 22, and 28°C), which will be particularly stressful to the animal, in comparison to its usual habitat temperature, in our lab, of 15°C. Furthermore, copepods were exposed to acute (24 hours) and chronic (1 week) level of light and dark exposure. This may help to determine whether AOX may play a role in the ability of *T. californicus* to tolerate environmental stress. These experiments may also provide insight into the loss of AOX in some animal species throughout the span of evolution (McDonald *et al*., 2009; McDonald and Vanlerberghe, 2004). For example, the human genome does not contain AOX, and the information gathered from our research may eventually contribute to the treatment of mitochondrial dysfunctions and disorders in humans using AOX gene therapy (Kemppainen *et al*., 2013). As well, it may lead to insights that could aid in the development of anti-parasitic drugs for use in fish aquaculture that can be used to kill parasitic copepods that live on the skin of economically valuable fish species.

2.1 Rationale and Objectives

In the past 30 years, there has been a large increase in the interest in the copepod genus *Tigriopus* as a model organism. Due to this increase in literature regarding *Tigriopus*, it makes *T. californicus* an excellent model organism for the study of protein level fluctuations when subjected to acute and chronic thermal and light stress. The objectives of this study are to:

1. Develop and use protocols to subject copepods to thermal and light stress control and experimental treatments in the lab.
2. Develop and use protocols for the isolation of DNA and its use in polymerase chain reactions (PCR) and for the isolation of total proteins from *T. californicus* animals subjected to control and experimental conditions and the analysis of protein levels of AOX using SDS-PAGE and Western blots.
3. Sequence the genes encoding AOX, GAPDH, EF1, β-tubulin, 16S and HSP 20, 70 and 90 in *T. californicus* using DNA isolation, polymerase chain reaction (PCR) using gene-specific primers, cloning vectors, and bacterial transformation and selection. These sequences will be translated into their amino acid equivalents and compared to other organisms' protein sequences for the same gene.
4. Identify any patterns present between the presence of AOX, the expression of the protein, and the thermal and light stress response of *T. californicus.* This will permit an investigation into how the translation of the AOX gene changes with fluctuations in environmental temperature and light exposure.

2.2 Hypothesis

Based on previous research conducted on AOX protein sequences and individual residue function in other organisms, I hypothesize that the AOX protein of *T. californicus* will be enzymatically active due to the presence of the conserved glutamate, histidine, tryptophan and alanine residues necessary for protein activity. Furthermore, I hypothesize that *T. californicus* possesses AOX in order to acclimate to a wide variety of environmental stressors. More specifically, I expect that AOX protein levels will change with exposure of the animals to fluctuating temperatures and light exposure.

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Figures

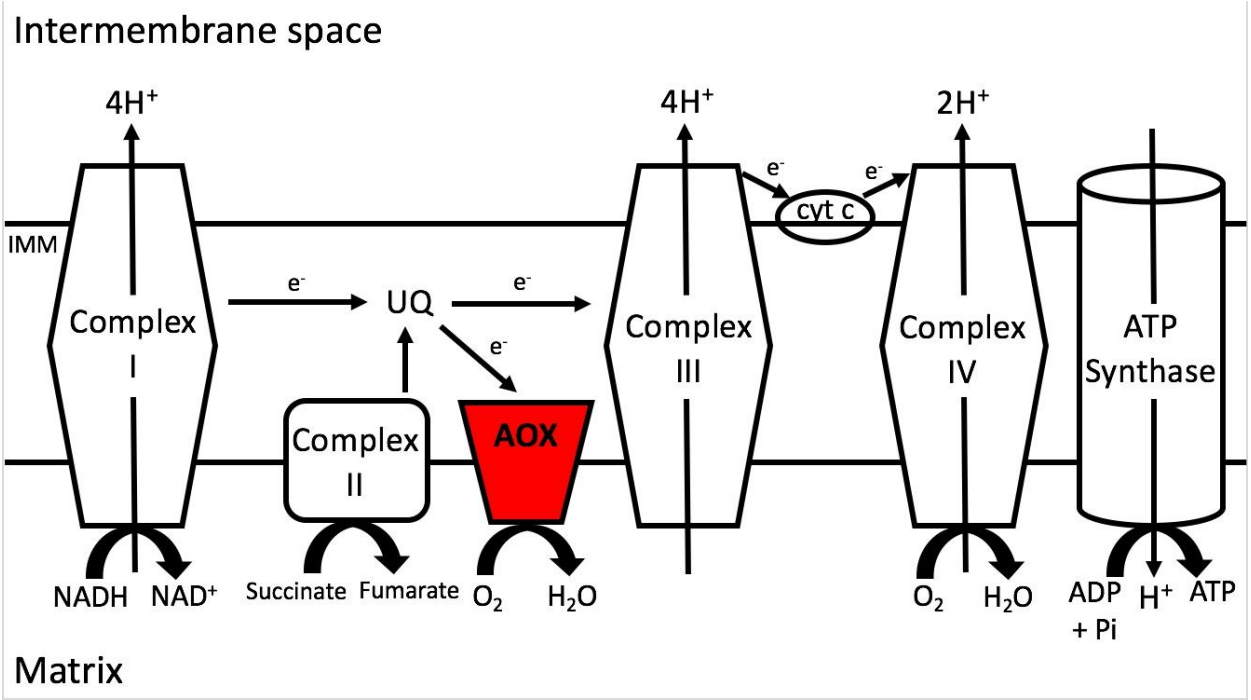

Figure 1: The electron transport system and the position of the AOX protein embedded in the inner mitochondrial membrane. Complex I, NADH dehydrogenase; Complex II, succinate dehydrogenase; Complex III, cytochrome bc1 complex; Complex IV, cytochrome c oxidase; e-, electrons; IMM, inner mitochondrial membrane; cyt c, cytochrome c; UQ, ubiquinol pool; e^-, electron, AOX, alternative oxidase.

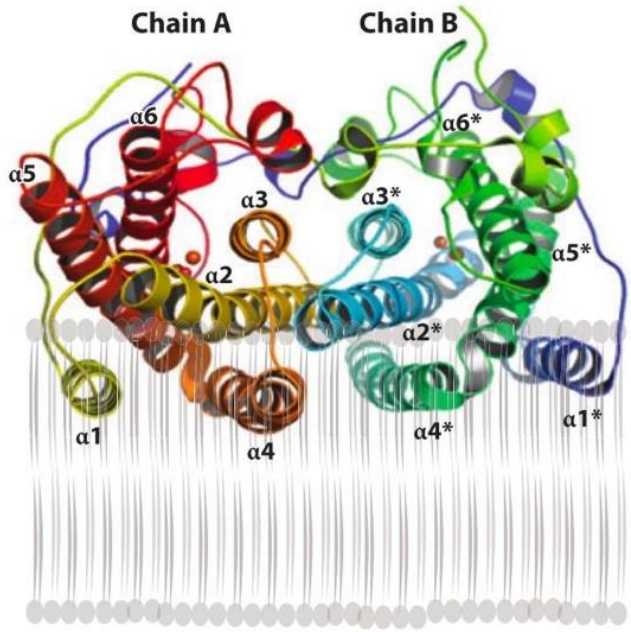

Figure 2: AOX dimer interaction with the mitochondrial membrane. Helices α1 to α6 are associated with chain A, and helices α1* to α6* are associated with chain B (taken from Moore *et al*., 2013).

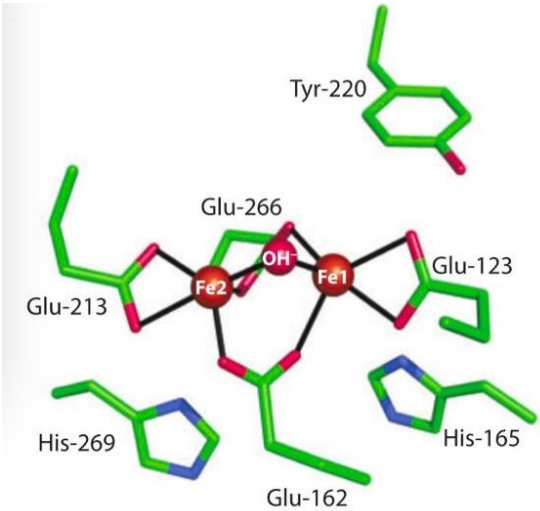

Figure 3: Structure of the active site in AOX. Diiron atoms are shown as spheres, and four glutamate and two histidine residues, which are important for diiron binding, are depicted as green sticks (taken from Moore *et al*., 2013).

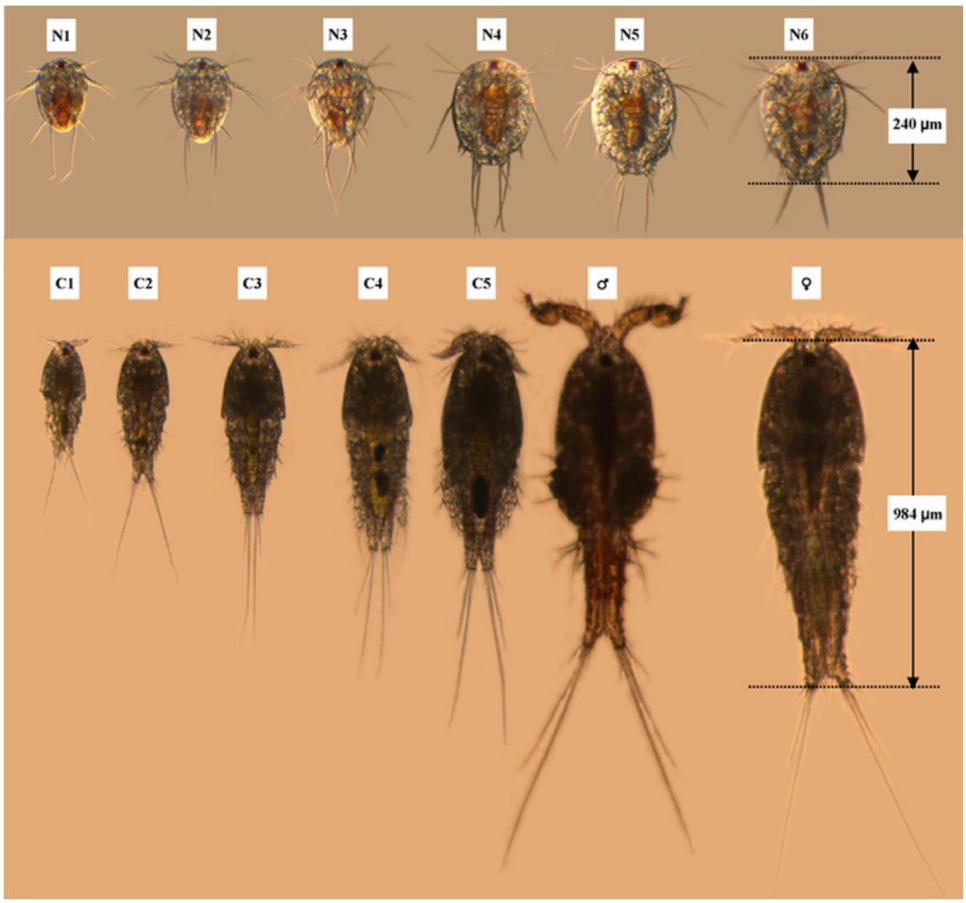

Figure 4: Different stages of development of the copepod Tigriopus japonicas, maintained under the following culturing conditions: 21 °C, 12 h light:12 h dark cycle and salinity 32ppt. The first 6 stages (N1-N6) are nauplius stages and the later six stages (second row) represent the copepodite stages (taken from Raisuddin *et al*., 2007).

CHAPTER 2

Optimizing the growth and cultivation of the copepod *Tigriopus californicus*

Abstract

The harpacticoid copepod, *Tigriopus californicus*, is easily maintained under lab conditions, has short generation times, and is typically used for experiments involving marine food chains, chemical ecology in aquatic environments, and providing predictions on the past history and future status of marine ecosystems (Sehgal, 1983). Optimal conditions for culturing include multiple 400 mL habitats on a 12 hour light:dark cycle at a salinity of 16 g/L while regulated at a temperature of 15°C. The habitats should be cleaned and fed weekly with 0.01 g of Nutrafin Basix Staple Tropical Fish Food and 0.005 g of Spirulina Natural fish food, per habitat. Due to the ease of maintaining these animals and the wide range of experimental usages, *T. californicus* is an excellent experimental model organism and can adapt to a wide range of environmental stressors. *T. californicus* is characterized by small space needs and genetically divergent populations which can be cross-bred in the laboratory; attributes which make *T. californicus* an exemplary model organism to be studied.

1. Introduction

Numerous marine fishes yield small pelagic eggs which larvae will hatch from and they require a source of live food once exogenous feeding begins. Research has indicated that harpacticoid copepods are an excellent alternative food resource in larval fish mariculture and can either replace or supplement brine shrimp and/or rotifers (Lee at al., 2005). Currently, there are over 3000 species in the order Harpacticoida, which is one of 10 orders in the subclass Copepoda (Huys and Boxshall, 1991). Similar to other crustaceans, copepods have separate sexes, with males and females differing based on sexually dimorphic characteristics that develop during the late stages of copepodid (Lee *et al*., 2008). Adult harpacticoid copepods are on average 1 mm in body length, 350 μm in width and possess a dry mass of approximately 3 μg (Rhee *et al*., 2009). Due to their small size, harpacticoid copepods serve as a pertinent food source. Fishes will eventually undergo an ontogenetic shift, which is a shift in their diet, from primarily consuming harpacticoids to larger-bodied prey. This shift normally occurs once the fishes reach a standard body length of approximately 35 mm, but this does not discount the fact that harpacticoid copepods may serve as prey for their entire life. This is due to the fact that similar to other types of copepods, harpacticoids promote rapid growth and a high reproductive rate in fishes and invertebrates. Copepods have invaded a significant variety of aquatic environments and microhabitats (Reid, 2001).

One study conducted by Volk *et al*. (1948) demonstrated that food conversion efficiency in juvenile *Oncorhynchus keta* fed the harpacticoid *Tigriopus californicus* was greater than when fed calanoid copepods or amphipods. Furthermore, Kreeger *et al*. (1991) looked at the nutritional value of newly hatched *Artemia* that were fed with either lipid microspheres and/or the harpacticoid copepod *T. californicus*. It was discovered that shrimp survival, growth, and the

number of females brooding offspring were not significantly different between feeding groups. What was found, was that the number of viable offspring produced was significantly improved by adding lipid microspheres and *T. californicus* the Artemia diet (Kreeger *et al*., 1991). This finding was attributed to the fact that both the lipid microspheres and *T. californicus* are a high source of essential fatty acids (Kreeger *et al*., 1991).

Interest in the copepod genus *Tigriopus* has escalated in an unprecedented manner and a considerable number of publications have focused on this genus (Raisuddin, *et al*., 2007). *T. californicus* is a marine invertebrate which inhabits high intertidal and supralittoral rock pools located along the west coast of North America (Burton and Lee, 1994). Given the location of *T. californicus'* intertidal habitat, it is subject to daily fluctuating abiotic stressors such as temperature, salinity, oxygen levels, and biotic stressors including competition (Burton and Lee, 1994; McAllen *et al*., 1999). The tidepools *T. californicus* resides in are particularly harsh habitats of supralittoral splash pools that can experience temperatures as high as 40°C and salinities of 139 ppt (Powlick 1999; Kelly *et al*., 2012).

T. californicus can be easily propagated in the laboratory over a multitude of generations (Leong *et al*., 2018). Harpacticoid copepods have high reproductive potential, rapid development rates and a low age of first reproduction (Cutts, 2002). Rates of egg production by female copepods are influenced by numerous factors, including temperature, which predominantly leads to an increase in the overall number of eggs produced per female per day until saturation is reached (Ianora, 1998). Beyond that optimal temperature increases lead to a decrease in egg production rates. The highest reproductive rate was seen at 17°C when female's longevity was intermediate (Lee *et al*., 2008). This is due to high temperatures causing reductions in female copepods longevity, so that even if egg production rates increase, the reproductive rate of

females declines (Uye, 1981).

Copepods are located in a vast array of aquatic environments, including oceans, ponds, hydrothermal vents and many other fresh and salt water conditions (Boxshall and Walter, 2019). Harpacticoid copepods are able to withstand extreme temperature and salinity conditions (Powlik *et al*., 2017). Most copepods, however, are unable to tolerate low oxygen conditions and aeration of cultures is very desirable to these organisms (Wetzel *et al*., 2001). All species of *Tigriopus* are prominently located in shallow supratidal rock pools which undergo irregular tidal flooding (Raisuddin *et al*., 2007). Research has demonstrated that *T. japonicas* demonstrates excellent survival in acute toxicity tests at temperatures up to 35°C and salinities ranging from 15 to 45 ppt (Kwok and Leung, 2005). These copepods' strong adaptability to a wide range of conditions is highly favourable when it comes to using this organism in ecotoxicity and environmental genomic studies to understand the natural variability in coastal areas (Raisuddin *et al*., 2007).

Not only are these organisms known to adapt to a wide variety of environments and withstand extreme temperature and salinity ranges, but they can also feed on an array of food items in their natural environments, including diatoms and particulate organic matter (Raisuddin *et al*., 2007). These aspects make copepods easy to maintain under lab conditions, in addition to their short generation times. Moreover, harpacticoid copepod densities in mass culture can be more than 100,000/L, thereby exceeding the success to date of calanoid copepods (Stottrup, 2000). This can be attributed to a multitude of traits the harpacticoid copepod possesses, including: high reproductive potential, rapid development rates, and a low age of first reproduction (Cutts, 2002).

One of the most important steps in being able to conduct any of these experiments is to

first find appropriate habitat parameters in which to grow the organism. Living organisms are products of their environment, changes in their habitat induces changes in the chemical composition of organisms (Corner and O'Hara, 1986). The ability to establish large cultures in the laboratory is a necessity for any organism that is going to be adopted as a model system. We provide a simple procedure for growing and cultivating copepods, specifically of the species *Tigriopus californicus*, for experimental purposes. The advantages of this protocol include the low maintenance and space requirements of *T. californicus*, as well as, their perseverance through unfavourable conditions.

Although a lot of research is done on copepods in the scientific community, there is not much information regarding habitat development and maintenance, and the rearing of the copepods in the lab. The purpose of this study was to optimize the growth conditions for *T. californicus* in the lab in order to generate sufficicient biomass to perform DNA and protein extractions. The main objective of this chapter is to give detailed descriptions for developing and maintaining copepod habitats in the lab. Furthermore, copepod maintenance procedures were developed that controlled the environment and mitigated stress on the organism.

2. Materials and Methods

2.1 Salt Water Preparation

A large (2L) container was filled with 1.5L of filtered Milli-Q water and subsequently placed onto a stir plate with a magnetic stir bar. The stir plate was turned on (moderate-high setting) and 84 g of instant ocean salt was added. An analytical or top loading balance was used to measure the mass of the salt. The solution was left to mix for approximately 5-7 minutes or until all the salt had dissolved. The entire solution was poured into a 2L graduated cylinder and

topped up to the 2L mark with Milli-Q filtered water. This salt water solution can be stored in large bottles for future use. (Note: This procedure creates 2L of 42g/L salt water) (Figure 1).

2.2 Habitat Set-Up

To start a new habitat for T. californicus a 400mL beaker with 150-200mL of prepared salt water is needed. Approximately 50-100 animals, at different life stages, should be added to the new habitat (Figure 2). *Tigriopus californicus* animals were obtained from J&L Aquatics Canada, Vancouver, British Columbia. The animals arrived in an approximately 150mL bottle containing approximately 1000-2000 individuals at various life stages. The habitat should be covered with a shallow petri dish in order to limit water evaporation and the prevent foreign substances from entering the habitat. Following habitat set-up, the beakers were placed into an incubator, regulated at 15°C on a 12 hour light:dark cycle.

2.3 Habitat Clean-up (Perform weekly)

First, an aspiration system is used to remove the top algae layer of each habitat, approximately the top 10 mL of each habitat (the algae layer appears as a film-like light green layer, due to the food source the copepods are fed). A disposable pipette is then used to remove the chunks of algae at the bottom of each habitat. This step is done with caution in order to not suck up and withdraw and copepods from the habitat. The contents of the beaker are poured over a filter into a waste container, allowing for the collection of animals in the filter. Pouring was halted prior to the remaining algae in the beakers started to flow into the filter (leaving approximately 40 mL of water in the habitat). The filter containing the copepods is placed into a deep petri dish containing prepared salt water. A new disposable pipette is used to transfer the extracted copepods from the filter back into the habitat. Each habitat is topped off with prepared salt water until the total volume of water in the beaker is approximately 200 mL. The disposable

pipette is used to flush the sides of the beaker with salt water from the habitat to ensure all copepods are submerged in the habitat (Figure 3). Each clean habitat is covered with a petri dish and placed back into an incubator. Note: When habitats start to become heavily populated with copepods (200 - 250 animals), create more habitats by transferring the copepods from the filter into two separate beakers to create one additional habitat (approximately every 3 weeks).

2.4 Copepod Feeding (perform weekly)

The prepared salt water solution from section 2.1 is used to make the food solution to be added into each beaker. 50 mL of salt water solution, for each habitat, is poured into a large beaker (2L). Beaker is placed on a stir plate with a magnetic stir bar. Using an analytical balance, 0.01 g of Nutrafin fish food is measured for each habitat. Measured Nutrafin fish food is then placed into a mortar and the pestle is used to grind it as fine as possible. A stir plate is turned on at a moderate to high speed and the ground-up Nutrafin is added to the salt water solution. Next, the analytical balance is used again to weigh out 0.005g of Spirulina Natural fish food for each habitat, and is subsequently added to the salt water solution already containing the Nutrafin fish food. The solution is left to thoroughly mix for approximately 7 minutes. A 50 mL graduated cylinder is used to administer 50mL of the prepared food mixture into each habitat (Figure 4).

3. Results

T. californicus was subjected to three temperature conditions: 6°C, 15°C and 28°C. When subjected to 6°C the copepods were slow moving, and reproduction rates were much lower in comparison to 15°C. When *T. californicus* habitats were held at 28°C, there was evaporation and salt crystals appeared on the beakers. These only appeared at 2 weeks if beakers were not cleaned and water not changed. This lead to decreased population size, with very few organisms being able to survive week to week. It was observed that a couple of days after the water change,

the water levels had been decreasing due to evaporation taking place. As a result, the copepods had become stressed from the external factor of evaporation; this was noted as stressful to the copepods because of the observed decrease in population density. In order to bring the habitat back to stable conditions (i.e. salinity of the water), the habitats had been cleaned and water changed every week. This was done to mitigate any environmental stressors that can contributing to decreases in population of the organism.

4. Discussion

The copepod *T. californicus* is found on the Pacific coast of North America. *T. californicus* inhabits splash/tide pools at seashores that are covered at high tide and uncovered at low tide. Since the splash pools are isolated from the ocean a number of environmental factors vary, such as temperature and salinity. *T. californicus* have evolved adaptive mechanisms, which allow the organism to survive in a wide range of environments (McDonough and Stiffler, 1981).

The reduction in water levels, due to evaporation, lead to an increase in salinity of the water. This is similar to the field based observation of dry out in intertidal pools (Burton and Feldman, 1981; Burton and Lee, 1994). The drying out of pools is caused by evaporation from solar heat and is seen as an abiotic stressor contributing to the overall survival of *T. californicus* in both field and laboratory cultures (Willett, 2010). The results from the temperature conditions demonstrated that *T. californicus* survived longer at 15°C than at 28°C and repopulated more at 15°C than at 6°C. Throughout the experiment there was never complete population death, a couple copepods were always able to survive as long as the water was changed weekly. A possible explanation for this is that *T. californicus* inhabits intertidal pools, which are constantly subjected to extensive fluctuations in salinity, evaporation and experiences a range of temperatures between 4°C - 35°C (Edmands and Deimler, 2004; Powlik, 1999; Schoville *et al*.,

2012). In support of the above finding that more copepods were present in habitats at 15°C compared to 28°C, a lab based study found that there was a decline in *T. californicus* survivorship when temperature levels increased from 15°C to 25°C (Edmands and Deimler, 2004; Kontogianis, 1973).

Zooplankton have developed methods to deal with extreme environmental stress, in order to sustain their populations in the future. In order for aquatic invertebrates to cope with the unpredictability of their environments, such as a change in temperature, oxygen levels, salinity, or drought, they produce resting eggs. This stage will allow the encysted embryos to withstand their harsh environment and they will remain in this dormancy stage until the resting eggs encounter favourable conditions (Caceres 1998). This fact can lend as a possible explanation for the small population size seen at 6°C. Copepods might have experienced immense environmental stress when exposed to 6°C, that instead of reproducing they lay resting eggs. However, Powlik *et al*. justified in their 1997 paper that an appropriate range for constant culturing of *T. californicus* was between 2°C- 5°C (Powlik *et al*., 1997). The documented range for culturing *T. californicus* was much more representative of the small scale changes the copepod experiences in the intertidal pools, compared to holding the species at a variable constant temperature at 15°C-25°C (Powlik *et al*., 1997).

5. Conclusions and Future Directions

This work demonstrates that the optimal temperature to keep copepod habitats at is 15°C. Furthermore, the habitat's water should be changed every week in order to remove any additional algae that may have grown and to mitigate increases in salinity levels, through evaporation. *T. californicus* is an emerging model organism due to its short generation time, low space requirements, and the fact that the species has numerous genetically divergent populations

that are capable of being crossed in the laboratory (Burton & Feldman, 1982). By developing a method to clean and maintain copepod habitats, that alleviates the amount of stress the organism experiences, it can allow for further study of genes' functions and regulation. For example, these procedures will allow for the study of AOX through manipulation of environmental factors in a laboratory setting that will increase stress and in turn cause fluctuations in AOX expression.

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Figures

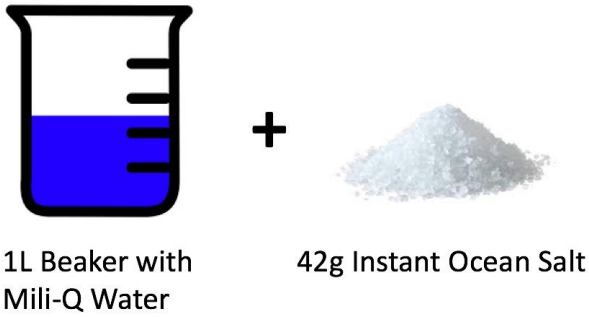

Figure 1: Materials needed to create salt water for copepod habitats.

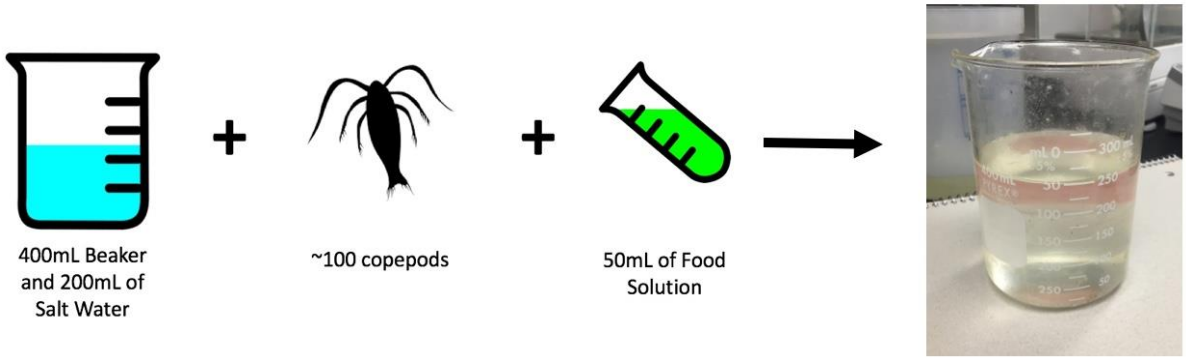

Figure 2: Materials required to construct a new copepod habitat.

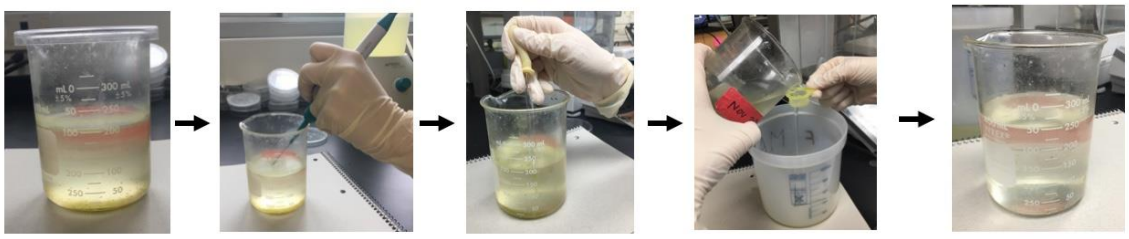

Figure 3: Procedure for cleaning a copepod habitat and removing as much algae as possible.

Figure 4: Food supplies needed to feed a 400 mL beaker copepod habitat each week.

CHAPTER 3

Identification, expression, and DNA sequence analysis of alternative oxidase in *Tigriopus californicus*

Abstract

In addition to the typical electron transport system in animal mitochondria responsible for oxidative phosphorylation, some species possess an alternative oxidase (AOX) pathway, which causes electrons to bypass proton pumping complexes. Although AOX appears to be energetically wasteful, studies have revealed its wide taxonomic distribution. AOX discovery in animals is recent, and further research into its expression, regulation, and physiological role has been impeded by the lack of an experimental model organism. DNA database searches using bioinformatics revealed an AOX sequence present in the arthropod *Tigriopus californicus*. Multiple sequence alignments compared AOX proteins and examined amino acid residues involved in AOX catalytic function and post-translational regulation. The AOX protein sequence of *T. californicus* contains amino acid residues required for catalytic activity. Furthermore, the AOX protein sequence of *T. californicus* contains a conserved C-terminal motif which is highly characteristic of animal AOXs. Identifying an effective housekeeping gene for use as a control is required to investigate the transcription levels of AOX in an animal model using real-time PCR. This research is making strides towards understanding the role and physiological function AOX plays in animal models, as AOX transcription levels have never been analyzed in an animal.

1. Introduction

Sequencing copepod genomes is of high importance due to copepods being one of the most abundant organisms on the planet and playing a critical role in marine and freshwater environments (Barreto *et al*., 2018). In spite of the diversity and abundance of copepods, the first copepod genomes were published only a few years ago (Kang *et al*., 2017). Some of the challenges in sequencing genes from copepods are due to their small size and hard exoskeleton. The majority of DNA extraction procedures require a large mass of tissue. In order to better understand the importance of copepods and study specific genes in copepods, such as AOX and heat shock proteins, a DNA extraction procedure that accounts for their small size and hard exterior needs to be developed.

Alternative oxidase (AOX) is a terminal oxidase that presents a branch point in the electron transport system (ETS) at the level of ubiquinone. AOX is an alternate route of electron exit by causing electrons to bypass complexes three and four, which are two of the three proton pumping complexes in the ETS, and using the electrons for its own reduction of oxygen. Overall, this causes less adenosine triphosphate (ATP) to be synthesized per oxygen molecule consumed and is energetically wasteful (Moore and Siedow, 1991). AOX is an interfacial membrane protein located on the matrix side of inner mitochondrial membrane (Rogov *et al*., 2016). In contrast to complexes I, III, and IV of the ETS, AOX does not translocate protons when electrons are transported through the complex. Therefore, AOX is not coupled with ATP synthesis and energy accumulation; instead the energy is released as heat (Rogov *et al*., 2016). Despite the fact that AOX is energetically wasteful, it has a wide taxonomic distribution in some plants, fungi, animals, protists and bacteria (McDonald and Vanlerberghe, 2004).

Even though there are certain areas pertaining to AOX that have been thoroughly researched, there are still numerous avenues for future research regarding this protein. The discovery of plant AOX was made in 1975, and since then plants, more specifically flowering plants, have been the main organism of study when it comes to AOX (McDonald *et al*., 2009). In 2003, AOX was first discovered in prokaryotes, and in 2004 the first animal sequence was discovered (McDonald and Vanlerberghe, 2004). Recently, an AOX gene was detected in the copepod *T. californicus* (Tward *et al*., 2019).

Public molecular databases indicate that AOX exists in several animal species due to either the presence of AOX DNA or mRNA (McDonald and Vanlerberghe, 2004; McDonald *et al*., 2009). Several studies have heterologously expressed the AOX from the sea squirt *Ciona intestinalis* in human cell lines, mice, and fruit flies and the AOX from the Pacific oyster *Crassostrea gigas* has been expressed in the yeast *Saccharomyces cerevisiae* (Robertson *et al*. 2016; Rodriguez-Armenta et al., 2018; Hakkaart et al., 2006; El-Khoury et al., 2013; Kemppainen et al., 2014). These studies have demonstrated that the heterologously expressed AOXs are correctly targeted to the mitochondria and are functional enzymes. While this is useful information, what is lacking is the study of an AOX enzyme in an organism that contains the gene in its genome and naturally expresses the protein. *T. californicus* has been identified as a suitable organism for such studies due to the presence of a naturally occurring AOX gene in this species. By naturally occurring, we mean that the AOX gene will be studied in the organism that produces it.

The AOX gene is encoded by the nuclear genome and the gene structure has been investigated most thoroughly in plants (Vanlerberghe & McIntosh, 1997). In plant genomes, AOX has two subfamilies, AOX1 and AOX2. The gene that encodes AOX1 is transcribed in

response to certain types of stress such as parasite attacks, oxidative, and temperature stress (Rogov *et al*., 2016, Costa *et al*., 2012). In contrast, AOX2 is often expressed during different stages of plant development, and its expression is inducible and controlled by other processes (Considine *et al*., 2002, Rogov *et al*., 2016). When it comes to the presence of AOX in fungi, it has been found to be less diverse in comparison to plants, containing only one of the two subfamilies (Albury *et al*., 2009). The expression of this gene is highly dependent on the functional state of the mitochondria in the fungus that is expressing it (Rogov *et al*., 2016). The existence of a multitude of AOX sequences found in evolutionary distant organisms can be helpful in determining which amino acids are important for the folding and the function of the protein (Rogov *et al*., 2016). In animals, the AOX sequence has been found to have a unique C-terminus that can be used to distinguish animal AOXs from those of other kingdoms (McDonald *et al*., 2009).

It has been confirmed, through electron paramagnetic resonance technology, that AOX belongs to the family of di-iron carboxylate proteins and is dimeric (Moore *et al*., 2008). Each monomer in AOX is composed of six long helices (α1-α6) and four short helices (αS1-αS4) which are arranged in an antiparallel fashion (Moore *et al*., 2013). Helices α2, α3, and α4 from both monomers make up the dimer interface, which contains six highly conserved residues in all AOX sequences and eight residues that indicate that a dimeric structure is common to all AOXs (Moore *et al*., 2013).

In AOX's oxidized state, the two iron atoms, located in each active site, are linked by a hydroxo bridge and ligated by four highly conserved glutamate residues (Moore *et al*., 2013). The di-iron carboxylate proteins are characterized by a series of highly conserved glutamate (Glu, E) and histidine (His, H) residues (Chapter 1, Figure 3), which are required for the co-

ordination of the di-iron centre of the AOX enzyme (Siedow *et al*., 1995). The AOX protein structure in organisms from a wide variety of kingdoms have highly conserved Glu and His residues at specific position in the protein sequence (McDonald, 2008). In addition to the Glu and His residues, a conserved tyrosine residue is required for AOX activity (Chapter 1, Figure 3) (Moore and Albury, 2008). One study observed a hydrophobic region of the AOX protein that is thought to play a key role in binding ubiquinol (Chapter 1, Figure 2) (Albury *et al*., 2009). Furthermore, Crichton *et al*. (2009) identified a conserved tryptophan (TrpI) residue which is thought to play a pertinent role in anchoring AOX to the inner mitochondrial membrane. Recent studies have proposed that ThrI and CysII affect the catalytic cycle of AOX with respect to its interaction with oxygen (Crichton *et al*., 2005). As well, Gln I (Q-27) and Tyr II (Y-60) are highly conserved and reside within the dimer interface and substitution of either of these residues with another residue would lead to significant inhibition due to their role in helix-helix interaction (Chapter 1, Figure 2) (Moore *et al*., 2013). The AOX gene possesses three highly conserved tyrosine (Tyr, Y) residues which each play a crucial role in electron transport (Moore *et al*., 2013). Tyrosine I (Y-38) is associated with ubiquinol binding, but it has been demonstrated that a mutation occurring to this Tyr does not lead to a complete loss of function (Moore *et al*., 2013). Due to its position in the AOX four-helix bundle, forming a hydrogen bond with a conserved His and being far enough away from the di-iron center, this suggests that it most likely stabilizes the structure of the oxidase rather than being directly involved in ubiquinol binding (Moore *et al*., 2013). Tyrosine III (Y-108), which is located on one of the helices, is likely involved in hydrogen bonding networks rather than in electron transport. This finding was supported by the observation that if tyrosine III (Y-108) was switched out with an alanine residue, AOX would still retain some of its activity, which would not be the case if the residue

was pivotal for electron transfer (Moore *et al*., 2013). Conversely, tyrosine II is highly conserved, as it is essential for AOX's catalytic cycle (Albury *et al*., 2002). Tyr II (Y-60) is buried deep within the four-helix bundle and close to the di-iron center. Mutational analysis has shown that this residue (Tyr II, Y-60) is crucial to the enzymatic activity of all AOXs by way of electron transport (Chapter 1, Figure 3) (Albury *et al*., 2002). Alanine (Ala, A) also plays a critical role in the substrate-binding cavity. Both Ala I (A-56) and Tyr II (Y-60) are situated very close to the active site (6 Å), hence why there can be no substitutions for these residues (Moore *et al*., 2013). The crystal structure of AOX in the protist *Trypanosoma brucei* has been examined and led to insights regarding the structure and catalytic cycle of AOX (Shiba *et al*., 2013).

The physiological role of AOX is still under investigation, but it is known to play a role in thermogenesis in some angiosperms (Grant *et al*., 2008; Seymour and Matthews, 2006). Furthermore, AOX has also been shown to reduce the generation of reaction oxygen species (ROS) (Møller, 2001) which can lead to oxidative damage to the mitochondria and other cellular components (Maxwell *et al*., 1999). In eukaryotic cells, the majority of ROS comes from the mitochondria (Maxwell *et al*., 1999). Therefore, AOX may play a role in aiding in the balancing of carbon metabolism, electron flow, and ATP biosynthesis.

Heat shock proteins

Heat shock proteins (HSP) are synthesized in all organisms and are highly conserved proteins that respond to various environmental stressors such as temperature fluctuations, chemical exposure, and hypoxia (Rhee *et al*., 2009). The majority of HSPs are involved in assembly and folding of proteins (Fink, 1999). When exposed to stressful conditions, HSPs move to the cell nucleus where they mend and protect nuclear proteins while consecutively minimizing protein aggregation to mitigate genetic damage (Rhee *et al*., 2009).

HSP20 contains an αA-crystallin and an αB-crystallin domain, which are domains that are shared by certain small heat shock proteins and cause them to share a significant degree of sequence homology (MacRae, 2000), and have been shown to play a role in modulating cellular defense systems in response to environmental stressors in *Tigriopus japonicus* (Seo *et al*., 2006). The HSP20 gene produces a transcript of 1014 base pairs and the protein consists of 174 amino acid residues (Seo *et al*., 2006). One study on the copepod *Tigriopus japonicus* showed that there is a positive correlation between the up-regulation of HSP20 mRNA expression and increasing temperatures (Seo *et al*., 2006). Furthermore, Seo *et al*. (2006) demonstrated that *T. japonicus* HSP20 mRNA expression increased when subjected to changes in water temperature, but not upon fluctuations in salinity. Another study confirmed this finding that expression of the heat shock protein family led to increases in thermal tolerance upon exposure of the organism to heat shock (Arrigo and Landry, 1994). Schoville *et al*. (2012) stated that the role of HSP20 in *Tigriopus californicus* is to respond specifically to heat stress. One study exposed *T. japonicas* to three different temperatures (4, 10 and 30°C) and took samples after 5, 10, 20, 30, 60, 90, 120, and 180 minutes (Seo *et al*., 2006). It was concluded then when *T. japonicas* was exposed to 4°C it showed the largest gene expression of HSP20 after 30 minutes and when exposed to 30°C the largest gene expression of HSP 20 was after 90 minutes (Seo *et al*., 2006).

In order to measure the transcription levels of any gene, a well-defined housekeeping gene is required to serve as a means of standardizing the data. Housekeeping genes, also known as reference genes, are expressed in a stable and non-regulated constant level, and are primarily involved in processes essential for the survival of cells (Kozera and Rapacz, 2013). Furthermore, the most important attribute of a reference gene is that its expression level is unaffected by experimental factors. Housekeeping genes will bring to light the variability resulting from

imperfections of the technology being used and ensure that any variation in the amount of genetic material will also affect the housekeeping gene to the same extent. The use of a housekeeping gene leads to an increase of resolution and greater accuracy of the results when making definitive statements regarding fluctuations in the transcription of other genes (Kozera and Rapacz, 2013).

We hypothesize that *T. californicus'* AOX protein sequence will possess the conserved residues, which are required in order for the protein to function. Prior to being able to test the hypothesis an effective and efficient method to isolate DNA from copepods has to be established. Furthermore, we hypothesize that when comparing *T. californicus'* AOX sequence to the AOX sequences of other organisms, such as plants and animals, the crucial amino acids required for enzymatic function will be conserved across domains.

The purpose of this study is to: 1) confirm the presence of an AOX gene in the copepod *Tigriopus californicus*; 2) sequence the AOX gene of *T. californicus*; 3) compare the amino acid composition of the AOX of *T. californicus* to those of other copepods, animals, and plants and 4) to identify if the AOX enzyme is active in *T. californicus* based on the presence or absence of the conserved glutamate, histidine, tryptophan, and alanine residues necessary for protein activity. Bioinformatics were used to perform a molecular database search to identify the AOX gene from closely related copepods and other organisms in order to address the above objectives. To validate our bioinformatics results, polymerize chain reaction (PCR) was utilized to amplify the HSP and housekeeping genes using gene specific primers.

2. Materials and Methods

2.1 Procedure of the HotSHOT DNA Isolation Method Applied to Copepods

Currently in the literature there is no effective and efficient method to isolate DNA from a small number of copepods. Montero-Pau *et al*. (2008) developed the HotSHOT (Hot Sodium Hydroxide and Tris) DNA extraction procedure, which provides DNA from resting eggs in a range of taxa that can be further used in PCR (polymerase chain reaction) amplification. The HotSHOT method is a reliable, simple, and cheap DNA extraction approach which can be altered for use in other aquatic invertebrates with resting eggs (Ishida *et al*., 2012). Resting eggs have a similar hard exterior to a copepod's exoskeleton. Therefore, the HotSHOT method may be able to be used in the isolation of DNA from copepods and enable the investigation of genes including AOX, heat shock proteins (HSP) and housekeeping genes.

In order to isolate DNA, ~20-30 copepods were individually isolated from a habitat. In a microcentrifuge tube, 150 µL of an alkaline lysis buffer (25 mM NaOH, 0.2 mM of EDTA, pH 8) was dispensed. Under a microscope, an individual copepod was transferred into the microcentrifuge tube, carrying over as little water as possible. A sterile pipette tip was used to crush open the copepod against the wall of the microcentrifuge tube. The sample was then incubated for 30 minutes at 95°C and subsequently cooled on ice for 3-4 minutes. 150 µL of neutralizing solution (40 mM of Tris-HCl, pH 5.0) was added to the microcentrifuge tube and the tube was vortexed. Afterwards, the tube was placed in a table top centrifuge for approximately 10 seconds. The DNA sample was placed in a freezer at -20°C for long-term storage. During every use, the sample was allowed to thaw and was centrifuged to get all of the debris from the copepod's exoskeleton at the bottom of the tube (pellet). The DNA sample was taken from the supernatant.

2.2 Quantification of DNA using a Spectrophotometre

The concentration of a DNA sample can be determined by the use of UV spectrophotometry. DNA absorbs UV light very efficiently making it possible to detect and quantify it at concentrations as low as 2.5 ng/µl. The nitrogenous bases in nucleotides have an absorption maximum at about 260 nm. Using a 1-cm light path, the extinction coefficient for nucleotides at this wavelength is 20. Based on this extinction coefficient, the absorbance at 260 nm in a 1-cm quartz cuvette of a 50 µg/ml solution of double stranded DNA is equal to 1. The concentration of DNA can be calculated using the following equation:

DNA concentration (µg/ml) = (OD_{260}) x (dilution factor) x (50 µg DNA/ml)/(1 OD_{260} unit)

The sample is prepared by placing 995µl of nuclease free water and 5µl of DNA sample in an eppendorf tube (dilution factor is 200). This sample was placed in the spectrophotometer along with a blank sample (1000µl of nuclease free water) to get a reading for each sample at A_{260} and A_{280} using quartz cuvettes.

2.3 Primer Design

In order to conduct PCR, primers were designed for *T. californicus* genes based on cDNA sequences. Molecular database searches revealed putative *T. californicus* AOX, β-Tubulin, 16S, EF1, GAPDH, HSP20, HSP70, and HSP90 sequences using BLAST (Basic Local Alignment Search Tool) at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The Primer3 program (http://frodo.wi.mit.edu/primer3) was utilized for the design of each of the primers for amplification of heat shock proteins 20, 70 and 90 and all housekeeping genes from *T. californicus*. The custom primers were sent to Invitrogen (Invitrogen Life Technologies, Carlsbad, CA, USA) to be produced for use in the PCR protocol.

All heat shock, AOX (developed by Jaspreet Singh) and housekeeping gene primers are summarized in Tables 1 and 2.

2.4 Polymerase Chain Reaction

In order to evaluate the validity and reliability of the DNA extraction protocol, a fragment of each heat shock gene was amplified. Three primers for each heat shock protein gene were developed and used in PCR. PCR was conducted using the GoTaq Green Master Mix (2x) (Promega), which contains a dye so the sample is ready for gel electrophoresis after PCR. Each PCR reaction contained: nuclease free water (25 µL), GoTaq Green Master Mix 2x (12.5 µL), forward and reverse primers (2 µL of each at 10 µM), and DNA extract (sample, 5 µL, all source DNA was taken from the same sample) (Table 3). These substances should be added in this order into a sterilized PCR tube, in order to prevent contamination. For the negative control the Taq DNA polymerase is left out. When adding these substances to the PCR tube the tubes should be held in a cold tube rack in order to keep all substances cold and to prohibit any reactions from occurring too early. To prevent and minimize the cross-contamination between samples, new sterilized pipette tips should be used between adding each substance to each PCR tube.

To amplify the heat shock protein and housekeeping genes, a PCR program was developed (Figure 1). First, the thermal cycler was heated to 94°C and held there for 3 minutes. To optimize amplification conditions, the thermal cycler was programmed for 40 cycles of amplification. Each cycle consisted of 30 seconds at 94°C for denaturation, 1 minute at 45°C for annealing, and 2 minutes at 68°C for elongation (the final elongation step was extended for 7 minutes during the last cycle in order to allow DNA strand synthesis to finish completely) (Table 4). While running/setting up the thermal cycler program the lid temperature was set to 100°C to prevent evaporation. For the annealing step the temperature was set at 45°C because the T_m

(melting temperature at which half of the DNA strands are single stranded) for all primers is 60°C.

2.5 DNA Gel Preparation

In order to make a DNA gel in order to visualize PCR products, a small gel tank, small gel tray, gel comb and gel caster are needed (Figure 2). The small gel tray is placed into the gel case and tightened until the tray is firmly in place. The gel comb is inserted into the gel case. Next a mixture of TAE buffer (1 mL), Milli-Q water (49 mL), agarose (0.60 g), and ethidium bromide (2 µL) is made in an Erlenmeyer flask. The Erlenmeyer flask is placed in the microwave for an initial 45 seconds, in order to dissolve the agarose. The flask was removed from the microwave using hot gloves, the contents were swirled, and placed back in microwave for 10 seconds. This step was repeated until the solution began to bubble. The hot flask is then left to sit until it is warm enough to touch without burning your hand. The gel solution is then slowly poured into the gel tray, to prevent any bubbles from being in the gel. It is left to sit for approximately 30 minutes, in order for it to solidify into a gel.

2.6 DNA Gel Electrophoresis

First, the gel tray was placed on the gel stage of the tank, with the gel comb still in place. Next, 1x TAE (Tris base, acetic acid and EDTA) running buffer (5 mL TAE buffer, and 245 mL MilliQ water) is poured into the tank until the gel was completely covered with solution (Figure 3). Following this step, the comb is removed and the wells were topped off with 1x TAE running buffer (a thin layer of buffer covering the gel). The first well was loaded with 3 µL of DNA ladder (GeneDirex, 100bp DNA Ladder Ready To Use), and the subsequent wells were loaded with 12 µL of each PCR sample. The gel was run at 80 V for approximately 1 hour and 20 minutes. Once the blue stain had run 75% of the way through the gel the run was stopped. The

DNA gel was imaged on a VersaDoc, with the following setting: Nucleic Acid Gels, Ethidium Bromide. PCR fragments were visualized on the DNA gel at different sizes, depending on the gene fragment (Refer to Table 1 and 2 for exact locations of the different gene fragments based on expected cDNA product size).

2.7 Extraction of DNA products From the DNA Gel

Prior to imaging the DNA gel a ruler was placed beside it in order to pinpoint exactly which part of the gel was going to be excised. A clean scalpel was used to cut the piece from the gel and follow-up pictures were taken in order to make sure of three things: 1. The right section of the gel was excised, 2. That the wanted DNA fragment was taken, and 3. That there is no excess agarose around the extracted section. Eppendorf tubes were weighed before and after placing the gel fragment in them. The QIAquick Gel Extraction Kit (Qiagen) was utilized for this procedure. For every 100 mg of gel, 300 µL of buffer QG is added into the eppendorf tube containing the piece of extracted DNA gel. The tubes were incubated at 50°C for 10 minutes, during which the tubes were vortexed every 2-3 minutes to help dissolve the gel. Following the dissolving of the gel pieces the solution should be yellow in color. 100 µL of isopropanol is then added to the eppendorf tube for every 100 mg of gel. Next, the QIAquick spin column is placed in a 2 mL collection tube and the solution is placed inside the spin column, in order to bind the DNA. The spin column and collection tube were placed in the centrifuge for 1 minute at 13,000 rpm. The flow through is discarded as hazardous waste. 500 µL of Buffer QG is added to the column and again centrifuged for 1 minute at 13,000 rpm, and flow through is discarded appropriately. The column is then washed with 750 µL of Buffer PE and centrifuged (1 minute and 13,000 rpm). The spin column is placed into a clean 1.5 mL Eppendorf tube and 50 µL of Buffer EB is added to the column in order to elute the DNA. Prior to centrifuging for 1 minute

(13,000 rpm), the column is left to stand for 4 minutes in order to increase the yield of purified DNA. DNA samples are then stored in the -20°C freezer until they are ready to be used.

2.8 Luria Broth Agar Plates Preparation

1 L of Milli-Q filtered water, 25 g of LB (Luria broth) medium, and 15 g of agar are added to a media jar (Figure 4). The media jar containing the solution is placed in the autoclave on a liquid 30 cycle. Once removed from the autoclave, the contents are left to cool to room temperature. 1 mL of ampicillin mixture (0.1 g of ampicillin stock and 1 mL of Milli-Q filtered water) is added to the media jar containing the solution. Prior to pouring the plates the work station was sufficiently cleaned with 70% ethanol, as well, a Bunsen burner was lit to remove any contaminants in the air. Furthermore, the lip of the media jar was placed on top of the flame to get rid of any contaminants that might have attached while the media jar is open. The lip of the media jar was placed on top of the flame between pouring every 5 plates to prevent any contamination. The LB solution was poured into each petri plate until it was approximately ¾ of the way full and plate lids were placed on top immediately after pouring. Plates were left to solidify and stored inverted in the fridge to prevent any condensation from getting onto the solidified media. Once the plates were ready to be used, they were placed in a laminar flow cabinet. 45 µL of X-Gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside) and 45 µL of Isopropyl β-D-1-thiogalactopyranoside (IPTG) are added to the plate, spread evenly around the whole plate and left until the substances have been absorbed by the media in the plate.

2.9 Plasmid pGEM-T Easy Vector Map

Please refer to Figure 5 for picture of pGEM-T Easy Vector map and sequence reference points. There are two points on this plasmid which are important for selection of transformed strains. The first is the ampicillin resistance gene; the media used for the plates contains

ampicillin to make sure that only a bacterial strain which contains a plasmid with an ampicillin gene (this acts as a selection marker) can grow. The second aspect of the plasmid which was manipulated is the LacZ gene. The LacZ gene codes for a protein called beta-galactosidase, which generates a blue coloured product when it reacts with the X-Gal substrate. In a successful transformation, the PCR product is inserted into the LacZ gene thereby halting the production of beta-galactosidase. Therefore, if the colony on the agar plate is blue, it indicates that the PCR product did not insert into the plasmid. Conversely, if the colony is a shade of white, it indicates that the PCR product has successfully inserted itself into the plasmid.

2.10 Ligation of PCR products into the pGEM-T-Easy Vector

First, the tubes containing the pGEM-T Easy Vector and Control Insert DNA were centrifuged to make sure all contents were at the bottom of the tubes. Next, standard ligation reactions were set up, on ice, by adding 1 µL of Nuclease Free Water, 5 µL of 2× Rapid Ligation Buffer, 1 µL of pGEM-T Easy Vector, 2 µL of each PCR product, and 1 µL of T4 DNA Ligase (added last). For the positive control, all contents are the same except, instead of adding 2 µL of PCR product, 2 µL of control insert DNA is added (Table 5). The contents in each tube were mixed, centrifuged and left to incubate for 1 hour at room temperature.

2.11 Transformation of *E. coli* Using the pGEM-T-Easy Vector Containing PCR Inserts

Following the incubation period, 2 µL of each ligation reaction is added to a sterile polypropylene tube while on ice. Next, 50 µL of JM109 High Efficiency Competent Cells (Promega) were added to each polypropylene tube. The tubes were very gently mixed and left in ice for 20 minutes. Each polypropylene tube containing cells was then heat shocked at 42°C for 45-50 seconds. Immediately following the heat shock step, samples were returned to ice for 2

minutes. 950 µL of Super Optimal broth with Catabolite repression (SOC) medium is then added to each tube and incubated for 1.5 hours at 37°C with shaking (150 rpm). Two plates were used to spread each sample, the first one contained 50 µL of the transformation culture and the second one had 100 µL of the transformation culture. Different amounts were used on the two plates to ensure that one plate would not have an over-abundance of growth and a white colony would be able to be isolated easily. Plates were wrapped with parafilm, incubated for 24 hours at 37°C and subsequently placed in a fridge at 4°C to facilitate blue colour development.

2.12 Liquid Culturing of Transformed *E. coli*

500 mL of Milli-Q filtered water and 12.5 g of LB medium were added to a media jar. The media jar containing the solution was placed in the autoclave on a liquid 30 cycle. Once removed from the autoclave the contents were left to cool to room temperature. Prior to isolation of a white colony from each plate and the placement of it in liquid LB medium, the work station was sufficiently cleaned with 70% ethanol, as well, a Bunsen burner was lit to remove any contaminants in the air. Furthermore, the lip of the media jar containing liquid LB media was placed on top of the flame to get rid of any contaminants that might have attached while the media jar was open. 5 mL of liquid LB media was added to each sterile polypropylene tube. Plates containing blue and white colonies were removed from the fridge and placed on the sterile bench top. An inoculation loop was used to isolate a white colony from each plate. Prior to the isolation, the inoculation loop was placed over the Bunsen burner flame in order to kill off any bacteria or contaminants. It was then left to cool for approximately 30 seconds and then used to scrape one white colony off of the plate. It was then placed inside the sterile polypropylene tube containing the SOC medium. These steps were repeated until each sterile polypropylene tube

contained one white colony from its corresponding plate. Tubes were placed in incubator for 24 hours at 37°C with shaking (150 rpm).

2.13 Isolation of Plasmid DNA

The 5 mL bacterial cultures contained in the polypropylene tube were spun down in an eppendorf tube 1mL at a time. In order to do this the centrifuge was set to 8000 rpm for 3 minutes at room temperature. Following each spin, the supernatant was disposed of as biohazardous liquid waste and the pellet remained at the bottom of the eppendorf tube. Once all five spins were done, all the liquid supernatant was removed and the bacterial pellet remained. The QIAGEN QIAprep Spin Miniprep Kit was used to isolate the plasmid DNA. First, 250 µL of Buffer P1, containing RNase A, was placed in the eppendorf tube to re-suspend the cells. 250 µL of Buffer P2 was subsequently added and mixed, by inverting the tube six times, until the solution became blue. Buffer P2 caused the cells to lyse and released the DNA into the solution. This reaction cannot last longer then 5 minutes because it will destroy other components of the cell such as lysosomes, which would release digestive enzyme that can destroy the DNA. To stop this reaction, 350 µL of Buffer N3 is added and mixed immediately by inversion 6 times until the solution becomes clear. The tubes are then placed in the centrifuge for 10 minutes at 13,000 rpm and room temperature, in order to move all the cellular debris to the bottom (DNA is located in supernatant). Following centrifugation, 800 µL of the supernatant is placed in the QIAprep 2.0 spin column. The spin columns are placed in the centrifuge for 60 seconds to spin at 13,000 rpm at room temperature. This centrifuge step will allow the DNA to stick to the column and the liquid waste will go into the collecting tube and is subsequently disposed of. The column is then washed with 0.5 mL of Buffer PB and placed back in the centrifuge for 60 seconds at 13,000 rpm and room temperature. This wash with Buffer PB will remove any endonucleases that were

present in the JM109 High Efficiency Competent Cells that were used during the transformation steps. The flow through was collected in the waste container and disposed of as biohazardous liquid waste. 0.75 mL of Buffer PE is then added and centrifuged for 60 seconds at 13,000 rpm and room temperature. This buffer washes out any unwanted particles that might be in the spin column. Flow through in the collected tube is again discarded appropriately, and the spin column is subsequently placed back in the centrifuge to remove any residual wash buffer. The waste container is disposed of properly and the spin column is placed in a clean eppendorf tube. 50 µL of nuclease free water is placed on the center of the spin column and left to sit for 1-2 minutes. Subsequently the sample is centrifuged for 1 minute at 13,000 rpm and room temperature. This will elute the DNA from the spin column and the DNA sample will now be contained in the eppendorf tube. The eppendorf tubes with the DNA samples are then stored in the fridge at 4°C. The DNA samples were sent to the SickKids TCAG DNA Sequencing Facility for sequencing. 7.0 µL sample containing 50 ng of purified template DNA was placed in a sterile eppendorf tube with 0.7 µL of one of the primers for the DNA sequence (either the forward or reverse primer).

3. Results

3.1 Molecular analysis of *T. californicus* AOX

The HotSHOT method, developed for diapausing eggs, proved to be an effective DNA isolation method for copepods when the isolated DNA was subsequently used to conduct PCR. Conversely, when performing ligations and inserting the DNA into a plasmid more DNA was needed. Therefore, the HotSHOT method was implemented with a couple of alterations. First, the number of copepods was increased to ~25-30 instead of from a single copepod. Second, all solutions added into the eppendorf tube were tripled in volume (alkaline lysis buffer and neutralizing solution). Third, the copepods are sonicated (10 seconds sonication, 10 seconds on

ice ×3) instead of broken open with a sterile pipette tip (Table 6).

3.2 Primer Design Efficacy

In order to conduct PCR, primers were designed for *T. californicus* genes based on cDNA sequences from closely related species. Overall, 28 primers were developed for nine different genes (Tables 1 and 2). The majority of the primer sets (17/28) either produced no PCR product or produced multiple PCR products as visualized on DNA gels. Successful primers were identified for 16S (Primer sets 16S-1, -3 and -4), HSP20 (HSP20A, B and C) HSP70 (HSP70A, B, and C) HSP90 (HSP90A) and elongation factor 1 (EF1) (EF1-B); Table 7 summarizes the success of each primer set. Furthermore, for the genes that displayed a single PCR product of the correct size on the DNA gel, only three were ligated and cloned successfully. Subsequently, when samples were sent to The Centre for Applied Genomics (TCAG) at Sick Kids Hospital to be sequenced, none of the genes came back successfully sequenced. Table 7 summarizes the success of each primer set.

3.3 Heat Shock Proteins' Primers Efficacy

HSP20-A, B and C primers (Table 1) were successful at isolating a specific segment of the copepod genome (Figure 6). These segments were found to be the correct length as based on the expected cDNA product size (HSP20-A, 376bp; HSP20-B, 308bp; HSP20-C, 307bp). When undertaking ligation and cloning, only the sample containing the HSP20-C primers worked. This product was then sent off to be sequenced at TCAG DNA Sequencing Facility, but they were unable to successfully sequence the gene.

The next heat shock protein that we attempted to sequence, in *T. californicus*, was HSP70. Three primers (Table 1) were developed (HSP70-A, B and C) based on other closely

related organisms' HSP70 gene sequences (Table 1). All three primers were able to successfully isolate gene segments that were the correct product size (HSP70-A, 205bp; HSP70-B, 219bp; HSP70-C, 206bp) (Figure 7). We were unable to ligate and clone the HSP70 products and therefore they could not be send off for sequencing.

Another heat shock protein that we looked at was HSP90. Three sets of primers were developed based on HSP90 gene sequenced in closely related organisms to our copepod, *T. californicus* (Table 1). Only one of the three primers sets was effective at replicating the correct segment of DNA (HSP90-A, 394bp). HSP90-B (320bp) and HSP90-C (306bp) produced multiple bands on the DNA gel and therefore could not be ligated and cloned (Figure 7). When attempting to ligate and clone the HSP90-A sample, we were unsuccessful.

3.4 Housekeeping Genes' Primers Efficacy

A multitude of different housekeeping genes were looked at in order to sequence one in *T. californicus*. The first housekeeping gene that was looked at was GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Three sets of primers were developed (GAPDH-1, 2 and 3) based on GAPDH sequences in closely related copepods (Table 2). All primers were unsuccessful at replicating any segments of DNA from *T. californicus*. Therefore, this led to no products being displayed on the DNA gel (Figure 8). This made us conclude that GAPDH would not work as a housekeeping gene in our model organism based on the primers that have been developed.

The next housekeeping gene we attempted to sequence was EF1α. Three primers were designed based on closely related organisms' sequences in the NCBI database (Table 2). When undertaking PCR, primer sets EF1α-1 and EF1α-2 produced multiple sequences on the DNA gels, while the EF1α-3 primers produced no products. Consequently, ligation, cloning and sequencing could not be attempted.

In addition, we tried to sequence another gene in the EF1 complex, in the copepod *T. californicus*. Of the three primer sets (Table 2) developed (EF1-A, B and C), only EF1-B generated one band on the DNA gel of the correct product size (241bp) (Figure 9). Primer sets EF1-A and C produced multiple bands down the DNA gel and therefore could not be analyzed further (Figure 9). The sample containing the EF1-B primers was ligated and cloned and set to the TCAG DNA Sequencing Facility at SickKids Hospital, but they were unable to successfully sequence the gene.

Another housekeeping gene that was looked at was β-Tubulin. All three primer sets that were developed were ineffective at isolating the specific product of interest (Table 2). Primer sets β-Tubulin-1, 2 and 3 produced multiple bands down their respected lanes of the DNA gel (Figure 9). Ligation, cloning and sequencing were therefore not attempted.

The next housekeeping gene that we attempted to sequence was 16S. Three primer sets were developed based on DNA sequences found in the NCBI database of closely related organisms (Table 2). All three primer sets did not work in isolated a DNA fragment (Figure 10). These primers could not be used to sequence the 16S gene from *T. californicus*. Next, we attempted to sequence this gene by using bioinformatics. A sequence alignment was done to align the specific 16S gene sequence found in closely related organisms to the entire genome of *T. californicus* (Figure 10). This led to the identification of one section of the *T. californicus* genome that had a high similarity of base pairs to the 16S gene sequence found in another closely related arthropod (Figure 10). This segment of the *T. californicus* genome was then used to develop primers to isolate the 16S gene (Table 2). Four primer sets were developed (T. cal 16S-1, 2, 3 and 4) and three of the four were successful at isolating the correct product through PCR (T. cal 16S-1, 330bp; T. cal 16S-3, 282bp; T. cal 16S-4, 286bp) (Figure 11). The T. cal

16S-2 primer set produced multiple products on the DNA gel (Figure 11). Ligation and cloning was not attempted for these samples, instead DNA samples containing the working primers were sent to be sequenced directly at the TCAG DNA Sequencing Facility at SickKids Hospital. The sequencing facility was unable to sequence the gene of interest.

3.5 Identification of Novel AOX Sequences in Copepods

A molecular database search, using the National Center for Biotechnology Information's (NCBI) BLAST (Basic Local Alignment Search Tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), revealed the presence of a putative AOX sequence in seven different copepods species (Table 8). The copepod species are representative of three different orders (Calanoida, Cyclopoida, and Harpacticoida) and five different families. All the predicted AOX protein sequences contain at least 3 or more of the 4 iron-binding motifs which contain conserved glutamate (Glu, E) and histidine (His, H) residues required for the enzyme's functional activity (Chapter 1, Figure 3) (McDonald *et al*., 2009).

3.6 *In Silico* Analyses of the T*igriopus californicus* AOX Sequence

A putative *T. californicus* AOX DNA sequence of 551bp was found through a molecular database search (Table 9). The putative *T. californicus* cDNA sequence (Table 9) was translated using the ExPASy Translate Tool (http://web.expasy.org/translate/) into the predicted protein sequence summarized in Table 10. The predicted protein from *T. californicus* contained 3 of the 4 iron binding motifs, which are highly conserved glutamate and histidine residues required for AOX's enzymatic function in other organisms (McDonald *et al*., 2009).

Comparison of the AOX protein sequence from *T. californicus* to AOX protein sequences in plants (Figure 12), animals (Figure 13) and other copepods (Figure 14), demonstrated highly conserved regions across a multitude of organisms (Table 11). Protein sequence comparison

indicates that the glutamate (Glu, E) and histidine (His, H) residues required for AOX activity are completely conserved (Figures 12-14). Other residues that may be important in AOX catalysis including Q27/GlnI, Y38/TyrI, H46/HisII, Y60/TyrII, and Y108/TyrIII (numbering as per the *T. californicus* sequence) are conserved in all organisms analyzed, except that *C. glacialis* does not possess TyrIII (Y-108) (Figure 14). When looking at the copepod protein sequence, the C-terminal motif (N-P-[YF]-X-P-G-[KQE]), specific to animals, is highly conserved (McDonald *et al*., 2009) (Figures 13 and 14). *T. californicus* and *T. japonicus* possess a very similar motif with two variations (N-P-F-**E**-**K**-G-K) (Figure 9).

3.7 Amino Acid Conservation in AOX

When analyzing the core regions of the AOX protein in all the organisms it can be seen that all of the iron-binding residues which are imperative for AOX activity are conserved (Figure 11-13). Our results confirm that ArgI (R-3), LeuI (L-6) and GlnI (Q-27), amino acids that have been shown to be universally conserved in the dimer interface, are present across all AOX sequences examined (Figure 12-14). As well, a key tyrosine residue (Y-60/TyrII) involved in AOX activity is conserved in all organisms examined (Figures 12-14). Conversely, Isoleucine-24, which was previously thought to be conserved in the AOX protein structure, was only present in 14 of the 25 AOX sequences analyzed. Arginine-19 is another amino acid originally thought to be conserved amongst all AOX protein sequences, but it is not present in two of the organisms looked at, *C. glacialis* and *M. tecatiformis* (Figures 13 and 14). Along with the above mentioned universally conserved residues required for AOX's function, there are several other residues that have been demonstrated in previous research studies to be conserved in the AOX protein sequence. Table 12 outlines 23 of the 29 universally conserved residues in AOX protein sequences (this list includes the aforementioned residues). Only 23 residues are outlined due to

other six not being present in the protein region that was being analyzed.

4. Discussion

4.1 DNA Isolation from the Copepod *T. californicus*

The HotSHOT method was used to isolate DNA from the study organism *T. californicus*. This method was originally invented to isolate DNA from diapausing eggs. Due to the similar hard exterior of both diapausing eggs and copepods I predicted that this method could be used to isolate DNA from copepods. Despite the fact that there are numerous other ways to isolate DNA from copepods, the HotSHOT method has superior features, such as being a single tube procedure, which allows for increased efficiency and minimized risk of cross contamination. Although following the HotSHOT method led to isolation of DNA, the sample did not contain enough DNA to successfully use in PCR. When alterations were made to the HotSHOT method (increase in the number of copepods, sonication, and increased volume of solution), DNA was able to be successfully used in PCR. After performing DNA quantification on samples obtained using the HotSHOT method and the adjusted HotSHOT method, there was a 25-fold increase in the amount of DNA isolated (20 ng/µL and 500 ng/µL, respectively) (Table 6). We have demonstrated that this method of DNA extraction is consistent and reliable for DNA quantification and PCR amplification in copepods. The HotSHOT method was originally thought to only be able to isolate DNA from a variety of resting eggs (Ishida *et al*., 2012), but with the above changes, this reliable, simple and cheap DNA extraction approach can be used for other aquatic invertebrates. In summary, the altered HotSHOT method is a rapid, inexpensive, high-performance technique for PCR-quality DNA extractions from copepods, which avoids cross-contamination and, as larger volumes are used, it allows for more PCR reactions per sample. This DNA extraction methodology will facilitate the application of large-scale screening

molecular techniques in several areas of molecular ecology, from population genetics to barcoding studies.

4.2 The Taxonomic Distribution of AOX in Copepods

It had been initially thought that the AOX gene was not present in arthropods due to a gene loss event and because arthropod and vertebrate species require maximal muscle force from their aerobic ATP stores to survive in stressful conditions (Fernandez-Ayala *et al*., 2009; McDonald and Vanlerberghe, 2006; McDonald *et al*., 2009; Vanlerberghe, 2013). We have demonstrated that multiple species of copepods, from around the world, possess AOX in their genomes (Table 8). The first discovery of AOX in arthropods was from the brine shrimp *Artemia franciscana* (Rodriquez-Armenta *et al*., 2018). Previously, it was hypothesized that due to a gene loss event, AOX was not present in arthropods (McDonald *et al*., 2009), but the data displayed in Figure 9 disproves that hypothesis.

Next, to ensure that the AOX sequence recovered from the arthropod *T. californicus* was from an animal species and not from contamination (e.g. from a microbial symbiont or pathogen), that could have taken place throughout the experiment, the sequence was translated to its predicted protein sequence and analyzed. All animal AOX protein sequences possess a specific C-terminal motif (N-P-[YF]-X-P-G-[KQE]), which is highly conserved (McDonald *et al*., 2009). *T. californicus* and *T. japonicus* possesses a very similar motif with two variations (N-P-F-**E**-**K**-G-K) (Figure 14) (McDonald *et al*., 2009). To confirm this finding, when comparing *T. californicus*' AOX sequence to plant AOX sequences (Figure 12), it can be seen that none of the plants possess the same C-terminal motif.

4.3 AOX Protein Similarities and Differences Between Organisms

When looking at all organisms' AOX core regions it can be seen that all of the iron-binding residues required for AOX activity are conserved in the sequences (Figures 12-14) indicating that these proteins should be catalytically active. Moore *et al.* (2013) identified six residues within the dimer interface that are universally conserved. Three of these six residues are located in the N-terminus and the other three are located in the core and C-terminal regions. In regard to the three in the core and C-terminal regions, our results confirm the finding (Figures 7-9) that ArgI (R-3), LeuI (L-6) and GlnI (Q-27) are highly conserved across all AOX sequence in all organisms. Furthermore, Moore *et al.* identified eight residues that are conserved and play an important role in the dimeric structure of all AOXs. The majority of these residues are located in the N-terminal region and cannot be seen in our sequence alignment, but the last two (R-19 and I-24) are located within the sequence alignment. Arginine-19 is preserved in all organisms looked at except *C. glacialis* and *M. tecatiformis* (Figures 13 and 14). In contrast, Isoleucine-24 is not as conserved as previously thought, with only 14 of the 25 analyzed AOX protein sequences containing this residue. Previous research stated that a dimeric structure is not universally conserved in all AOX sequences; in order to explore this feature, the other six residues would have to be analyzed for conservation in the AOX protein sequence (Chaudhuri *et al*., 2005; Umbach and Siedow, 1993).

A key tyrosine residue (Y-60/TyrII) involved in AOX activity is conserved in all organisms examined (Figures 12-14). Therefore, at the functional level it is likely that the AOXs of all organisms, in all kingdoms, share a similar catalytic mechanism (McDonald, 2009). At a structural level, it is evident that a tryptophan residue (Y-38/TyrI) that is thought to a play a role in AOX structure is conserved amongst all organisms analyzed (Figures 12-14). All organisms'

AOX sequences, used in the multiple sequence alignment, display three of the highly conserved amino acid residues that have been thought to be involved with ubiquinol-binding (Q-27/GlnI, Y-38/TyrI, H-46/HisII) (Moore *et al*., 2013). The binding site for ubiquinol on AOX is the reducing substrate of the protein (Moore *et al*., 2013).

Along with the above mentioned universally conserved residues, there are numerous other residues that have been shown in previous studies to be preserved in AOX protein sequences in numerous organisms. Table 12 outlines 23 of the 29 universally conserved residues in AOX protein sequences (this list includes the aforementioned residues). Only 23 residues are outlined due to other six not being present in the protein region that was being analyzed. Of the 23 residues listed in the table almost all of them are completely preserved in all protein sequences except three organisms: *E. messerschmidtii*, *C. glacialis*, and *M. californianus* (Figures 12-14). *E. messerschmidtii* was only missing one of the 23 residues, Proline I (P-15) (Figure 13). *C. glacialis* was only missing one residue in its sequence (Glycine I/G-50) and the last three of the 23 residues (Histidine III/H-131, Asparagine II/N-135) due to the AOX gene only being a partial sequence (Figure 9). When comparing *T. californicus'* and *C. glacialis'* nucleotide sequences more closely it can be seen that there is a single nucleotide insertion mutation. There is an additional adenine nucleotide in the *C. glacialis* sequence (caaagtatt) when compared to the *T. californicus* sequence (caagtatt). This insertion mutation has led to a frameshift when translating the RNA to protein. When a single adenine is removed from the *C. glacialis* sequence it allows for the conservation of the reading frame and preserves the highly conserved tyrosine amino acid residue. *C. glacialis* is read caaagtatt instead of caagtatt as translated in *T. californicus*, this leads to the amino acid output in *C. glacialis* being KV instead of KY, as seen in *T. californicus*. *M. californianus* does not possess the correct last two

conserved residues, Asparagine II/N-135 and Histidine IV/H-136 (Figure 8). When further examining the differences between the *T. californicus* and *M. californianus* there appears to be a nucleotide deletion in the *M. californianus* DNA sequence towards the C terminus. There is one less guanine nucleotide in the *M. californainus* sequence (cgggggtcaatcaccccc) when compared to the *T. californicus* sequence (cggcttgtgaatcacacc). By inserting a guanine into the *M. californainus* nucleotide sequence (cgggggggtcaatcaccccc) it not only preserves the last two conserved amino acid that are characteristic of all AOX enzymes, but it also restores the last half of the C-terminal motif which is characteristic of animal AOXs (PGK) (Figure 15). When a single guanine is added to the *M. californainus* sequence it allows for the conservation of the reading frame and preserves the highly conserved asparagine and histidine amino acid residues (Figure 10). *M. californainus* is read cgggggggtcaatcac instead of cgggggtcaatcacc. With the addition of this single nucleotide it leads to the amino acid output in *M. californainus* being RGVNH instead of RGSIT. It is unknown whether these mutations exist in the animals themselves, or whether they are the result of poor sequencing methods.

When comparing AOX protein sequences amino acid by amino acid, it can be seen that *T. californicus'* sequence was similar to sequences from other Maxillopoda sequences (Table 13). But when comparing the *T. californicus* sequence to both plant and other animal AOX protein sequences, there was comparable percent similarities (Table 13).

5. Conclusions

In summary, we have altered a previously developed effective and efficient protocol in order to extract PCR-quality DNA from *T. californicus*, which avoids cross-contamination and, as larger volumes are used, it allows for more PCR reactions per sample. This technique can be applied to large-scale screening of organisms in several areas of research, such as: molecular ecology,

population genetics and barcoding studies. We have successfully developed primers for 16S (Primer sets T. cal 16S-1, -3 and -4), HSP20 (HSP20A, B and C) HSP70 (HSP70A, B, and C) HSP90 (HSP90A) and EF1 (EF1-B). When comparing *T. californicus'* AOX sequence to other organisms' AOX sequences, we found that the protein structure possessed the highly conserved amino acids required for the functionality of AOX. Furthermore, *T. californicus'* AOX protein sequence possessed the C-terminal motif that is highly characteristic of all animal AOX sequences studied to date. *T. californicus* has been demonstrated to be a good organism for the investigation of animal AOX when attempting to gain deeper understanding of its physiological function. Future work should endeavor to sequence the entire AOX gene from *T. californicus*. A full sequence would allow for the testing of hypotheses and predictions regarding conserved residues in the N-terminal region of the protein.

6. Future Directions

By developing a multitude of working primers for both housekeeping genes and other genes known to play a role in helping the organism survive under different environmental stressors, this brings researchers one step closer to being able to perform real-time PCR (RT-PCR) on *T. californicus*. Finding an effective housekeeping gene is necessary to investigate the transcription levels of AOX in an animal model. This research is making strides towards understanding the role and physiological function AOX plays in animal models, as AOX transcription levels have never been analyzed in an animal. By understanding why *T. californicus* possesses AOX, we can better understand why some other organisms, such as humans, do not express it or have lost the AOX gene. The study of animal AOX may ultimately lead to the treatment of mitochondrial dysfunction and disorders in humans using AOX gene therapy.

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Figures

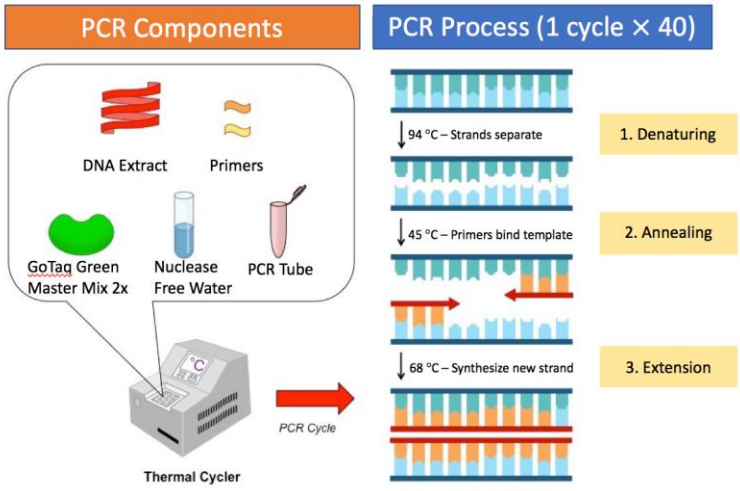

Figure 1: Polymerase chain reaction (PCR) is a method to detect and amplify specific DNA sequences based on the primers used. GoTaq Green Master Mix 2x, nuclease free water, DNA extract and forward and reverse primers are all added into a single PCR tube. The PCR tube is placed in a thermal cycler where it is subjected to changing temperatures to amplify a specific DNA sequence. 1) Solution is heated to 94°C to cause the DNA strands to separate (denature). 2) Solution is then cooled to 45°C to allow primers to bind to the template DNA strands (annealing). 3) Solution is heated to 68°C to permit the synthesis of the new strands (extension).
DNA, Deoxyribonucleic acid; PCR, Polymerase chain reaction
(lb.bioninja.au, 2018)

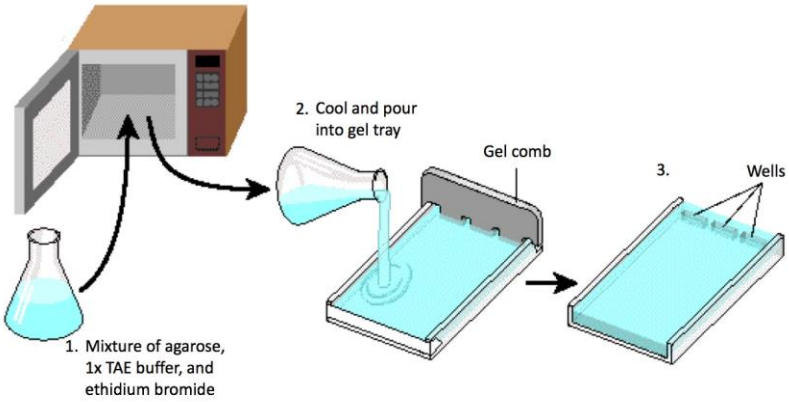

Figure 2: DNA gel preparation. 1) A mixture of agarose, 1x TAE buffer and ethidium bromide is placed in an Erlenmeyer flask in the microwave. 2) Once the solution begins to bubble it is removed from microwave and left to cool and subsequently poured into the gel tray with gel comb. 3) Left to solidify (~30 minutes) and gel comb is removed (Nslc.wistl.edu, 2018).
TAE buffer, Tris base, acetic acid and EDTA buffer

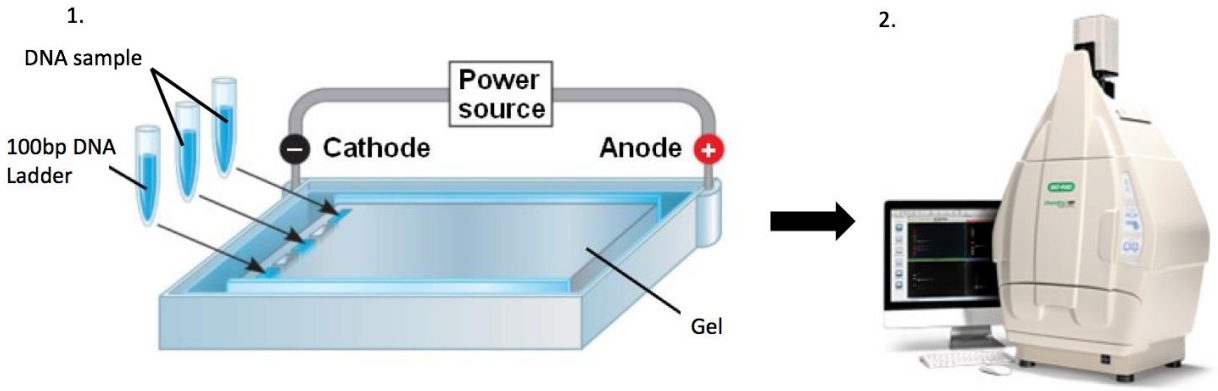

Figure 3: DNA gel electrophoresis. 1) Gel is placed into gel tank and the DNA samples and 100bp DNA ladder is loaded into the wells. The gel is run at 80V until the DNA is approximately 75% down the gel (running from cathode side to anode side) (Bio1151.nicerweb.con, n.d.). 2) The gel is taken out of the gel tank and placed in the VersaDoc (Boi-Rad) and imaged (Diamet.lv, n.d.).

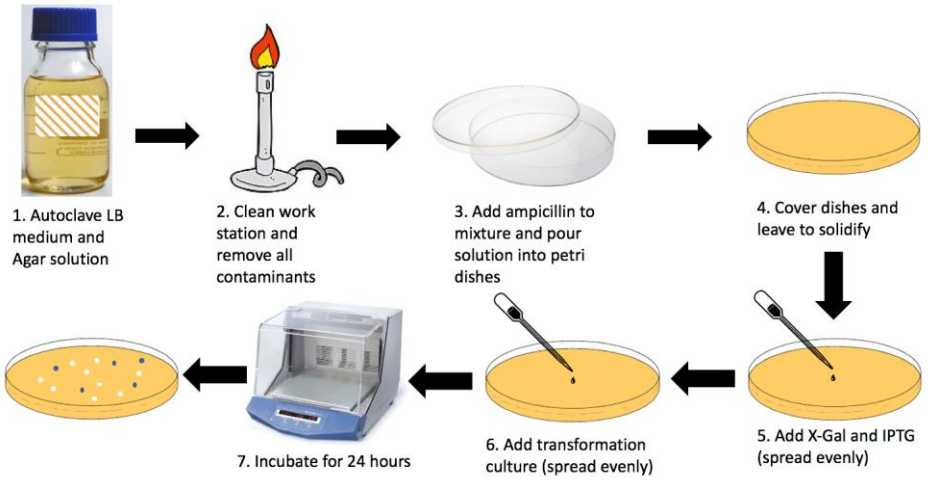

Figure 4: Preparation of Luria Broth agar plates. 1) Luria Broth medium and agar solution are mixed in a media jar and autoclaved to remove any contaminants. 2) Sufficiently clean the work station where the plate will be poured and light a Bunsen burner to remove and contaminants from the air. 3) Add ampicillin into the medium and pour the solution into petri dishes (3/4 full). 4) Cover petri dishes and leave it to solidify. 5) Add X-Gal and IPTG to each plate and evenly spread it around the whole plate (leave to absorb into media). 6) Add the transformation culture and spread it evenly around the whole plate, cover plate and wrap in parafilm. 7) Plates are incubated for approximately 24 hours at 37°C. The final product will have both light white (DNA inserted properly) and blue (DNA did not insert) colonies.
IPTG, Isopropyl β-D-1-thiogalactopyranoside; LB, Luria Broth; X-Gal, 5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside

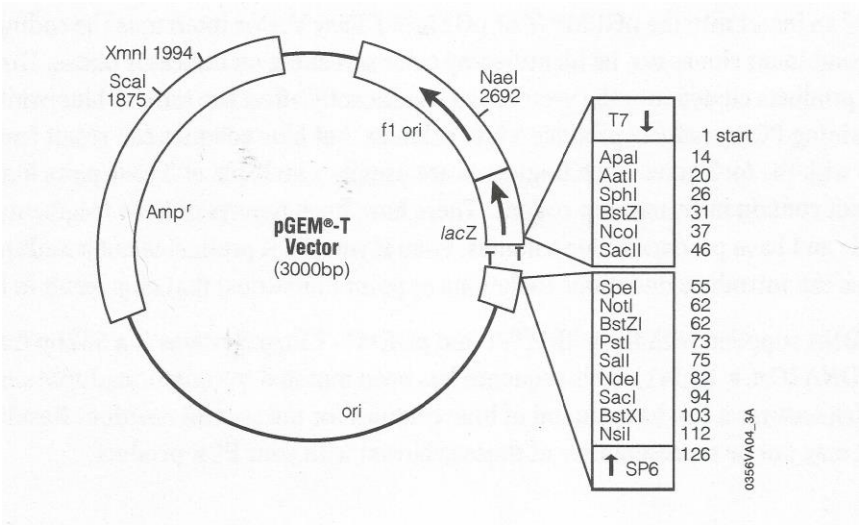

Figure 5: pGEM-T Easy Vector map and sequence reference points. When DNA insertion is successful, the DNA inserts into the LacZ gene thereby halting the production of β-galatosidase (colony is light white). If DNA insertion is unsuccessful, the LacZ gene continues to make β-galatosidase (colony is blue). (pGEM-T and pGEM-T Easy Vector Systems, 2015)

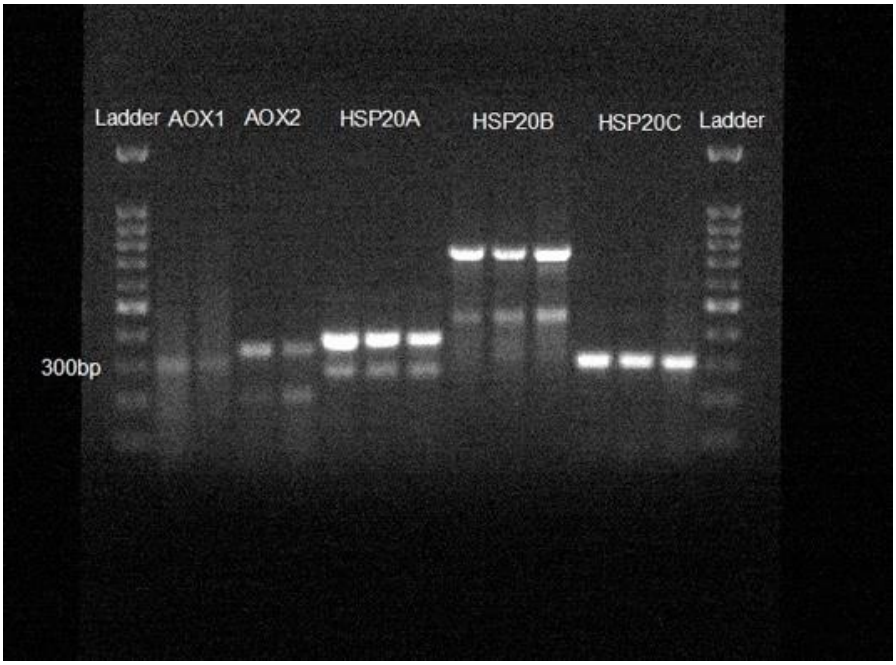

Figure 6: Agarose gel electrophoresis (2% agarose) of PCR amplified products using AOX and HSP20 primers and *T. californicus* isolated DNA. 3 µL of DNA ladder (GeneDirex, 100bp DNA Ladder Ready To Use), and the subsequent wells were loaded with 12 µL of each PCR sample. The gel was run at 80 V for approximately 1 hour and 20 minutes. Expected product sizes are displayed in Table 1.

<u>Legend</u>

1. 3 µL DNA ladder (GeneDirex, 100bp DNA Ladder Ready To Use)
2. 12 µL of PCR sample (DNA sample A, AOX1 primers)
3. 12 µL of PCR sample (DNA sample B, AOX1 primers)
4. 12 µL of PCR sample (DNA sample A, AOX2 primers)
5. 12 µL of PCR sample (DNA sample B, AOX2 primers)
6. 12 µL of PCR sample (DNA sample A, HSP20A primers)
7. 12 µL of PCR sample (DNA sample B, HSP20A primers)
8. 12 µL of PCR sample (DNA sample C, HSP20A primers)
9. 12 µL of PCR sample (DNA sample A, HSP20B primers)
10. 12 µL of PCR sample (DNA sample B, HSP20B primers)
11. 12 µL of PCR sample (DNA sample C, HSP20B primers)
12. 12 µL of PCR sample (DNA sample A, HSP20C primers)
13. 12 µL of PCR sample (DNA sample B, HSP20C primers)
14. 12 µL of PCR sample (DNA sample C, HSP20C primers)
15. 3 µL DNA ladder (GeneDirex, 100bp DNA Ladder Ready To Use)

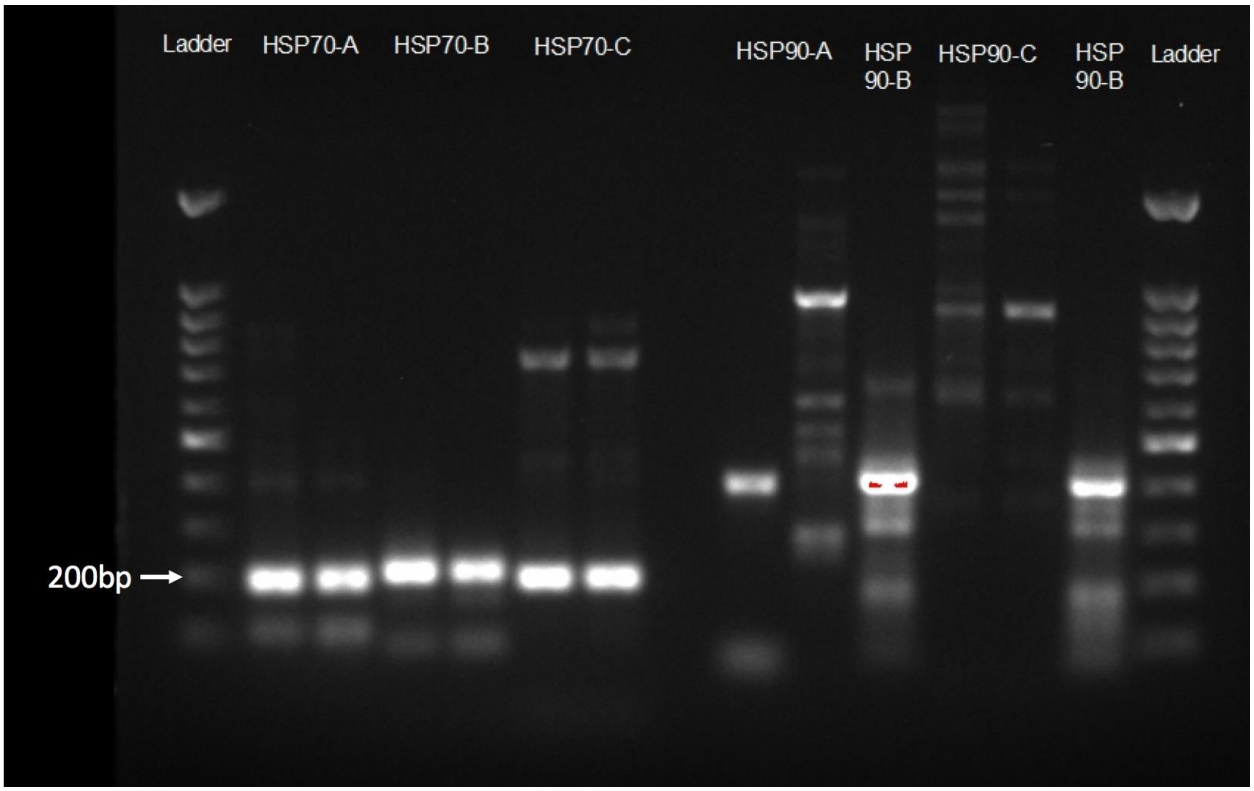

Figure 7: Agarose gel electrophoresis (2% agarose) of PCR amplified products using HSP70 and HSP90 primers and *T. californicus* isolated DNA. 3 µL of DNA ladder (GeneDirex, 100bp DNA Ladder Ready To Use), and the subsequent wells were loaded with 12 µL of each PCR sample. The gel was run at 80 V for approximately 1 hour and 20 minutes. Expected product sizes are displayed in Table 1.

<u>Legend</u>

1. 3 µL DNA ladder (GeneDirex, 100bp DNA Ladder Ready To Use)
2. 12 µL of PCR sample (DNA sample A, HSP70A primers)
3. 12 µL of PCR sample (DNA sample B, HSP70A primers)
4. 12 µL of PCR sample (DNA sample A, HSP70B primers)
5. 12 µL of PCR sample (DNA sample B, HSP70B primers)
6. 12 µL of PCR sample (DNA sample A, HSP70C primers)
7. 12 µL of PCR sample (DNA sample B, HSP70C primers)
8.
9. 12 µL of PCR sample (DNA sample A, HSP90A primers)
10. 12 µL of PCR sample (DNA sample B, HSP90A primers)
11. 12 µL of PCR sample (DNA sample A, HSP90B primers)
12. 12 µL of PCR sample (DNA sample A, HSP90C primers)
13. 12 µL of PCR sample (DNA sample B, HSP90C primers)
14. 12 µL of PCR sample (DNA sample B, HSP90B primers)
15. 3 µL DNA ladder (GeneDirex, 100bp DNA Ladder Ready To Use)

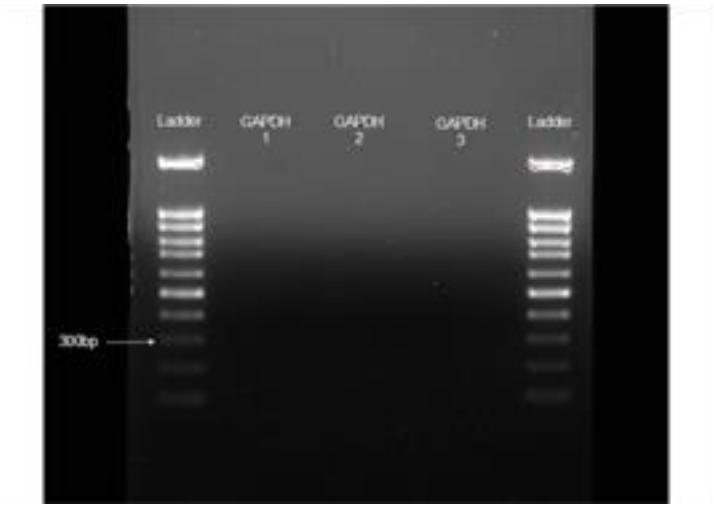

Figure 8: Agarose gel electrophoresis (2% agarose) of PCR amplified products using GAPDH primers and *T. californicus* isolated DNA. 3 µL of DNA ladder (GeneDirex, 100bp DNA Ladder Ready To Use), and the subsequent wells were loaded with 12 µL of each PCR sample. The gel was run at 80 V for approximately 1 hour and 20 minutes. Expected product sizes are displayed in Table 2.

<u>Legend</u>

1. 3 µL DNA ladder (GeneDirex, 100bp DNA Ladder Ready To Use)
2. 12 µL of PCR sample (DNA sample A, GAPDH-1 primers)
3. 12 µL of PCR sample (DNA sample B, GAPDH-1 primers)
4. 12 µL of PCR sample (DNA sample A, GAPDH-2 primers)
5. 12 µL of PCR sample (DNA sample B, GAPDH-2 primers)
6. 12 µL of PCR sample (DNA sample A, GAPDH-3 primers)
7. 12 µL of PCR sample (DNA sample B, GAPDH-3 primers)
8. 3 µL DNA ladder (GeneDirex, 100bp DNA Ladder Ready To Use)

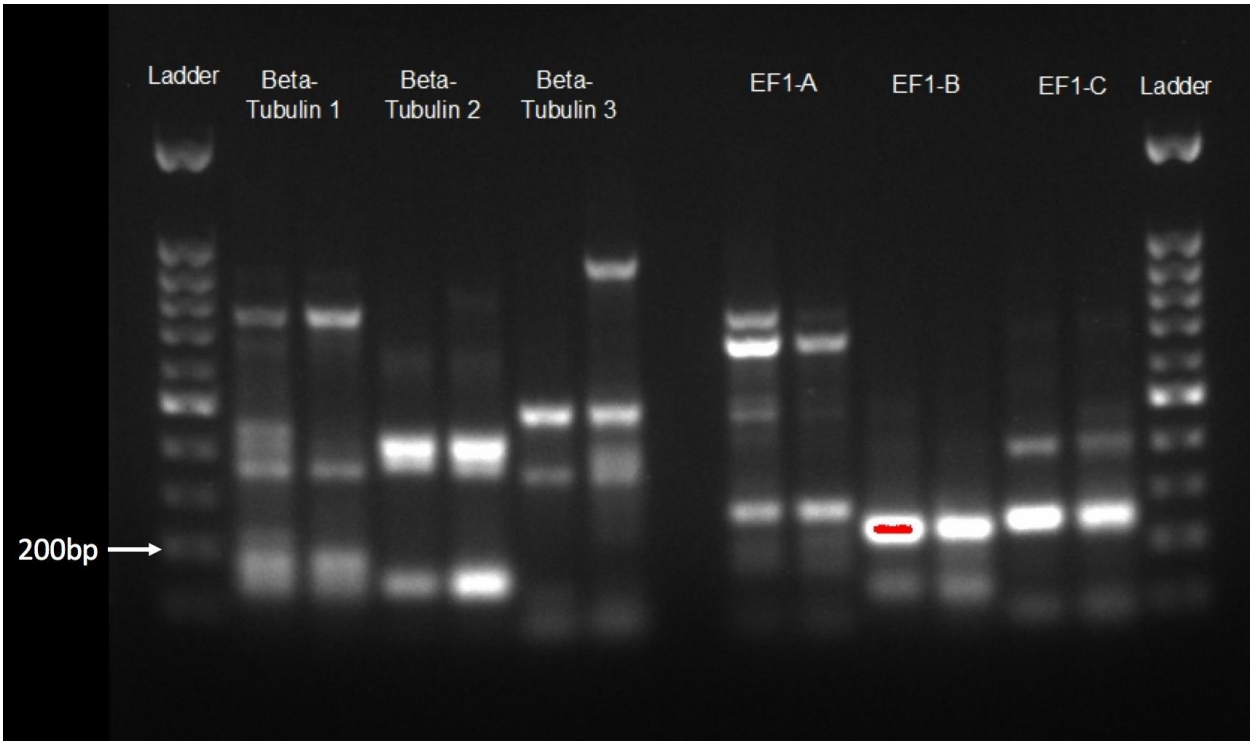

Figure 9: Agarose gel electrophoresis (2% agarose) of PCR amplified products using β-Tubulin and EF1 primers and *T. californicus* isolated DNA. 3 µL of DNA ladder (GeneDirex, 100bp DNA Ladder Ready To Use), and the subsequent wells were loaded with 12 µL of each PCR sample. The gel was run at 80 V for approximately 1 hour and 20 minutes. Expected product sizes are displayed in Table 2.

<u>Legend</u>

1. 3 µL DNA ladder (GeneDirex, 100bp DNA Ladder Ready To Use)
2. 12 µL of PCR sample (DNA sample A, β-Tubulin-1 primers)
3. 12 µL of PCR sample (DNA sample B, β-Tubulin-1 primers)
4. 12 µL of PCR sample (DNA sample A, β-Tubulin-2 primers)
5. 12 µL of PCR sample (DNA sample B, β-Tubulin-2 primers)
6. 12 µL of PCR sample (DNA sample A, β-Tubulin-3 primers)
7. 12 µL of PCR sample (DNA sample B, β-Tubulin-3 primers)
8.
9. 12 µL of PCR sample (DNA sample A, EF1-A primers)
10. 12 µL of PCR sample (DNA sample B, EF1-A primers)
11. 12 µL of PCR sample (DNA sample A, EF1-B primers)
12. 12 µL of PCR sample (DNA sample B, EF1-B primers)
13. 12 µL of PCR sample (DNA sample A, EF1-C primers)
14. 12 µL of PCR sample (DNA sample B, EF1-C primers)
15. 3 µL DNA ladder (GeneDirex, 100bp DNA Ladder Ready To Use)

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T. californicus, Mitochondrial Genome   GCCCGGTGATAAAAATTAAACGGCTTGACAAGGTAGCAAAATCAATTGTTCTTTAATTGA
C. sinicus 16S                          ---------------CCGCGTTAGT-GCTAAGGTAGCATAATAATTAGTTTTTTAATTGG
                                                             * *  ********* *** * * *** ********

T. californicus, Mitochondrial Genome   GAACCAGAATGAACGGCTTAATTAACTTAAAATATCAGACCTGTTTCGTTTAAATTAATA
C. sinicus 16S                          AAAATGGAATGAATGGTTTCACTAAAATATAGCATTTACACTAGTAAGTGAAATTT--TA
                                         **   ******* ** ** * ***  ** *  **     **  *  **  ** **  **

T. californicus, Mitochondrial Genome   TTCTGAGTGCAAATACTTAGACTAAATTCAGGGACGAGAAGACCCTA-AAATCTTATTTA
C. sinicus 16S                          ATTTAAGTGAAAATTCTTAAAAGACCCACTTAGACGAGAAGACCCTATGAATCTGGTAAA
                                         * * **** **** **** *  *    *   ***************  *****  *  *

T. californicus, Mitochondrial Genome   CT-----TATTTAGATTATTATAAAAAATTAATTGGTTGGGGCAAC--CTAATAAATGTA
C. sinicus 16S                          CTAAGAATGTGCCTACAATAGTTAATTGTTTATTTTTTGGGGTAAAAATTAATAAAAATA
                                        **     * *    *  **  * **   ** ***  ****** **    *******  **

T. californicus, Mitochondrial Genome   AATACTTTTTATAGTCTAAACTTGAACTCTGCAACTTATTACCAAGATACTTTAGGGATA
C. sinicus 16S                          TTAATAAATACTTATTTAACTTAATCCTCTAGGAAACATGAAGAAGATCCTCTAGGGATA
                                           *    *  *  * ***  *    ****   *   ** *  ***** ** ********

T. californicus, Mitochondrial Genome   ACAGCATTAAGGGCCTTGGAGTTCATATCT-ACTGTGCCTGAATGACCTCGATGTTGAAT
C. sinicus 16S                          ACAGCATTATACTTAAAAGAGTTCTTATCAGAATAAGTGTTTGTGACCTCGATGTTGAAT
                                        *********         ****** ****  * *  *  *   ****************

T. californicus, Mitochondrial Genome   TAAGAAACCTTCTAAGAGAAAAAGCCTAGAAGTT-CAGTCTGTTCGACTGGTATTTTCTT
C. sinicus 16S                          TAAATACTCCCGTGTGTGTAGGAGCTCACTGGAGACGGTCTGTTCGACCTATAATAT-TT
                                        ***  *  *   *  * * *  ***  *   *   * ***********   ** * * **

T. californicus, Mitochondrial Genome   ACATGATTTGAGTTAAGATCGACGTAAGTCAGATTGGTTTCTATCTTGAATTTTCTTTGA
C. sinicus 16S                          ACATGATTTGAGTTAAAATCGACGTAAGTCAGATTGGTTTC------------------
                                        *************** ************************
```

16.

Figure 10: DNA Sequence alignment between *T. californicus'* entire mitochondrial genome (only portion aligned shown) and *C. sinicus's* 16S gene sequence.

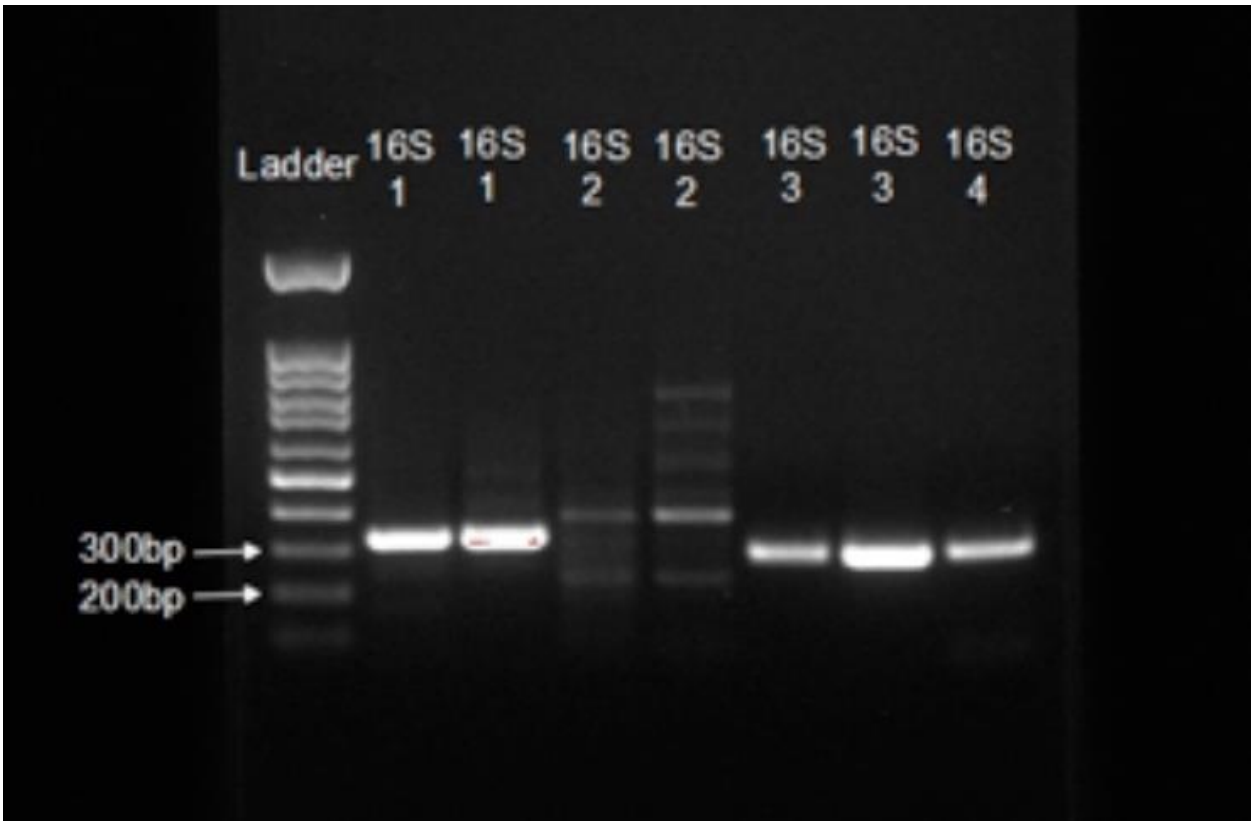

Figure 11: Agarose gel electrophoresis (2% agarose) of PCR amplified products using 16S primers and *T. californicus* isolated DNA. 3 µL of DNA ladder (GeneDirex, 100bp DNA Ladder Ready To Use), and the subsequent wells were loaded with 12 µL of each PCR sample. The gel was run at 80 V for approximately 1 hour and 20 minutes. Expected product sizes are displayed in Table 2.

<u>Legend</u>

1. 3 µL DNA ladder (GeneDirex, 100bp DNA Ladder Ready To Use)
2. 12 µL of PCR sample (DNA sample A, T. cal 16S-1 primers)
3. 12 µL of PCR sample (DNA sample B, T. cal 16S-1 primers)
4. 12 µL of PCR sample (DNA sample A, T. cal 16S-2 primers)
5. 12 µL of PCR sample (DNA sample B, T. cal 16S-2 primers)
6. 12 µL of PCR sample (DNA sample A, T. cal 16S-3 primers)
7. 12 µL of PCR sample (DNA sample B, T. cal 16S-3 primers)
8. 12 µL of PCR sample (DNA sample A, T. cal 16S-4 primers)

```
T. californicus     NERMHLMTFMRLRRPGPIFRGTVILTQWLFTFTFSFAYILSPNFCHRFVGYLEEQAVVTYTHILEEIDAG
C. orbiculans       NERMHLMTFMEVSQPKWWERMLVMAVQGVFFNAYFVIYLMAPRVAHRVVGYLEEEAIHSYTEFLKEIDNG
A. capillus-veneris NERMHLMTFMEVSQPKWWERMLVMAVQGVFFNAYFVIYLMAPRVAHRVVGYLEEEAIHSYTEFLKEIDNG
N. hyalina          NERMHLMTFMEVSQPRWWERALVLVTQGIFANAFFVLYIISPRVAHRVVGYLEEEAIISYTGYLEAIDSG
P. banksiana        NERMHLMTFMEVAKPRWYERALVFTVQGIFFNAYFLMYILSPKLAHRITGYLEEEAIHSYTEFLKELDKG
K. subtile          NERMHLMTFVEITKPRLWERALVFGVQGVFFNAYFLAYILWPKVCHRIVGYLEEEAVISYTHYLNDIDSG
C. incerta          NERMHLITFLQLRQPGPAFRAMVIVAQGVFFNAYFLAYLLSPRTCHAFVGFLEEEAVKTYTHALEEIDAG
C. reinhardtii      NERMHLITFLQLRQPGPAFRAMVILAQGVFFNAYFIAYLLSPRTCHAFVGFLEEEAVKTYTHALVEIDAG
A. acetabulum       NERMHLLTFLKLREPGPLFRGFVILTQGIFFNTFFLAYLVSPTLCHRMVGYLEEEAIKTYSHCLHDIETG
                     GluII HisI                    GlnI        TyrI      HisII  GluIII AlaI TyrII

T. californicus     RLPMWKTLPAPELAIKYWRLPEDAKMREVILAIRADEAHHRLVNHTLGSMDLKDNPFEKGK
C. orbiculans       ---NIPNVPAPQIAIDYWSLPKNAKLRDVVVVVRADEAHHRDVNHFASGVLRDGKKLREVG
A. capillus-veneris ---NIPNVPAPQIAIDYWSLPKNAKLRDVVVVVRADEAHHRDVNHFASGVLRDGKKLREVG
N. hyalina          ---KIENSPAPQIAIDYWKLPKDARLREVVVAVRADEALHRDVNHFASDIKQHGKELKEVP
P. banksiana        ---NIPNVPAPAIAIDYWRLPKDSTLRDVVVVVRADEAHHRDVNHFASDIHYQGKELREAA
K. subtile          ---KIANTAAPAIAIDYWRLPKDAKLRDVVLAVRADEAHHRDVNHFAANIKTEGKQLKESP
C. incerta          --RLWKDTPAPPVAVQYWGLKQGATMRDLILAVRADEACHAHVNHTLSQLNPSTDANPFAT
C. reinhardtii      --RLWKDTPAPPVAVQYWGLKPGANMRDLILAVRADEACHAHVNHTLSQLNPSTDANPFAT
A. acetabulum       --LGWAERPAPPIAIEYWKLPADASMRDVVLAVRADEACHSHVNHTFASMGP-KDTNPFSP
                                  TyrIII                  GluIV HisIII
```

Figure 12: A multiple-sequence alignment from the second iron-binding site to the end of the AOX protein from a variety of plant models. The black arrows denote the iron-binding residues (GluII, GluIII, GluIV, HisI, HisIII), the green arrows point to residues important for AOX activity (GlnI, TyrI, HisII, AlaI, TyrII, TyrIII).

```
T. californicus     NERMHLMTFMRLRRPGPIFRGTVILTQWLFTFTFSFAYILSPNFCHRFVGYLEEQAVVTYTHILEEIDAG
E. messerschmidtii  NERMHLMICMGLRKMGPFLRTIVFAAQMGIVPFYAIGYFFSPRFCHSFVGYLEEEAVKTYTHVLQLCDAG
O. niloticus        NERMHLLTAMTMKKPGLFMRSMIIGAQGVFVTGFSLAYLLSPRFCHRFVGYLEEEAVKTYTHCLKELDAG
L. decemlineata1    NERMHLMTFMTLKNPSPLFRAMVIAAQGVFYNAFFLCYIVSPSTCHRFVGHLEEEAVITYTRCIKEIEAG
H. tuberculata      NERMHLMTFMTLKNPSPLFRAMVIAAQGVFYNAFFLCYIVSPSTCHRFVGHLEEEAVITYTRCIKEIEAG
T. domestica        NERMHLMTALVLRKPTPIFRAGVLMTQGLFLSAFSLSYLLSPRFAHRFVGYLEEEAVITYTKLLVEIDAG
R. venosa           NERMHLMTALQLKQPSLLFRTAVIGSQGLFVTMFSVWYMFSPRFCHRFVGYLEEEAVKTYTKCLQDIDQG
C. cathayensis      NERMHLMTALQIKKPSMLFRLSVIGSQGVFVTMFFVWYAISPKFCHRFVGYLEEEAVKTYTKCLEDIDHG
P. maximus          NERMHLMTALQLRQPTWLFRGCVIGAQGIFVTFFSFAYLLSPRFCHRFVGYLEEEAVITYSKCLKDIKDG
M. californianus    NERMHLMTALQLRRPTKIFRLGVVLSQGAFVTMFSLAYLISPRFCHRFVGYLEEEAVITYTRCVQDIHHG
M. tectiformis      NERMHLMTALRIANPGLFMKLSIAGAQGIFVSLFSLAYFISPNYCHRFVGYLEEEAVVTYTSCLESIDSG
                     GluII HisI                    GlnI        TyrI      HisII  GluIII AlaI TyrII

T. californicus     RLPMWKTLPAPELAIKYWRLPEDAKMREVILAIRADEAHHRLVNHTLGSMDLKDNP----------FEKG
E. messerschmidtii  KLPKFD-TPAHVSARNYYHLPANATMRDVITNIRADEAHHRELNHCLAHLTKKECN------PFPPGY--
O. niloticus        KLPMWQNTIAPEVARNYWKLPEDASMRDLIAQIRADEAHHRVVNHNFSDLAVVEKKTGVAVNPYGPGR--
L. decemlineata1    HLPLWTNLPAPEIAKDYWRLPEDAKFLDVVYAVRSDESTHRFVNHTLASLDYKNDVNPFALREPDMTTKG
H. tuberculata      HLPLWTNLPAPEIAKDYWRLPEDAKFLDVVYAVRSDESTHRFVNHTLASLDYKNDVNPFALREPDMTTKV
T. domestica        HLPLFESLPAPPVARDYWALSHEANFRDVVLAIRADESHHREVNHTFAGMQAHQPNP---------FPPG
R. venosa           PMKHWQTQVAPEVAIRYWKLSEDATMRDVILAIRADEAHHRLVNHTLA----------------------
C. cathayensis      SMKHWNTKSAPPLAITYWKLPETAVMRDVILAIRADEAHHRVVNHTLASLNYNDFNPYKPGQ--------
P. maximus          PMKHWQTQEAPDLAIRYWKLDKNATMLDVILAIRADEAHHRVVNHTLASLKEDDYNPYKPGK--------
M. californianus    SHAHWKTQSAPESAISYWKLPDNAQMLDVIFANRADEAQHRGSITP------------------------
M. tectiformis      HLKMWSRMKAPEIAIEYWKLSDDAMMRDVILAIRADEAHHRAVNHTLGSKKSDEANPFGPGE--------
                                  TyrIII                  GluIV HisIII
```

Figure 13: A multiple-sequence alignment from the second iron-binding site to the end of the AOX protein from a variety of animal models. The black arrows denote the iron-bonding residues (GluII, GluIII, GluIV, HisI, HisIII), the green arrows point to residues important for AOX activity (GlnI, TyrI, HisII, AlaI, TyrII, TyrIII).

```
T. californicus     NERMHLMTFMRLRRPGPIFRGTVILTQWLFTFTFSFAYILSPNFCHRFVGYLEEQAVVTYTHILEE
T. japonicus        NERMHLMTFMRLRRPGPIFRGTVILTQWLFTFTFSFAYLLSPNFCHRFVGYLEEQAVVTYTHILEE
P. nana             NERMHLMTFMQMRKPGPLFRYTVILTQYLFTIAFSLGYLISPNFCHRFVGYLEEQAVVTYTHILEE
E. serrulatus       NERMHLMTFLQLKKPGPFFRGTVIVSQWIFTAFFSFAYLVSPQFCHRFVGYLEEQAVVTYSHILEE
C. finmarchicus     NERMHLMTFLQLRHPGPLFRGSVILTQLVFTAAFSMAYVVSPHFCHRFVGYLEEQAVVTYSDILQK
C. glacialis        NERMHLMTFLXLRHPGPLFXGSVXLTQLVFTAAFSMAYVVSPHFCHRFVXYLEEQAVVTYSDILQK
E. affinis          NERMHLLTFLELKKPGPIFRGVVVFTQLSFTAGFSLAYLISPHFCHRFVGYLEEQAVVTYTQILNK
                    GluII HisI                    GlnI        TyrI      HisII  GluIII AlaI  TyrII

T. californicus     IDAGRLPMWKTLPAPELAIKYWRLPEDAKMREVILAIRADEAHHRLVNHTLGSMDLK-DNPFEKGK
T. japonicus        IDAGRLPMWKTLPAPELAIKYWRLPENAKMREVILAIRADEAHHRLVNHTLGSMDLKSDNPFEKGK
P. nana             LDSGRLPMWEKLPAPEIAVKYWELPKDAMMRDVILAIRADEAHHRLVNHTLGSMKLHDENPFKPGH
E. serrulatus       LDKGNLPMWKVLPAPPIAIDYWQLPKDALMRDVILAIRADEAHHRLVNHTLGSMNVRDSNPFKPGE
C. finmarchicus     MDEGKLPMWSRLPAPEIAAKYWKLGEGAMMRDVILAIRADEAHHRVVNHTLGDMDLKQKNPFGPGE
C. glacialis        MDEGKLPMWSXLPAPEIAAKVLEARKGL--------------------------------------
E. affinis          IDEGKLPMWKNLPAPVIAVKYWKLDEKAVMRDVILAIRADEAHHRQVNHTLGDLDHNQDNPYGPGQ
                                    TyrIII                GluIV HisIII
```

Figure 14: A multiple-sequence alignment from the second iron-binding site to the end of the AOX protein from a variety of arthropods belonging to Maxillopoda. The black arrows denote the iron-bonding residues (GluII, GluIII, GluIV, HisI, HisIII), the green arrows point to residues important for AOX activity (GlnI, TyrI, HisII, AlaI, TyrII, TyrIII).

```
T. californicus     NERMHLMTFMRLRRPGPIFRGTVILTQWLFTFTFSFAYILSPNFCHRFVGYLEEQAVVTYTHILEEIDAG
M. californianus    NERMHLMTALQLRRPTKIFRLGVVLSQGAFVTMFSLAYLISPRFCHRFVGYLEEEAVITYTRCVQDIHHG
M. californianus+g  NERMHLMTALQLRRPTKIFRLGVVLSQGAFVTMFSLAYLISPRFCHRFVGYLEEEAVITYTRCVQDIHHG
                                                                      C-terminal
                                                                        motif
T. californicus     RLPMWKTLPAPELAIKYWRLPEDAKMREVILAIRADEAHHRLVNHTLGSMDLKDNPFEKGK---------
M. californianus    SHAHWKTQSAPESAISYWKLPDNAQMLDVIFANRADEAQHRGSITP------------------------
M. californianus+g  SHAHWKTQSAPESAISYWKLPDNAQMLDVIFANRADEAQHRGVNHPLTFIKEMTTFLLTRKRKLILLPGK
                                                   AsnII HisIV                  C-terminal
                                                                                  motif
```

Figure 15: Sequence alignment from the second iron-binding site to the end of the AOX protein from *T. californicus* and *M. californianus*. As well, *M. californianus*+g accounts for the deletion mutation. Black arrows denote two highly conserved residues characteristic of AOX.

Tables

Table 1: The custom primers that were made to amplify the AOX and HSP 20, 70 and 90 genes from *T. californicus*. Three different forward and reverse primer sets were created for each sequence in order to generate cDNA products ranging from 200- 500bp in size. AOX primers were developed by Jaspreet Singh.
AOX, Alternative Oxidase; HSP, Heat Shock Protein

Primer Name	**Forward Primer Sequence (5' → 3')**	**Reverse Primer Sequence (5' → 3')**	**Expected cDNA Product Size (bp)**	**Tm**
AOX1	CCTGACTCAATGGCTGTTCA	CAAAATGACTTCCCGCATCT	232	60
AOX2	TGCCACAGATTTGTTGGGTA	TTCTCAACCTCCGTTCGTTT	344	60
AOX3	GGATCCACACTCTCCTGGAA	CTCAACCTCCGTTCGTTTTT	503	60
HSP 20A	AATGAGCTCAGAACGCGACT	TTGAAGAACTCCTCCGCTGT	376	57
HSP 20B	AAAGCTCTGCCTCAAACCAA	ATCCTTTTCTCAGGCGCATA	308	60
HSP 20C	AAGCTCTGCCTCAAACCAAA	ATCCTTTTCTCAGGCGCATA	307	60
HSP 70A	GCGATCTGCTTCTTTTGGAC	CCCAACAAATGGTTGTCCTT	205	60
HSP 70B	AGCGATCTGCTTCTTTTGGA	CAAGTCAAATTTGCCCAACA	219	60
HSP 70C	AGCGATCTGCTTCTTTTGGA	CCCAACAAATGGTTGTCCTT	206	60
HSP 90A	GTGATCCGCAAGAACTTGGT	CGAGGTTCTTGCCATCGTAT	394	60
HSP 90B	CGAGGATGAAGGAGAACCAA	CACGATCACCTTCTCCACCT	320	60
HSP 90C	GAACATCAAATTGGGCATCC	GTGACGGACACGAGGTTCTT	306	60

Table 2: The custom primers that were made to amplify different housekeeping genes from *T. californicus*. Three different forward and reverse primer sets were created for each sequence in order to generate cDNA products ranging from 100- 500bp in size.
GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; EF1, Elongation Factor 1; T. cal, *T. californicus*

Primer Name	Forward Primer Sequence (5' → 3')	Reverse Primer Sequence (5' → 3')	Expected cDNA Product Size (bp)	Tm
GAPDH - 1	CAATGCCTCTTGCACCACTA	CCCATTCAGCTCAGGGATAA	235	60
GAPDH - 2	CAATGCCTCTTGCACCACTA	GTCAGCTTCCCATTCAGCTC	243	60
GAPDH - 3	GGGAAAGGTTATCCCTGAGC	GACCTGGTGTCTCCCAAGAA	228	60
EF1α - 1	AGACTTCCCCTCCAGGATGT	ACCTGTCCAGGATGGTTCAG	320	60
EF1α - 2	GAACGGTCAGACCAGAGAGC	CATCTCCACGGACTTGACCT	496	60
EF1α - 3	TTGGGTGGAATGAAGTGTGA	TGATAAGGACACCGCAATCA	420	60
EF1 - A	ACCAGCTGACCACTGAGGTT	CAGCGAACTTGCAAGCAATA	273	60
EF1 - B	GGTTAAGTCCGTGGAGATGC	AATATGGGCGGTGTGACAAT	241	60
EF1 - C	GGTTAAGTCCGTGGAGATGC	CAGCGAACTTGCAAGCAATA	257	60
β-Tubulin 1	GACCCTGCCTCGTCGTAGTA	CAGATCGGAGCCAAGTTTTG	125	60
β-Tubulin 2	CCCTGCCTCGTCGTAGTAGA	CAGATCGGAGCCAAGTTTTG	123	59
β-Tubulin 3	ACCCTGCCTCGTCGTAGTAG	GGAGCCAAGTTTTGGGAGAT	118	59
16S - 1	TTGGAAAATGGAATGAATGG	TCAACATCGAGGTCACAAACA	300	58
16S - 2	CGAGAAGACCCTATGAATCTGG	TCGAACAGACCGTCTCCAGT	253	60
16S - 3	TGGAAAATGGAATGAATGGTT	TCAACATCGAGGTCACAAACA	299	59
T. cal 16S 1	AACGGCTTGACAAGGTAGCA	CAACATCGAGGTCATTCAGG	330	60
T. cal 16S 2	AACGGCTTGACAAGGTAGCA	CCAGTCGAACAGACTGAACTTCT	383	60
T. cal 16S 3	GACGAGAAGACCCTAAAATCTTATT	CGTCGATCTTAACTCAAATCATGT	282	60
T. cal 16S 4	GACGAGAAGACCCTAAAATCTTATT	CTTACGTCGATCTTAACTCAAATCA	286	60

Table 3: Summary of contents in PCR mixtures.

Reagent	Volume (µL)
Nuclease free water	25
GoTaq Green Master Mix 2x	12.5
Primer set – Forward Primer	2
Primer set – Reverse Primer	2
DNA extract	5
Total	**46.5**

Table 4: Thermal cycler program for PCR

Step	Temperature (°C)	Time (seconds)	Number of Cycles
Initial denaturation	94	180	1
Denaturation	94	30	40
Annealing	45	60	40
Elongation	68	120	40
Final elongation	68	420	1

Table 5: Summary of contents in a tube for a standard ligation reaction.Note: For the positive control, all contents are the same except,instead of adding 2 µL of PCR product, 2 µL of control insert DNA is added.

Reagent	Volume (µL)
Nuclease free water	1
2× Rapid Ligation Buffer	5
pGEM-T Easy Vector	1
PCR product or Control insert DNA	2
T4 DNA Ligase	1
Total	**10**

Table 6: Summary of differences between the original HotSHOT DNA isolation method, outlined in Montero-Pau *et al*., 2008, and the DNA isolation method employed for this research project.

Original HotSHOT Method	Adjusted HotSHOT Method
- 1 copepod - 150 µL alkaline lysis buffer and 150 µL neutralizing solution - Pipette tip	- ~20-30 copepods - 3× volumes of solutions added - Sonication

Table 7: Summary of primer set development and efficacy at each step of DNA cloning in *T. californicus*. MS, multiple signals; NP, no product.

Gene Name	Detection via Bioinformatics	Primer Set Design	Successful PCR?	Successful Ligation & Cloning?	Successful Sequencing of PCR product?
HSP20	✔	HSP20-A	✔	✘	✘
		HSP20-B	✔	✘	✘
		HSP20-C	✔	✔	✘
HSP70	✔	HSP70-A	✔	✘	✘
		HSP70-B	✔	✘	✘
		HSP70-C	✔	✘	✘
HSP90	✔	HSP90-A	✔	✘	✘
		HSP90-B	✘ – MS	✘	✘
		HSP90-C	✘ – MS	✘	✘
GAPDH	✔	GAPDH - 1	✘ – NP	✘	✘
		GAPDH - 2	✘ – NP	✘	✘
		GAPDH - 3	✘ – NP	✘	✘
EF1α	✔	EF1α - 1	✘ – MS	✘	✘
		EF1α - 2	✘ – MS	✘	✘
		EF1α - 3	✘ – NP	✘	✘
EF1	✔	EF1 - A	✘ – MS	✘	✘
		EF1 - B	✔	✔	✘
		EF1 - C	✘ – MS	✘	✘
β-Tubulin	✔	β-Tubulin - 1	✘ – MS	✘	✘
		β-Tubulin - 2	✘ – MS	✘	✘
		β-Tubulin - 3	✘ – MS	✘	✘
16S	✔	16S - 1	✘ – NP	✘	✘
		16S - 2	✘ – NP	✘	✘
		16S - 3	✘ – NP	✘	✘
T. californicus 16s	✔	T. cal 16S - 1	✔		✘
		T. cal 16S - 2	✘ – MS	✘	✘
		T. cal 16S - 3	✔		✘
		T. cal 16S - 4	✔		✘

Table 8: Putative AOX sequences in copepods recovered using bioinformatics searches of public databases and the number of iron-binding sites that are present in the AOX protein.

Order	**Family**	**Species**	**Accession Number**	**Iron-binding sites present in AOX protein**
Calanoida	Calanidae	*Calanus nmarchicus*	GAXK01135432	All 4
		Calanus glacialis	HACJ01021827	1, 2 & 3
	Temoridae	*Eurytemora affinis*	GBGO01037152	All 4
Cyclopoida	Cyclopettidae	*Paracyclopina nana*	GCJT01014574	All 4
	Cyclopidae	*Eucyclops serrulatus*	GARW01011119	All 4
Harpacticoida	Harpacticidae	*Tigriopus californicus*	JW502496	All 4
		Tigriopus japonicus	GCHA01002206	All 4

Table 9: *Tigriopus californicus* 551bp AOX cDNA sequence (Tward et al., 2019).

GAATGCACGTGATCATGGATGGATCCACACTCTCCTGGAAGAAGCGGAGAATGAAAGGATGCATCTG
ATGACCTTCATGCGACTCCGAAGACCCGGGCCCATTTTCCGAGGCACCGTGATCCTGACTCAATGGCT
GTTCACATTTACGTTCTCATTCGCTTACATACTGTCGCCCAATTTTTGCCACAGATTTGTTGGGTATTTG
GAAGAGCAAGCAGTGGTCACTTACACTCACATCCTGGAAGAAATCGACGCAGGACGATTGCCCATGT
GGAAGACCTTGCCAGCTCCGGAATTGGCCATCAAGTATTGGAGATTGCCCGAAGACGCCAAGATGCG
GGAAGTCATTTTGGCAATCCGAGCCGATGAAGCTCATCATCGGCTTGTGAATCACACCCTTGGATCGA
TGGACCTCAAATCAGACAATCCTTTTGAGAAAGGGAAATAACTTTTGTCTTCGGCTCAGATACAAATTT
AATGGTCAATAACAAGTATTCCATGGTAAAAACGAACGGAGGTTGAGAAATATAAATAAATTGTCGA
GTCTTTTC

Table 10: *Tigriopus californicus* predicted AOX protein sequence (Tward et al., 2019).

NERMHLMTFMRLRRPGPIFRGTVILTQWLFTFTFSFAYILSPNFCHRFVGYLEEQAVVTY
THILEEIDAGRLPMWKTLPAPELAIKYWRLPEDAKMREVILAIRADEAHHRLVNHTLGS
MDLKDNPFEKGK

Table 11: Alternative oxidase (AOX) sequences from various organisms that were retrieved from molecular database searches and used in multiple sequence alignment analyses.

Phylum/Group	**Order**	**Family**	**Species**	**Accession Number**
Plant				
Coleochaetophyceae	Coleochaetales	Coleochaetaceae	*Coleochaete orbiculans*	GW591598
Pteridineae	Pteridaceae	Vittarioideae	*Adiantum capillus-veneris*	DK948381
Charophyceae	Charales	Characeae	*Nitella hyalina*	HO503263
Pinales	Pinaceae	Pinus	*Pinus banksiana*	GW754219
Klebsormidiophyceae	Klebsormidiales	Klebsormidiaceae	*Klebsormidium subtile*	JG441912
Chlorophyceae	Chlamydomonadales	Chlamydomonadaceae	*Chlamydomonas incerta*	DQ122873
	Chlamydomonadales	Chlamydomonadaceae	*Chlamydomonas reinhardtii*	AF047832
Ulvophyceae	Dasycladales	Polyphysaceae	*Acetabularia acetabulum*	CF258325
Animal				
Arthropoda	Crustacea	Malacostraca	*Eulimnogammarus messerschmidtii*	GEPZ01019091
	Hexapoda	Insecta	*Leptinotarsa decemlineata*	GEEF01172609
			Thermobia domestica	GASN02050936
Chordata	Tunicata	Ascidiacea	*Molgula tectiformis*	CJ360866

Mollusca	Gastropoda	Vetigastropoda	*Haliotis tuberculata*	GEAU01104081
		Caenogastropoda	*Rapana venosa*	GDIA01153225
			Cipangopaludina cathayensis	GCEL01042596
	Bivalvia	Pteriomorphia	*Pecten maximus*	GAOX01004715
			Mytilus californianus	ES402065
Arthropods – Crustacea – Maxillopoda				
Arthropoda	Crustacea	Maxillopoda	*Tigriopus japonicus*	GCHA01002206
			Paracyclopina nana	GCJT01014574
			Eucyclops serrulatus	GARW01011119
			Calanus finmarchicus	GAXK01135432
			Calanus glacialis	HACJ01021827
			Eurytemora affinis	GBGO01037152

Table 12: Roles and locations of universally conserved AOX residues in the core and C-terminal regions of the protein (numbered based on the *T. californicus* protein sequence).

***T. californicus* AOX Conserved Residues**	**Role**	**Helix Number**
N-1/AsnI	Secondary ligation sphere; hydrogen bond network	α3
E-2/GluI	Fe-Fe ligand	α3
R-3/ArgI	Membrane binding region	α3
M-4/MetI	Dimer interface; interaction with N-terminal arm	α3
H-5/HisI	Fe-Fe ligand	α3
L-6/LeuI	Dimer interface	α3
P-15/ProI	Dimer interface	-
Q-27/GlnI	Dimer interface	α4
Y-38/TyrI	Dimer interface; hydrogen bonds to HisII	α4
H-46/HisII	Membrane binding region	α5
G-50/GlyI	Forms kinks in helix α5	α5
E-53/GluII	Fe-Fe ligand	α5
E-54/GluIII	Interaction with N-terminal arm	α5
A-56/AlaI	Substrate binding channel 2	α5
Y-60/TyrII	Catalytic cycle	α5
A-105/AlaII	Hydrophobic interactions with helix α3	αS3
Y-108/TyrIII	Secondary ligation sphere; hydrogen bond networks	αS3
R-125/ArgII	Interaction with helix α5 and N-terminal arm	α6
D-127/AspI	Secondary ligation sphere; hydrogen bond networks	α6
E-128/GluIV	Fe-Fe ligand	α6
H-131/HisIII	Fe-Fe ligand	α6
N-135/AsnII	Interaction with helix α5	α6
H-136/HisIV	Interaction with helix α5	α6

Table 13: Percent similarities between *T. californicus'* AOX protein sequence and AOX protein sequences of other organisms in the plant and animal kingdoms.

	Similarity Fraction of amino acids when compared to *T. californicus*	**Percent Similarity between this protein and the AOX of *T. californicus***
Plants		
C. orbiculans	57/128	44.5%
A. capillus-veneris	57/128	44.5%
N. hyalina	61/128	47.7%
P. banksiana	61/128	47.7%
K. subtile	64/128	50.0%
C. incerta	72/129	55.8%
C. reinhardtii	72/129	55.8%
A. acetabulum	74/129	57.4%
Animals		
E. messerschmidtii	64/131	48.9%
O. niloticus	73/138	52.9%
L. decemlineata1	77/140	55.0%
H. tuberculata	76/140	54.3%
T. domestica	77/131	58.8%
R. venosa	76/118	64.4%
C. cathayensis	75/132	56.8%
P. maximus	76/132	57.6%
M. californianus	67/116	57.8%
M. tectiformis	76/132	57.6%
Arthropods – Crustacea – Maxillopoda		
T. japonicus	129/132	97.7%
P. nana	104/132	78.8%
E. serrulatus	100/132	75.8%
C. finmarchicus	96/132	72.7%
C. glacialis	59/94	62.8%
E. affinis	92/132	69.7%

CHAPTER 4

The effect of varying temperature and light on the levels of alternative oxidase protein in *Tigriopus californicus*

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Abstract

Despite being a non-energy conserving pathway, AOX has a vast taxonomic distribution and is present in many plants, fungi, and some protists. It has been most thoroughly characterized in a number of plants; however, the physiological function of a native AOX has never been identified in an animal that produces it. This is only the second study to confirm AOX protein expression in an animal and is the first study to look at a native AOX protein in an animal and its response to environmental stress. This study will examine the roles of AOX in the tolerance to environmental stressors in *T. californicus.* This will be tested by subjecting the copepods to five different temperatures (6-28°C), and extended periods of light/dark exposures. Samples will be taken after 24 hours (acute) and 1 week (chronic) of incubation at each stress. We demonstrate that levels of the AOX protein fluctuate in *T. californicus* in response to cold and heat stress compared to normal rearing temperature. Furthermore, we demonstrate that AOX protein levels are constitutively expressed when exposed to changes in their circadian rhythm. We predict that a functional AOX pathway is present in *T. californicus*, propose that this species will be a useful model organism for the study of AOX in animals, and discuss future directions for animal AOX research. By understanding why *T. californicus* possesses AOX, we can better understand why some other organisms, including humans, do not express or have lost the AOX gene.

1. Introduction

Copepods are a prominent link in the transfer of energy from plankton to organisms in higher trophic levels in the marine food web (Sehgal, 1983). Copepods are the preferred prey for predators as they provide a larger source of nutrients when compared to other zooplankton, such as shrimp and rotifers (Sehgal, 1983). Their high abundance, wide distribution across the world, and ecological importance has led to a plethora of studies being conducted on copepods (Corner and O'Hara, 1986). Given the substantial abundance of copepods and their importance in the marine ecosystem, understanding how stressors affect copepods is of high ecological importance (Nilsson *et al*., 2018). Copepods are widely used for monitoring environmental changes and act as indicators of ecosystem health (Beaugrand, 2009). *T. californicus* is a marine invertebrate copepod that inhabits high intertidal and supralittoral rock pools located along the west coast of North America (Burton and Lee, 1994). Given the location of *T. californicus'* intertidal habitat, these animals are subjected to daily fluctuating environmental stressors such as temperature, changes in light duration, and predation (Burton and Lee, 1994).

All living organisms are influenced by their environments, therefore a change in their environment often leads to changes in their chemical composition (Corner and O'Hara, 1986). Numerous studies have shown that generation times of temperate copepods, and biotic and abiotic factors affect development (Mauchline, 1998). One of the most important factors determining development of copepods is temperature (Lee *et al*., 2008). Many studies have indicated a correlation between the environmental temperatures in which these organisms reside and their adaptation towards these changing temperatures in order to maintain fitness (Angilletta *et al*., 2002; Hochachka and Somero, 2002; Angilletta, 2009). Fitness is the ability to survive to reproductive age, find a mate, and produce offspring. Under stressful temperatures organisms

might engage in temperature adaptation as a trade-off with fitness in order to survive (Willett, 2010). Trade-offs occur when there are competing requirements placed on an organism that hinders fitness from being maximized (Kelly *et al*., 2012). The genus *Tigriopus* demonstrates varying limits of upper temperature tolerance among its different populations that suggests that temperature adaptation is taking place (Willett, 2010). Research has indicated that northern populations of *T. californicus* were unable to survive at 37°C for 1 hour, but individuals from more southern populations only showed modest mortality (Willett, 2010). When placing *T. californicus* under chronic high temperature stress, populations demonstrated a south to north gradient in their capacity to survive at 32°C (Willett, 2010).

T. *californicus* copepods inhabit tidepools with temperatures ranging from 4°C to 35°C (Edmands and Deimler, 2004). Changing the temperature of the habitats, which the copepods reside in, can cause immense stress on the organism. Edmands and Deimler (2004) found that when there is an increase in temperature from 15°C to 25°C it proved stressful for *T. californicus* when looking at its effect on hybridization. Furthermore, a study conducted by Smith *et al*. (1978) found that there was a significant increase in the rate of oxygen consumption by copepods when subjected to the four commonly encountered pool temperatures in which the copepods reside (10, 15, 25 and 30°C), especially between 25 and 30°C. Smith *et al*. (1978) concluded that there was a positive correlation between increasing temperature and oxygen consumption rate Furthermore, it has been found that metabolic rates increase with an increase in temperature over the range of 5-30°C in this species (Smith *et al*., 1978).

Another environmental stress that is becoming increasingly prominent for intertidal and near-shore ecosystems is light pollution (Longcore and Rich, 2004; Smith, 2009). In all environments, the presence of an artificial light source can cause changes in an organism's

circadian rhythm. This alteration in the timing of an organism's day/night cycle leads to changes in the physiological processes and behaviors that would normally be cued to the solar day and night (Nesbit and Christie, 2014). One of the major biological networks that is affected by modifications to the light/day cycle is the circadian system: a complex of interacting genes and proteins that act as the molecular pacemaker for timing physiological and behavioral processes that run on an approximately 24-hour cycle and are cued by light and darkness (Nesbit and Christie, 2014).

In the last 50-80 years light pollution has become a significant issue. Light pollution is defined as affecting organisms that are exposed to light at the wrong place, at the wrong time, or at the wrong intensity (Depledge *et al.*, 2010). There has been a growing concern regarding the damaging effects light pollution can exert on aquatic species living in lakes, rivers, seas, and especially coastal areas. Light exposure intensity causes many species of planktonic copepods to make daily vertical movements (Sehgal, 1983). This phenomenon of vertical migration during different hours of the day is called diurnal migration. Copepods and cladocerans, a minute branchiopod crustacean of the order *Cladocera*, are the main groups that exhibit diurnal movement (Sehgal 1983). Even though light intensity is the most prominent cause of vertical distribution of copepods, other physical and chemical aspects of a body of water can cause vertical distribution (Sehgal, 1983). Normally, copepods come up towards the surface in the late afternoon, which is due to them swimming towards a light source of decreasing intensity (Sehgal, 1983). Throughout the night (hours of darkness), the upward migration continues. At dawn, there is an increase in the density of copepods at the surface; this is ascribed to the early morning light. As the sun continues to rise and become more intense there is a downward movement of copepods in a body of water (Sehgal, 1983).

AOX is an enzyme to helps organisms respond to stress. The alternative oxidase (AOX) is an inner mitochondrial protein that diverges at the ubiquinone pool in the respiratory electron transport system and bypasses the last two complexes in the cytochrome c oxidase (COX) pathway (Chapter 1, Figure 1). Respiration by the AOX pathway has been termed as 'cyanide-resistant' because of its tolerance to all COX inhibitors, such as cyanide and nitric oxide (Vanlerberghe *et al*., 1994; Huang *et al*., 2002). However, it has been demonstrated that AOX activity is compromised by salicylhydroxamic acid (SHAM) and n- propyl gallate (Vanlerberghe *et al*., 1994; Yip and Vanlerberghe, 2001). Similar to complex IV of the COX pathway, the AOX protein is a terminal oxidase and reduces oxygen to water (Berthold *et al*., 2002). However, unlike the COX complexes, AOX is non-proton motive and as a result, fewer ATP are yielded per oxygen consumed during its activity (Moore and Siedow, 1991). AOX is a homodimeric protein that has been categorized within the group of di-iron carboxylate proteins (Berthold *et al*., 2002). Structurally, AOX is made up of a di-iron centre and four helices that associate with the IMM (McDonald, 2008). Highly conserved amino acids have been identified within the four helices that are thought to play a role in AOX enzymatic function and regulation.

AOX has been demonstrated to be a stress responsive protein. The majority of research demonstrating that AOX helps mitigate stress experienced by the organism has been done in plants. The induction of AOX is triggered by a range of conditions and treatments including: light (Escobar *et al*. 2004), nutrient availability (Escobar *et al*. 2006), and a variety of biotic or abiotic stresses (Finnegan *et al*., 2004). Previous research on AOX in plants demonstrated that AOX expression increases when plants experience stressful conditions such as temperature changes or light stress (Giraud *et al*., 2008, Zhang *et al*., 2010). Recent work has demonstrated that when AOX is active it will heat the plant during thermogenesis, thereby allowing it to

survive at low temperatures (Watling *et al*., 2006). With respect to the role of AOX in defining the equilibrium of the defense systems in plants, it was shown in tobacco (*Nicotiana tabacum*) that plants, which lacked AOX, were vulnerable to the induction of programed cell death (Van Aken *et al*., 2009).

Despite being a non-energy conserving pathway, AOX has a vast taxonomic distribution and has been predominately characterized in the plant kingdom. AOX multigene families, transcript expression, protein regulation, and enzymatic activity have been thoroughly investigated in several plants (McDonald, 2008). AOX discovery in animals is recent, and further research into its expression, regulation, and physiological role has been impeded by the lack of an experimental model organism. Previous research has demonstrated the presence of the alternative oxidase protein in the arthropod *Artemia franciscana* (brine shrimp) through immunodetection (Rodriguez-Armenta *et al*., 2018). DNA database searches using bioinformatics revealed an AOX sequence present in the arthropod *T. californicus*. The physiological function of a native AOX has never been identified in an organism that produces it, such as *T. californicus*. Furthermore, *T. californicus* is characterized by a short generation time, small space needs, and many genetically divergent populations which can be cross-bred in the laboratory (Burton and Feldman, 1981). These attributes make *T. californicus* an emerging model organism in biology for the study of environmental stress responses in animals and AOX.

The purposes of this study were to: 1) Develop a protein isolation method that is efficient and effective for the copepod *T. californicus* and 2) examine changes in the levels of AOX protein in the copepod *T. californicus* during temperature and light stress. Copepods were exposed to four different treatment temperatures (6, 10, 22, and 28°C) for acute (24 hours) and chronic (1 week) periods of times. As well, copepods were exposed to varying periods of light

and darkness for short (24 hours) and extended (1 week) periods of times. Using SDS-PAGE and Western blots, AOX expression during each of the stressors was compared to a control (15°C and 12-hour light/dark cycle respectively), in order to examine changes in the levels of AOX protein. This is the first ever study to look at a native AOX protein in an animal and its response to environmental stress.

2. Materials and Methods

2.1 Isolation of Copepods

Following each temperature and light/darkness experiment, three samples of approximately 0.05 g of copepods were isolated from their habitats and placed in an Eppendorf tube. The volume of copepods in each sample collected was approximately 0.05 g (Table 1). Copepods were collected by filtration and a stainless-steel laboratory spatula was used to scrape the copepods off the filter and transfer them into an Eppendorf tube.

2.2 Protein Isolation without Quantification

In initial experiments that were used to test the effectiveness of AOX antibodies, a 350 μL solution comprised of the 2× Laemmli sample buffer (Bio-Rad) and β-mercaptoethanol was pipetted into each Eppendorf tube. This mixture was composed of 20 μL of β-mercaptoethanol for every 1 mL of 2× Laemmli sample buffer. Each sample was then sonicated in order to extract the proteins from the copepods. The sonicator was set to a frequency of 60kHz and each sample was sonicated for 10 seconds.

2.3 Protein Analysis: Gel Electrophoresis

Protein samples were analyzed using gel electrophoresis and Western blotting. For gel electrophoresis, a 15 well mini-PROTEAN TGX Stain-Free gel (Bio-rad), was loaded with 15

μL of each of the copepod samples. In order to determine the molecular weight of the proteins in our samples, two wells were loaded with 5 μL of the Precision Plus Protein WesternC Standards (Bio-rad). Following the loading of the wells the gel was run at a constant voltage (200 V) for 35 minutes. Pacific oyster AOX recombinant protein (Robertson et al., 2016) served as a positive control in order to verify the cross-reactivity of the AOX antibody with our copepod samples. A negative control blot was run using proteins isolated from the algal food source to rule out the possibility of an algal AOX being detected in our copepod samples (Tward et al., 2019). After the run was complete, the gel was removed from the apparatus and placed in the VersaDoc (settings: protein gel, stain- free) so an image could be taken to check that the protein samples ran correctly and were effectively separated on the gel.

2.4 Protein Analysis: Western Blot

Next, the proteins were transferred from the protein gel onto a nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-rad) and Trans-Blot Transfer Medium (Bio-rad). This process takes 3 minutes in the Trans-blot Turbo Transfer System. In one of the cassettes of the machine, a sponge is placed down first, followed by the gel, then the membrane and then another sponge. The roller is used to eliminate any bubbles between the layers, therefore allowing for good contact between the membrane and gel. Following protein transfer, the membrane is placed in 5% dry milk in 10 mL of 1X TBS-T (Tris Buffered Saline with Tween 20) on a shaking platform for 1 hour. The membrane is then subsequently washed three times in TBS-T. 2 μL of the primary AOX antibody (Plant AOX1/2, Cat. # ABIN3197483, Agrisera Antibodies) is then added with 20 mL of 1X TBS-T for 1 hour (dilution 1:10,000). Following this, the membrane is again washed three times (15 minutes, 10 minutes, and 10 minutes) with TBS-T buffer. The membrane is then incubated in 1 μL of a goat anti-rabbit IgG secondary

antibody (Cat. # ABIN101988, Agrisera Antibodies, dilution 1:25,000) in order to detect the AOX protein, 2 μL of the Precision Protein StrepTactin HRP Conjugate (Bio-rad) in order to visualize the protein standards ladder, and 20 mL of 10 TBS-T for 1 hour. The membrane is again washed three times in 1X TBS-T. Following the washes, the membrane is subsequently placed in a mixture of luminol/enhancer and peroxide buffer solution, from the Immun Star Western C Chemiluminescent kit (Bio-rad), in a 1:1 ratio for 5 minutes. The membrane was then imaged in a VersaDoc (settings: blot, Chemi high resolution), and the chemiluminescent signal was detected and imaged.

2.5 Temperature Experiments

The control temperature selected for all habitats is 15.0°C. Temperatures are adjusted in order to see if an acute and/or chronic change in temperature causes a change in the amount of AOX protein translated. Five temperature experiments will be run in order to test our hypothesis (Chapter 1, 2. Purpose) (Figure 1).

2.6 Light Exposure Experiments

The control day/night cycle for all habitats is 12 hours light and 12 hours darkness. Light cycles are adjusted in order to see if an acute and/or chronic change in light exposure causes a change in the amount of AOX protein translated. Two treatment experiments will be run in order to test our hypothesis (Chapter 1, 2. Purpose). The light treatment will consist of the copepods being exposed to light with no darkness period for up to a week. The dark treatment will consist of the copepods being exposed to only darkness for up to a week (Figure 2).

2.7 Protein Isolation for Quantification from Copepods

Protein samples were isolated using two solutions; the copepod extraction buffer and the 100 mM PMSF (phenylmethylsulfonyl fluoride) stock solution. PMSF is used to inhibit protease enzymes in order to prevent the degradation of extracted proteins. The copepod extraction buffer is composed of 100mM Tris (pH 7.5), 100 mM NaCl, and 5mM EDTA. Using a graduated cylinder, the total volume is brought up to 100 mL using Milli-Q water. This solution can be kept in a media jar and stored in the fridge (4°C). The 100 mM PMSF stock solution is composed of 0.174 g PMSF and 10 mL of ethanol and was made just prior to use. For each copepod sample, (isolated according to the 2.1 Isolation of Copepods methodology), 500 μL of the copepod extraction buffer and 5μL of the 100 mM PMSF stock solution is added to the eppendorf tube containing the animals. The contents of the tube are mixed very well using a vortexer. The sample is then placed on ice and undergoes three sonication cycles. One cycle is composed of the sample being sonicated for 10 seconds and then subsequently placed on ice for 10 seconds. Following the last cycle, the sample remains on ice (Figure 3). The samples are then centrifuged for 15 minutes at 15000 x g and 4°C. The supernatant containing the isolated proteins is transferred into a fresh Eppendorf tube and left on ice prior to use in SDS-PAGE.

2.8 Protein Quantification of Copepod Samples

In order to quantify the protein levels in each copepod sample the Bio-Rad Quick Start Bovine Serum Standards were used. 20 μL of each kit standard and each copepod sample was placed in its own Eppendorf tube. A blank tube was made containing 20 μL of the copepod protein extraction buffer and 100 mM PMSF stock solution mixture. Each one of these tubes was replicated, for a total of two, in order to get an average protein concentration for each sample. 1 mL of the Quick Start Bradford 1x Dye Reagent was added to each Eppendorf tube. The initial

dye starts out as a dark brown colour, and when exposed to increasing protein levels the dye becomes a darker blue in colour. Once the dye is added to each tube it is mixed by inversion and left to sit for at least 5 minutes before any analysis can take place. Each sample was dispensed into its own protein cuvette, and placed in the spectrophotometer to obtain an absorbance reading at 595 nm. The absorbance readings were then recorded and entered into a Microsoft Excel spreadsheet. The absorbance readings were averaged for the two sets of samples that were used (Tables 2-4). The concentration and average absorbance were plotted on a scatter plot and a linear trendline was added along with the equation for the trendline (Figures 4-6). This equation was then utilized to calculate the concentrations of the isolated copepod protein samples.

2.9 AOX Protein Expression: SDS-PAGE and Western Blot

Protein samples were analyzed using SDS-PAGE and Western blotting techniques. In order to determine an effective protein concentration to load for detection of proteins using these techniques, a 10 well mini-PROTEAN TGX Stain-Free gel (Bio-rad), was loaded with a concentration of protein that increased by a factor of 2 with each subsequent well. The first well possessed 2.5 μg of protein, with subsequent wells containing 5, 10, and 20 μg of protein.

For SDS-PAGE, a 10 well mini-PROTEAN TGX Stain-Free gel (Bio-rad), was loaded with 20 μg of protein from each of the copepod samples. This volume was calculated based on using the equation for the trendline and solving for x, while inserting the absorbance reading as the y value. Before moving on to the Western blot portion of the protocol an image of the gel was taken using the VersaDoc (settings: protein gel, stain free) to make sure all the lanes possessed a similar amount of protein. The same procedure as stated in section 2.4 “Protein Analysis: Western Blot” was followed for the Western Blot portion of this experiment.

2.10 Protein Isolation from *Drosophila melanogaster*

Proteins were isolated from *D. melanogaster* by first gassing them with carbon dioxide and them brushing them into a sterile eppendorf tube. Six samples were taken containing different masses of the organism. The masses of fruit flies in each Eppendorf tube can be found in Table 5. Proteins were isolated using the same technique explained in Methods 2.7 "Protein Isolation for Quantification in Copepods". Protein samples were quantified following the same protocol in Methods 2.8 "Protein Quantification".

2.11 SDS-PAGE and Western Blot for optimal detection using a Tubulin Antibody

20 μg of each protein sample, from *D. melanogaster*, was loaded on three different 15 well mini-PROTEAN TGX Stain-Free gels (Bio-rad). Before moving on to the Western blot portion of the experiment an image of the gel was taken using the VersaDoc to make sure all the lanes possessed a similar amount of protein. The same procedure as stated in section 2.4 "Protein Analysis: Western Blot" was followed for the Western Blot portion of this experiment, with slight alterations. A primary tubulin antibody (Anti-α-Tubulin antibody, Mouse monoclonal, Sigma T6199) was used and the secondary antibody was an Anti-Mouse IgG1 (γ-chain specific) a peroxidase conjugated antibody produced in rabbit (Sigma-Aldrich, SAB3701171). Each blot was exposed to a different concentration of antibody, in order to find the optimal amount for detection of the tubulin protein. The first blot had 2 μL of primary antibody (1:5000) and 1 μL of secondary antibody (1:10000), the second blot had double the amount of antibodies (1:2500 and 1:5000, respectively) and the third blot possessed four times as much of the antibodies (1:1250 and 1:2500, respectively).

3. Results

3.1 Detection of Alternative Oxidase Protein in *T. californicus*

Proteins were successfully isolated from *T. californicus* using 0.05 g of copepods and 350 μL of SDS-PAGE sample buffer containing β-mercaptoethanol and using sonication (Figure 7). The *T. californicus* AOX was recognized by a plant AOX antibody and was ~ 50 kDa in size (Figure 7). A yeast sample expressing the Pacific oyster alternative oxidase (Robertson et al., 2016) served as a positive control in order to verify the cross-reactivity of the AOX antibody with our copepod samples (Figure 8). The antibody did not cross-react with the AOX protein that is likely present in the algae provided to the copepods as a food source (Figure 9).

3.2 Protein Quantification and Equal Loading of Gels

Proteins were successfully quantified using the absorbance readings at 595nm in the spectrophotometer (Tables 2-4) and plotting the standards on a graph to obtain an equation for the line of best fit (Figures 4-6). In Microsoft Excel this equation was used to calculate the concentrations of the copepod protein samples. An SDS-PAGE was run to confirm that the protein isolation and quantification was successful by increasing the amounts of protein sample in each well by a factor of 2 (Figure 10).

3.3 Response of AOX Protein Levels to Temperature Treatments

Copepods were acclimated to 15°C for 2-4 weeks and then subjected to one of four different temperatures (6, 10, 22, or 28°C) for 24 hours or 1 week and 3 samples were taken for each treatment. Protein gels demonstrated that an equal amount of each protein was loaded into each well (Figures 11a-14a). Western blots indicate that AOX protein levels are low under control conditions (15°C) and are elevated when subjected to fluctuations in temperatures (6, 10,

22 and 28°C) for 24 hours and 1 week (Figures 11b-14b). Furthermore, as can be seen in Figures 11b-14b, there is a molecular mass shift taking place in terms of size of the AOX protein size.

3.4 Response of AOX Protein Levels to Light Treatments

Copepods were acclimated to a 12-hour light/12-hour dark for 2-4 weeks and then subjected to one of two different light treatments (constant exposure to light or constant exposure to darkness) for 24 hours or 1 week and 3 samples were taken for each treatment. Western blots indicate that AOX protein was detectable under all treatments, but that AOX protein levels did not change throughout all treatments (light and dark, acute and chronic) (Figures 15-20).

3.5 Tubulin Protein Expression in *Drosophila melanogaster*

In an attempt to normalize copepod AOX protein levels to a housekeeping protein, *D. melanogaster* protein samples were taken, quantified, and utilized to test a tubulin antibody's efficiency and efficacy. Three membranes containing isolated fruit fly proteins were exposed to different concentrations of primary and secondary antibody, in order to determine which concentrations allowed for optimal visual imaging of the tubulin protein on the Western blot. When exposing the membrane to higher concentrations of each antibody (1:1250 of primary antibody and 1:2500 of secondary antibody), the tubulin protein was easier to visualize (Figure 21). Tubulin was detected in fruit fly sample number 4 as two bands that cross-reacted with the tubulin antibody at ~25 kD and ~ 37 kD sizes (Figure 21).

4. Discussion

4.1 Development of a Scalable Protein Isolation Technique for the Copepod *T. californicus*

In order to accomplish several of my research goals a protein extraction protocol had to be developed for copepods. My methodology allows for the quantification and the analysis of

copepod proteins and is also scalable in terms of volume of buffer and mass of copepods used. This technique also worked in another arthropod of similar size and body composition the fruit fly *Drosophila melanogaster* (Figure 21a). This protein isolation procedure can be applied to a multitude of organisms, including fruit flies. The successful development of a protein isolation technique and the detection and identification of an AOX protein in *T. californicus* permitted this study to look at a native AOX in an animal and its response to environmental stress.

4.2 Detection of the AOX Protein in the Copepod *T. californicus*

Western blot analysis detected the presence of the AOX protein of *T. californicus* as a single protein band at ~50 kD (Figure 7). The AOX protein from isolated mitochondria from the yeast *Saccharomyces cerevisiae* overexpressing the *Crassostrea gigas* (Pacific oyster) AOX is also ~50 kD in size (Figure 8) (Robertson *et al.* 2016). This is only the second research study that has successfully looked at a native AOX protein in an animal using SDS-PAGE and Western blot techniques. A study conducted by Rodriguez-Amenta *et al*. (2018) showed that *Artemia franciscana* (brine shrimp) nauplii possesses an AOX protein that is between 25-37 kD in size. It is unknown at this time why the AOXs from *C. gigas* and *T. californicus* differ in size from that of *A. franciscana*. It can be concluded that AOX proteins can be different sizes (25-50 kD) in different animals even if they belong to the same taxonomic phylum and order. This size difference can be attributed to alternative splicing of the RNA sequence prior to it being translated into a protein sequence.

Due to the fact that many algae have AOX, we performed a Western blot using the AOX antibody in order to ensure that the algae that we feed the copepods was not the source of the AOX protein that we detected. Our results confirmed that the algae that we use as a food source does not contain an AOX that cross-reacts with the antibody (Figure 9).

4.3 The Effects of Temperature Stress on AOX Protein Levels in *T. californicus*

The copepod *T. californicus* has been shown to be able to acclimate to a wide variety of temperatures. However, the presence of the AOX protein, which copepods possess, raises the question as to whether it plays a role in the animal's ability to respond to temperature stress. In the current study, we determined whether AOX protein expression varies in *T. californicus* with exposure to a wide variety of temperatures over different time points.

Due to the lack of research on the physiological function of AOX in animal models, we can examine what is known about the enzyme in other organisms in order to understand the role AOX may play in animals. Available data regarding the role of alternative oxidase in copepods is limited, but it is well understood in other organisms, such as plants, fungi and protists (McDonald and Vanlerberghe, 2004; McDonald 2008).

Previous studies have indicated that AOX plays a role in the ability of organisms to tolerate various biotic and abiotic stressors (Juárez *et al*., 2006). One of the highly confirmed benefits of AOX in plants is thermogenesis, which is the production of heat by an organism (Angioy *et al*., 2004). Furthermore, when plants are exposed to cold stress, there is an increase in the transcription (mRNA) of AOX, which permits plants to be able to tolerate a colder environment (Li *et al.*, 2011). Another study conducted by Fiorani *et al.* (2005) demonstrates that the AOX pathway is upregulated in *Arabidopsis* plants when exposed to cold stress, and leads to a 20% reduction in plant growth. Other than plants, AOX has been studied in some fungi models, such as the fungal phytopathogen *Ustilago maydis*. Similar to other cosmopolitan organisms, *U. maydis* is subjected to numerous changes in environmental parameters. During *U. maydis* cell culturing, when assay temperatures were increased to 28°C, there was a 3.5-4.3× increase in AOX capacity (Juárez *et al*., 2006). Juárez *et al*. (2006) concluded that AOX allows the

mitochondrial metabolism to be active when subjected to biotic and abiotic stressors that can limit the activity of the cytochrome pathway.

Previous research has demonstrated that, in copepods, adaptation to environmental stressors, such as temperature, involves changes in protein expression (Kimmel and Bradley, 2001). Bradley *et al*. (1988) found that *E. affinis* adapted at the protein level to salinity and temperature changes. The first protein to be identified to help this matter was the upregulation of HSP70. Following this work, Gonzalez and Bradley (1994) demonstrated differential protein expression, in several metabolic pathways, in response to fluctuations in salinity and temperature in zooplankton. Kimmel and Bradley (2001) demonstrated that protein changes rise monotonically (always increasing; never remaining constant or decreasing), up to a certain point, with stress and that protein changes do not occur individually but as part of various metabolic pathways in the copepod *Eurytemora affinis*.

The copepods are normally reared at 15°C and subjected to a 12-hour day/night cycle and have been cultured at this temperature for several years in the laboratory. Therefore, they have likely adapted to this growth temperature and light regime over many generations in the lab, which is why it was used as the control condition. In our laboratory, we found that the copepods were able to survive and reproduce at temperatures ranging from 6°C to 28°C. This was confirmed when rearing copepods in the lab at 6°C and 28°C for approximately 1 month, and observing copepods at different stages in their life cycles (based on size of copepods). One recent study reported that, in agreement with our findings, copepods can survive in temperatures ranging from 6°C to 28°C (Edmand & Deimler, 2004). For the temperature experiments, we subjected our treatment groups to either 6°C (cold), 10°C, 22°C or 28°C (hot) for an acute period of time (24 hours) or a chronic period of time (1 week) prior to sampling. The control sample

(15°C) exhibited very low levels of detectable AOX protein (Figures 8b-11b). In contrast, animals grown at different temperatures from the control (6, 10, 22 and 28°C) exhibited higher detectable levels of AOX protein after 24 hours of exposure (Figures 11b and 12b) and 1 week of exposure (Figures 13b and 14b) to each temperature. Furthermore, as can be seen in Figures 11b-14b, when the copepods are subjected to varying temperatures, there seems to be a shift in the molecular mass in terms of size of the AOX protein when exposed to the warmer temperatures (22 and 28°C). These experiments are the first to demonstrate that AOX protein levels change in an animal in response to an environmental stressor.

Similar to what we see in *T. californicus*, an increase in AOX protein levels in response to cold stress has been reported in a variety of plant species (McDonald, 2008). Furthermore, research conducted by Zalutskaya *et al*. (2015) demonstrated an increase in AOX expression levels in the green alga *Chlamydomonas reinhardtii* when the organism was subjected to heat stress. In support of the above finding that there are fluctuations in AOX expression when exposed to changing environment temperatures, a lab based study found that there was a decline in *T. californicus* survivorship when temperature levels increased from 15°C to 25°C (Edmands and Deimler, 2004). This decline can be attributed to increased stress in copepods at temperatures outside of 15°C. Our data support our hypothesis that AOX protein expression changes when copepods are exposed to changing temperatures, due to *T. californicus* experiencing an abiotic stress in their environment.

4.4 The Effects of Light Stress on AOX Protein Levels in *T. californicus*

Another environmental stress that we examined in *T. californicus* was light stress and its effects on AOX protein levels. Almost all living organism are sensitive to fluctuations in the quality and the intensity of light in their environments (Longcore and Rich, 2004). Light is a key

regulator of gene expression of several pertinent respiratory enzymes, including cytochrome oxidase in the electron transport chain (Zhang *et al*., 2010). For some organisms, such as algae and seaweeds, photosynthetic activity is dependent on availability of light. Marine animals experience daily, monthly, and seasonal cycles in natural light intensity and quality which is reflected in rhythmical fluctuations in their physiology and behaviour (Depledge *et al.*, 2010). The next goal of this project was to see if exposure to 24 hours and 1 week of complete light or darkness caused changes in the expression of AOX protein.

Our results indicate that AOX protein is constitutively expressed in *T. californicus* under all light regimes examined (Figures 15b-20b). In contrast to temperature stress, which caused the levels of AOX protein to change compared to controls, light stress did not result in changes in AOX protein expression (Figures 15b-20b).

Due to the lack of knowledge about the physiological function of AOX in animals, we looked at experiments conducted in plants to compare to our findings. One study conducted on tobacco leaves demonstrates that AOX protein levels increased when exposed to periods of darkness rather than extended periods of light (Dessi and Whelan, 1997). This is contrary to our findings which demonstrate that there is no visible difference in the expression of AOX protein when exposed to extended periods of darkness (24hrs and 1 week) (Figures 15-20). Another study showed that when *Arabidopsis* photoreceptor mutants were exposed to light stress greater then 4 hours, there was a significant increase in the transcription level of AOX (Zhang *et al.*, 2010). This finding, regarding AOX protein expression, is not only in opposition of our finding but as well the previous finding by Dessi and Whelan (1997). It is hard to compare plants to animals with regards to light stress, as plants have a different mechanism than animals to deal with it as excess light energy is harmful to plants and leads to disruptions of the photosynthetic

apparatus (Zhang *et al.*, 2010). Due to the contradictory findings between all three studies, it can be seen that AOX may play a different role in the response to light stress depending on the organism.

5. Conclusion

In summary, we developed an effective and efficient protocol in order to extract proteins from *T. californicus* that is scalable. Furthermore, this methodology enables us to quantify the amount of protein in each sample and run both SDS-PAGE and Western blots to analyze the expression of AOX protein in copepods. This is only the second research study that has successfully looked at a native AOX protein in an animal. More importantly, this is the first and only study to look at a native AOX in an animal and its response to different environmental stressors. When *T. californicus* were exposed to varying environmental temperatures (6, 10, 22 and 28°C) they produced different levels of AOX protein compared to the control temperature (15°C). In contrast, when copepods were exposed to changes in their typical light regime (12 hours light: 12 hours dark cycle) they showed no visible change in the expression of AOX protein. We therefore hypothesize that AOX plays a role in helping *T. californicus* acclimate to a variety of environmental temperatures, but not to changes in its light:dark cycle. By understanding why *T. californicus* possesses AOX, we can better understand why some other organisms, such as humans, do not express it or have lost the AOX gene entirely.

6. Future Directions

By developing a protein isolation protocol that allows for protein quantification, and successful SDS-PAGE and Western blot protocols in *T. californicus*, researchers are closer to understanding the physiological function of AOX in an animal. Finding that temperature stress

causes changes in the expression of AOX protein in *T. californicus* indicates that AOX may help the animal to tolerate or acclimate to hot or cold environmental temperatures that they may encounter daily. Our results with light stress indicate that not all abiotic stressors influence AOX protein expression. Future research must quantify the expression of AOX protein in order to make definitive statements regarding the exact contribution of AOX in copepods towards surviving fluctuating temperatures.

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Figures

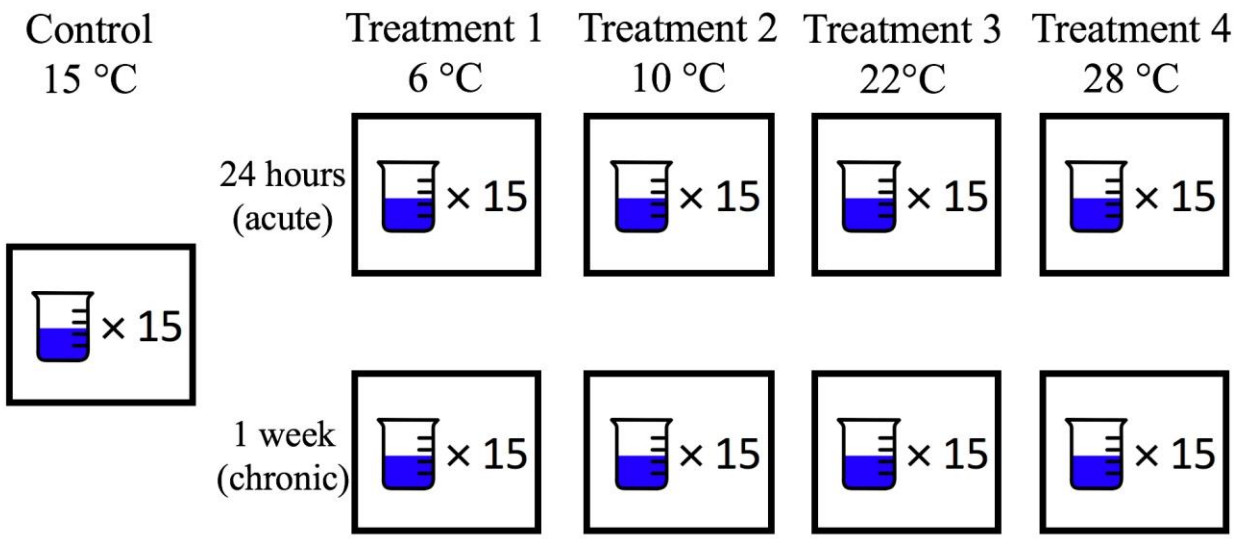

Figure 1: Visual representation of the control and treatment groups in the temperature experiments. Three samples, each consisting of maximum 5 beakers of animals, were taken for each control and treatment temperature.

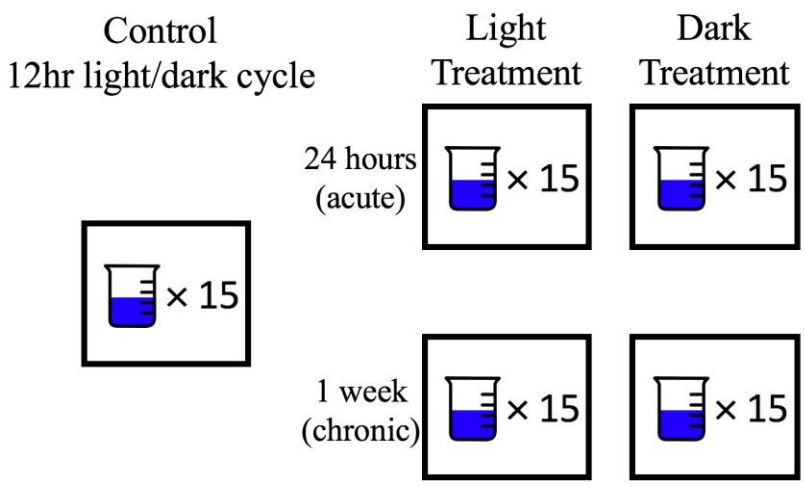

Figure 2: Visual representation of the control and treatment groups in the light exposure experiments. Three samples, each consisting of maximum 5 beakers of animals, were taken for each control and treatment group.

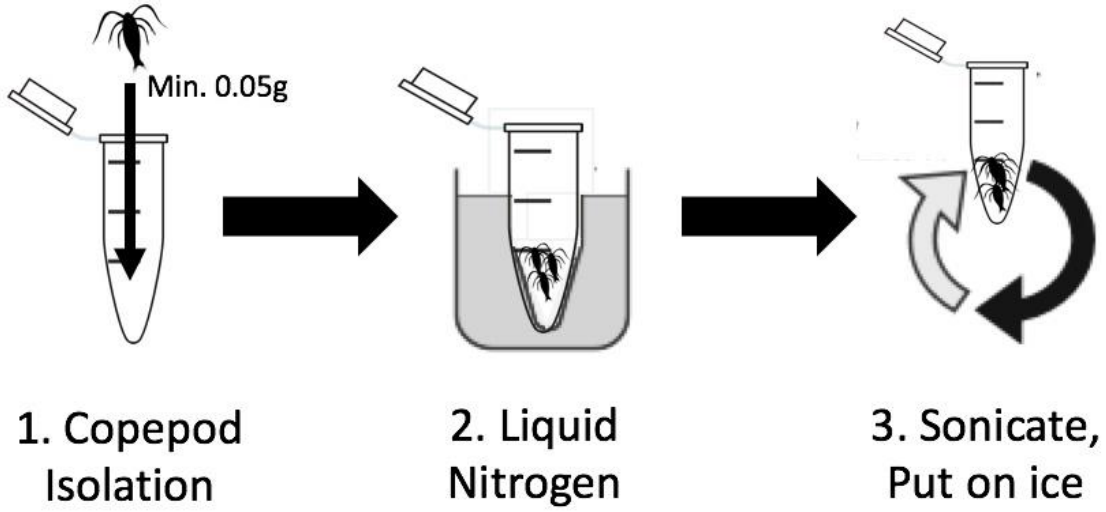

Figure 3: Procedure for extracting proteins from copepods.

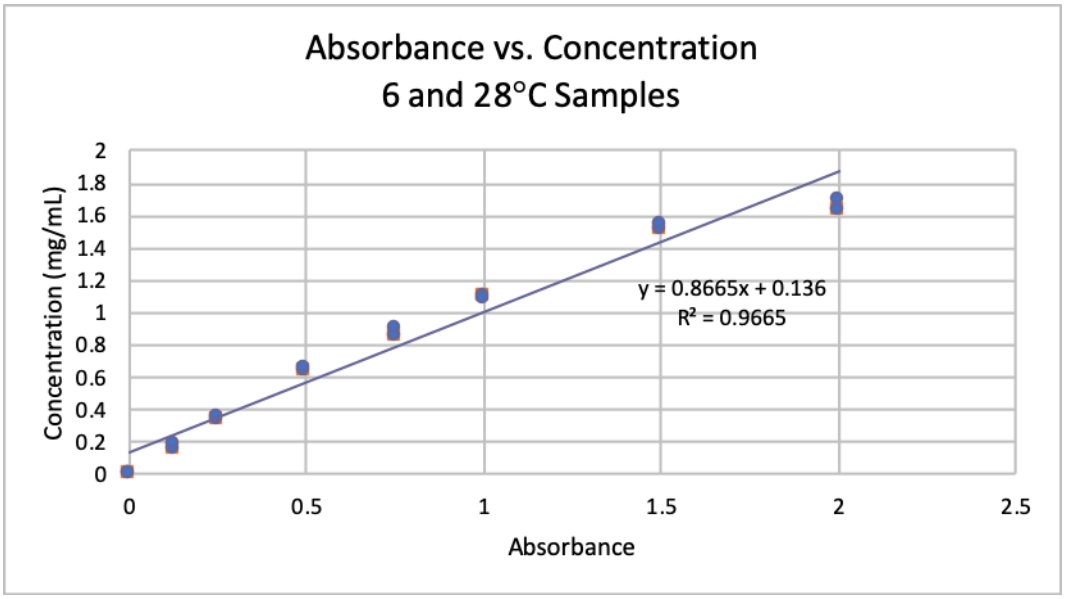

Figure 4: Protein absorbance at 595nm for Bio-Rad Quick Start Bovine Serum Standards. Trendline equation used to calculate concentrations of proteins in acute and chronic samples at temperatures 6 and 28°C

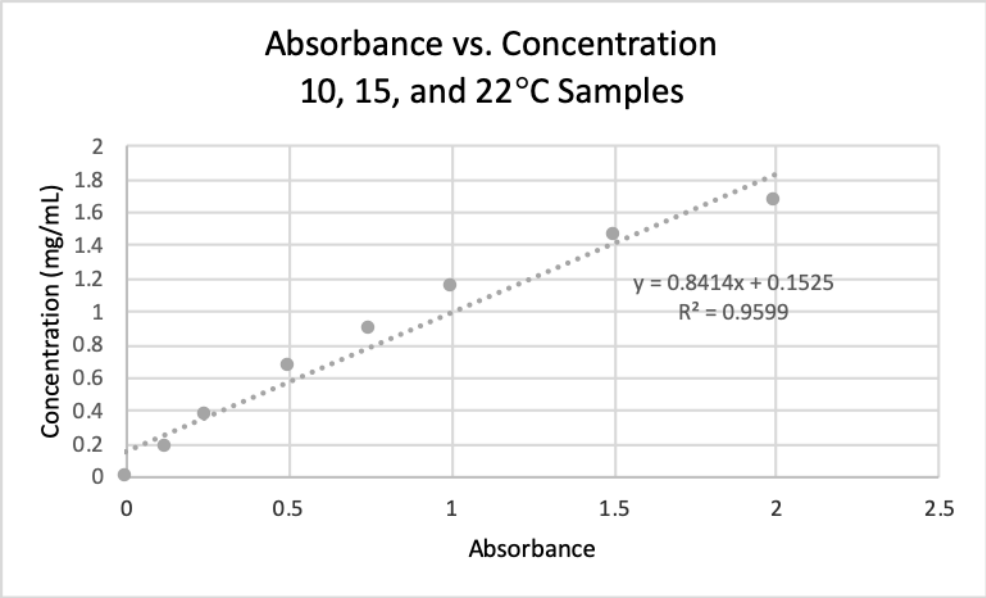

Figure 5: Protein absorbance at 595nm for Bio-Rad Quick Start Bovine Serum Standards. Trendline equation used to calculate concentrations of proteins in acute and chronic samples at temperatures 10, 15 and 22°C

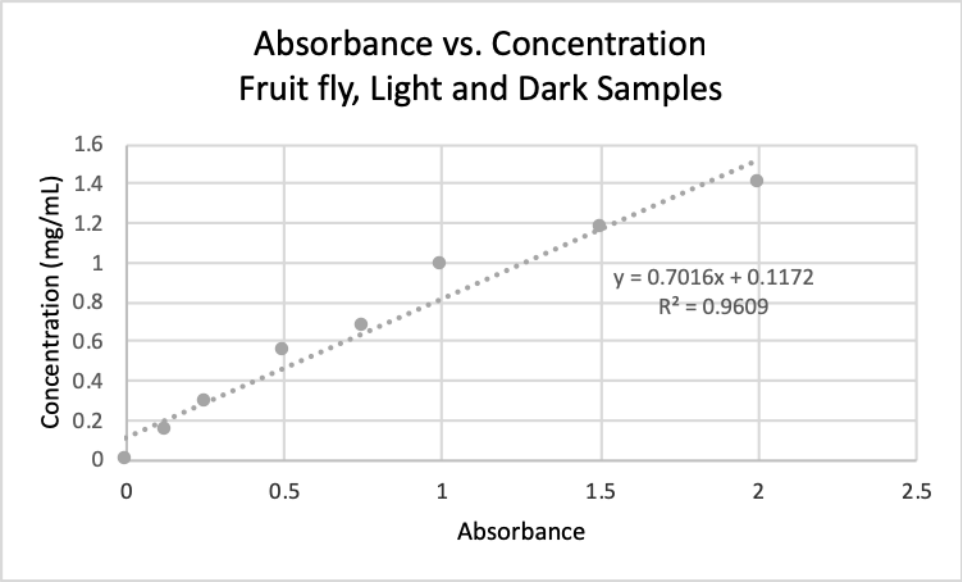

Figure 6: Protein absorbance at 595nm for Bio-Rad Quick Start Bovine Serum Standards. Trendline equation used to calculate concentrations of protein in fruit fly samples, acute and chronic light and dark samples

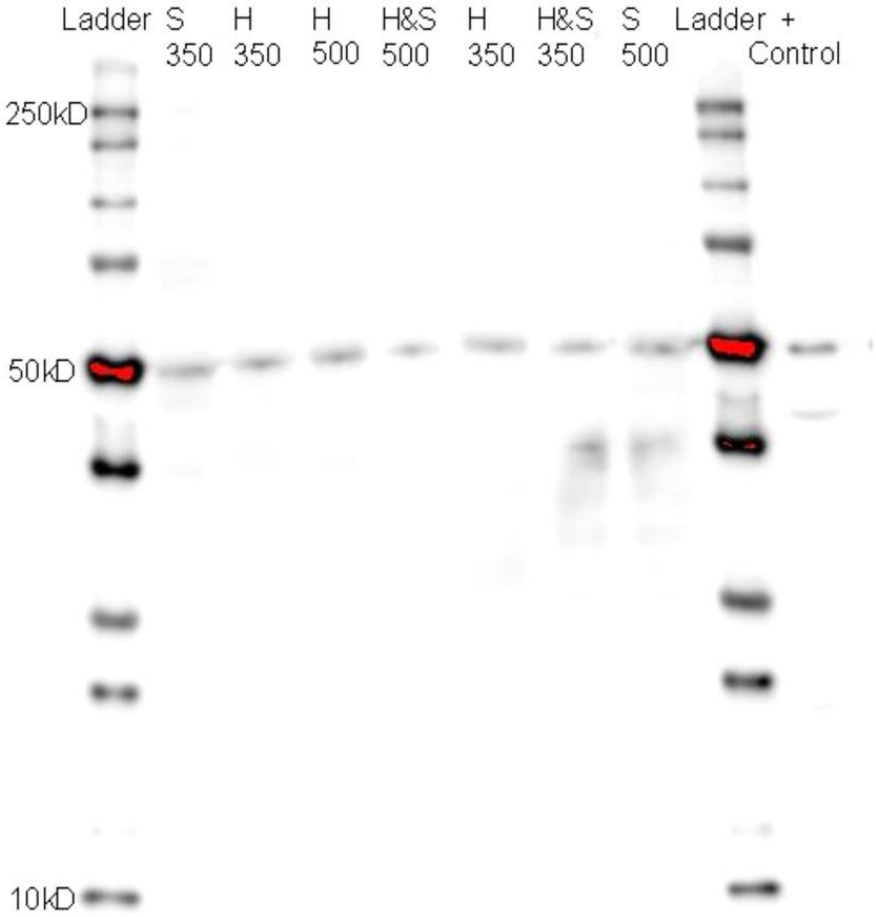

Figure 7: Detection of *T. californicus* AOX protein by reducing SDS-PAGE and Western blotting using a plant AOX antibody. All *T. californicus* samples per well are 14 µL of protein samples derived from 0.05 g of copepods of mixed developmental stages subjected to either sonication (S), homogenization (H), or a combination of the two (H & S). The protein sample buffer volume used (in microliters) is indicated by the number below the isolation method. Ladder lanes contain 5 µL of the Precision Plus Protein Western C Standards. The positive control is 14 µL of a protein sample from isolated mitochondria from *Saccharomyces cerevisiae* overexpressing the *Crassostrea gigas* AOX (Tward et al., 2019).

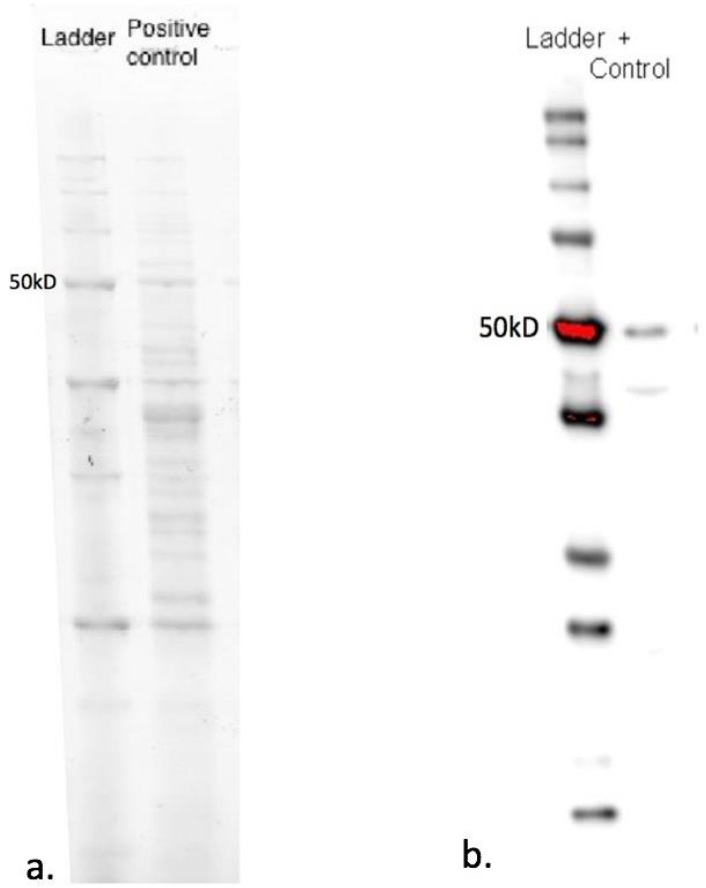

Figure 8: **a.** Protein sample run on reducing SDS-PAGE. Ladder lanes contain 5 µL of the Precision Plus Protein Western C Standards. The positive control is 14 µL of a protein sample from isolated mitochondria from Saccharomyces cerevisiae overexpressing the *Crassostrea gigas* AOX. **b.** Detection of *Crassostrea gigas* AOX protein by reducing SDS-PAGE and Western blotting using a plant AOX antibody. Ladder lanes contain 5 µL of the Precision Plus Protein Western C Standards. The positive control is 14 µL of a protein sample from isolated mitochondria from *Saccharomyces cerevisiae* overexpressing the *Crassostrea gigas* AOX.

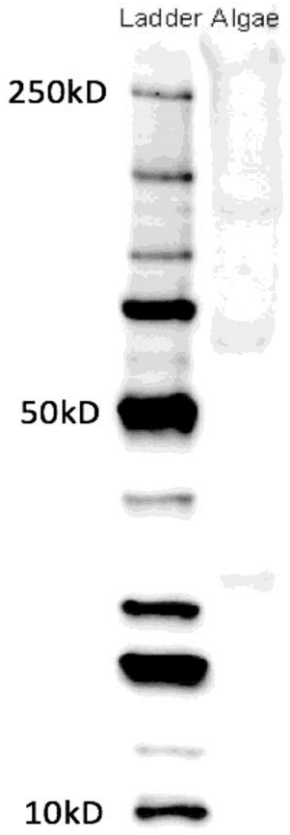

Figure 9: Protein samples run on reducing SDS-PAGE and Western blotted using a plant AOX antibody. The ladder lane contains 5 µL of the Precision Plus Protein Western C Standards. The algae lane contains a sample derived from the Spirulina used to feed the copepods. This sample was sonicated in the same manner as the copepod samples and 14 µL were loaded into the well.

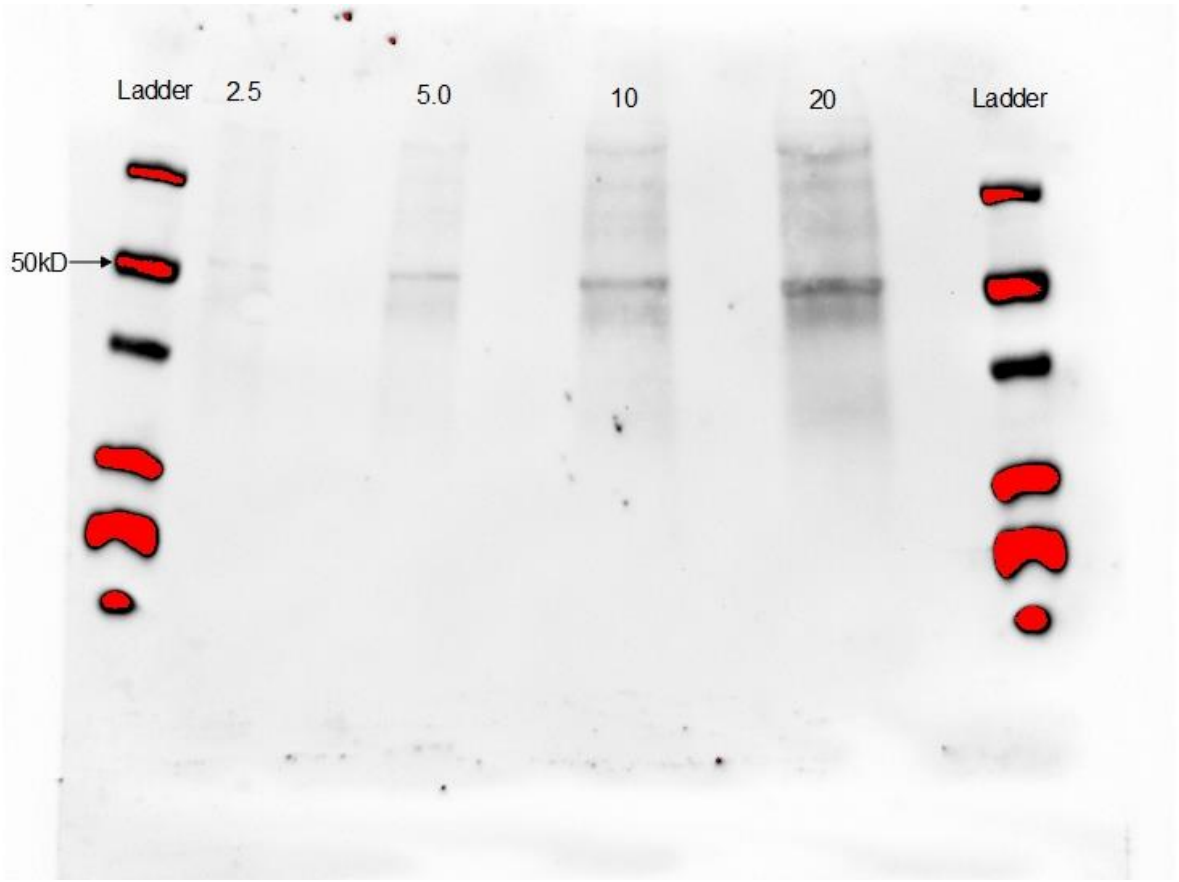

Figure 10: Western blot showing increasing concentrations of proteins and expression of AOX in order to check protein quantification protocol. The ladder lane contains 5 µL of the Precision Plus Protein Unstained Standards (Bio-Rad). The first well possessed 2.5 µg of protein, with succeeding wells of 5, 10, and 20 µg of protein.

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (5 µl)
2. 2.5 µg of copepod protein
3.
4. 5 µg of copepod protein
5.
6. 10 µg of copepod protein
7.
8. 20 µg of copepod protein
9.
10. Precision Plus Protein Unstained Standards (5 µl)

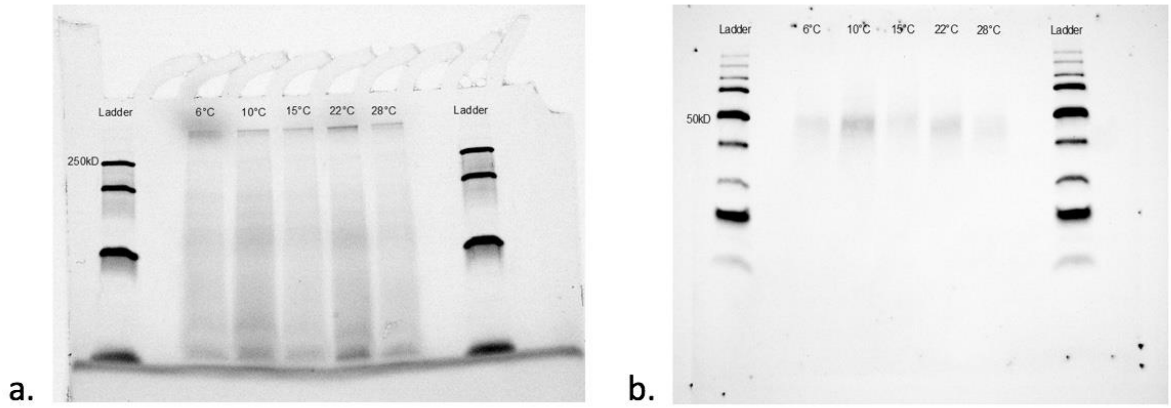

Figure 11: The first and seventh wells on the mini-PRTOEAN TGX Stain-Free gel (Bio-rad) were loaded with 2µL of the Precision Plus Protein Unstained Standards (Bio-rad). The other wells were loaded with 20 µg of protein from each protein sample. Following the loading of the wells the gel was run at a constant voltage (200V) for 35 minutes. Then the proteins were transferred onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad). **a.** 2nd acute temperature protein samples run on reducing SDS-PAGE. **b.** Western blot displaying 2nd acute temperature samples and expression of AOX

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. 6°C acute sample 2 (20 µg protein)
4. 10°C acute sample 2 (20 µg protein)
5. 15°C control sample 2 (20 µg protein)
6. 22°C acute sample 2 (20 µg protein)
7. 28°C acute sample 2 (20 µg protein)
8.
9. Precision Plus Protein Unstained Standards (2 µl)

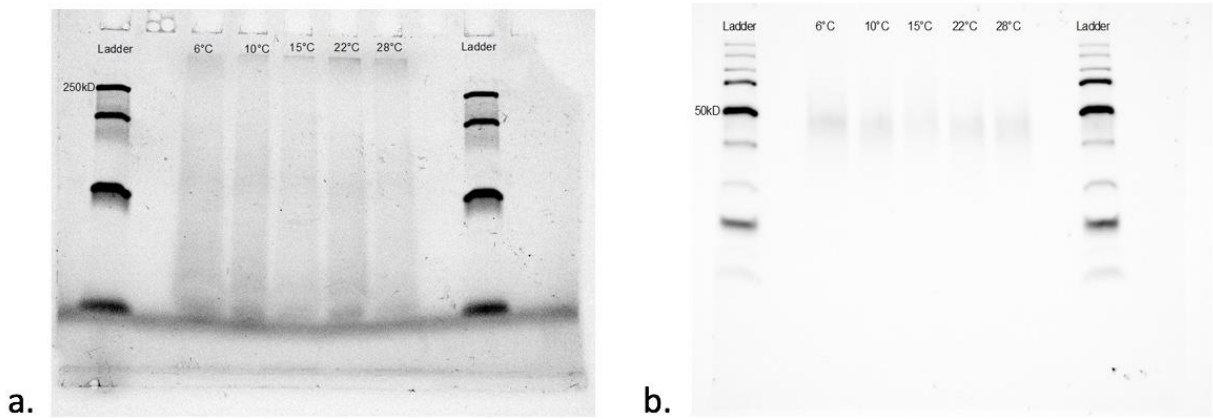

Figure 12: The first and seventh wells on the mini-PRTOEAN TGX Stain-Free gel (Bio-rad) were loaded with 2µL of the Precision Plus Protein Unstained Standards (Bio-rad). The other wells were loaded with 20 µg of protein from each protein sample. Following the loading of the wells the gel was run at a constant voltage (200V) for 35 minutes. Then the proteins were transferred onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad). **a.** 3rd acute temperature protein samples run on reducing SDS-PAGE. **b.** Western blot displaying 3rd acute temperature samples and expression of AOX

Legend

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. 6°C acute sample 2 (20 µg protein)
4. 10°C acute sample 2 (20 µg protein)
5. 15°C control sample 2 (20 µg protein)
6. 22°C acute sample 2 (20 µg protein)
7. 28°C acute sample 2 (20 µg protein)
8.
9. Precision Plus Protein Unstained Standards (2 µl)

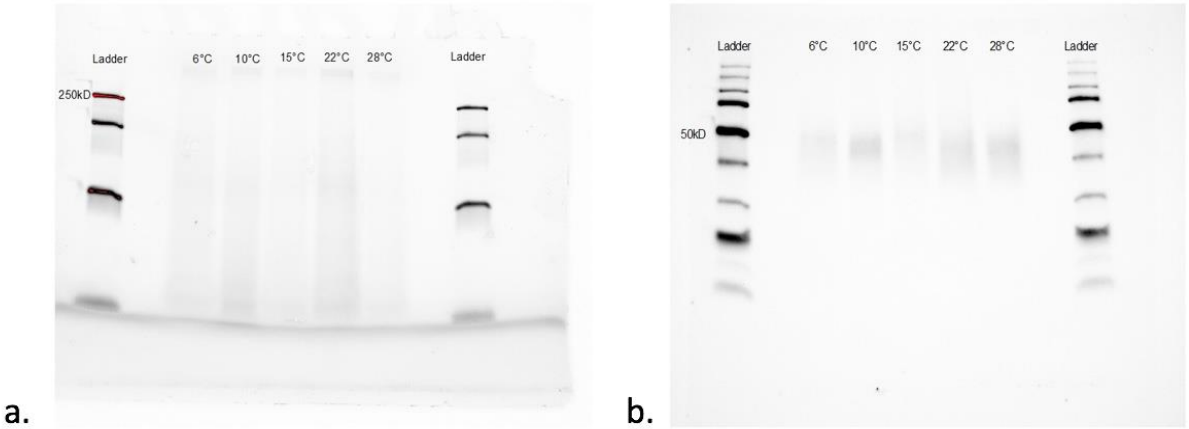

Figure 13: The first and seventh wells on the mini-PRTOEAN TGX Stain-Free gel (Bio-rad) were loaded with 2µL of the Precision Plus Protein Unstained Standards (Bio-rad). The other wells were loaded with 20 µg of protein from each protein sample. Following the loading of the wells the gel was run at a constant voltage (200V) for 35 minutes. Then the proteins were transferred onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad). **a.** 2nd chronic temperature protein samples run on reducing SDS-PAGE. **b.** Western blot displaying 2nd chronic temperature samples and expression of AOX

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. 6°C acute sample 2 (20 µg protein)
4. 10°C acute sample 2 (20 µg protein)
5. 15°C control sample 2 (20 µg protein)
6. 22°C acute sample 2 (20 µg protein)
7. 28°C acute sample 2 (20 µg protein)
8.
9. Precision Plus Protein Unstained Standards (2 µl)

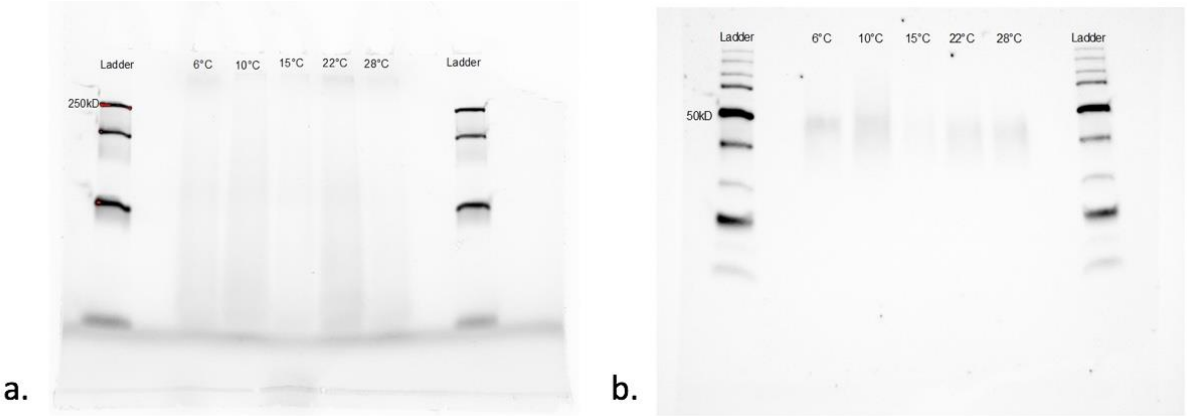

Figure 14: The first and seventh wells on the mini-PRTOEAN TGX Stain-Free gel (Bio-rad) were loaded with 2µL of the Precision Plus Protein Unstained Standards (Bio-rad). The other wells were loaded with 20 µg of protein from each protein sample. Following the loading of the wells the gel was run at a constant voltage (200V) for 35 minutes. Then the proteins were transferred onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad). **a.** 3rd chronic temperature protein samples run on reducing SDS-PAGE. **b.** Western blot displaying 3rd chronic temperature samples and expression of AOX

Legend

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. 6°C acute sample 2 (20 µg protein)
4. 10°C acute sample 2 (20 µg protein)
5. 15°C control sample 2 (20 µg protein)
6. 22°C acute sample 2 (20 µg protein)
7. 28°C acute sample 2 (20 µg protein)
8.
9. Precision Plus Protein Unstained Standards (2 µl)

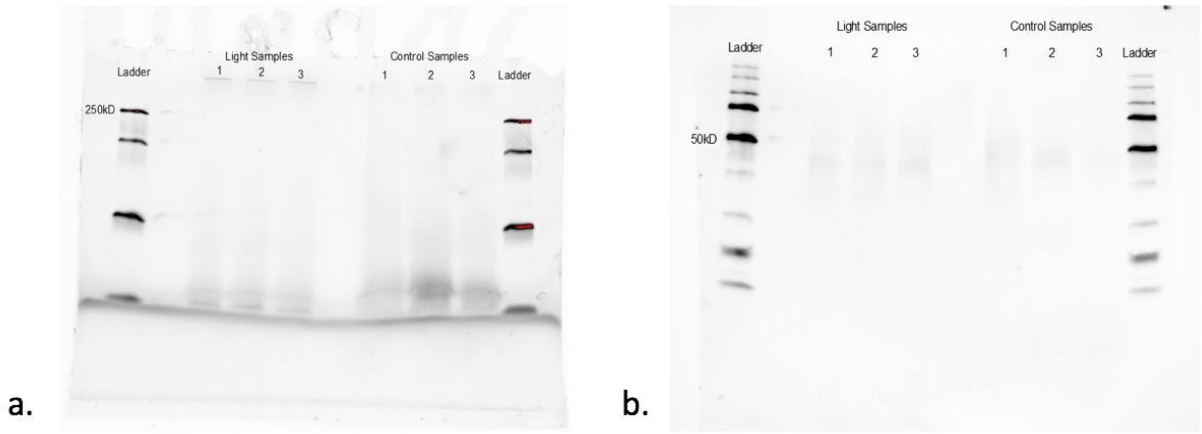

Figure 15: The first and seventh wells on the mini-PRTOEAN TGX Stain-Free gel (Bio-rad) were loaded with 2µL of the Precision Plus Protein Unstained Standards (Bio-rad). The other wells were loaded with 20 µg of protein from each protein sample. Following the loading of the wells the gel was run at a constant voltage (200V) for 35 minutes. Then the proteins were transferred onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad). **a.** Acute light exposure and control protein samples run on reducing SDS-PAGE. **b.** Western blot displaying acute light exposure samples and control samples and expression of AOX

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Acute light sample 1 (20 µg protein)
4. Acute light sample 2 (20 µg protein)
5. Acute light sample 3 (20 µg protein)
6.
7. Control light:dark sample 1 (20 µg protein)
8. Control light:dark sample 1 (20 µg protein)
9. Control light:dark sample 1 (20 µg protein)
10. Precision Plus Protein Unstained Standards (2 µl)

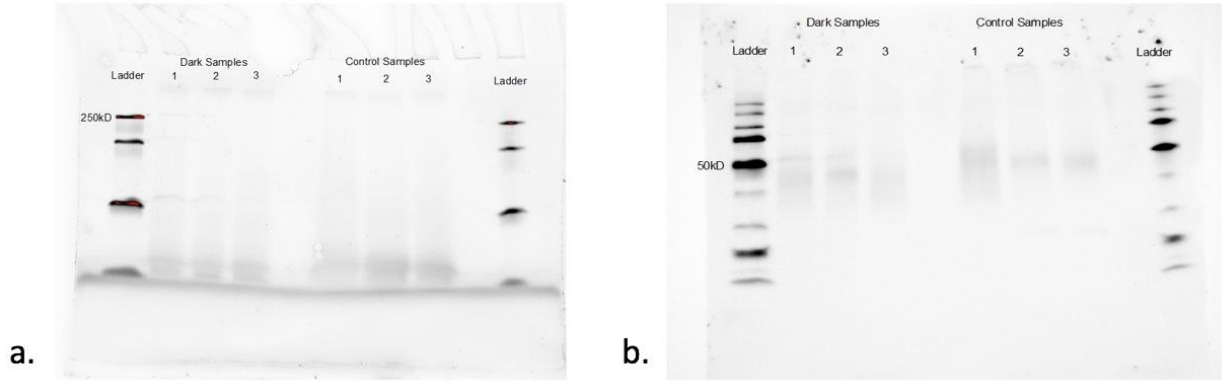

Figure 16: The first and seventh wells on the mini-PRTOEAN TGX Stain-Free gel (Bio-rad) were loaded with 2µL of the Precision Plus Protein Unstained Standards (Bio-rad). The other wells were loaded with 20 µg of protein from each protein sample. Following the loading of the wells the gel was run at a constant voltage (200V) for 35 minutes. Then the proteins were transferred onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad). **a.** Acute dark exposure and control protein samples run on reducing SDS-PAGE. **b.** Western blot displaying acute dark exposure samples and control samples and expression of AOX

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Acute dark sample 1 (20 µg protein)
4. Acute dark sample 2 (20 µg protein)
5. Acute dark sample 3 (20 µg protein)
6.
7. Control light:dark sample 1 (20 µg protein)
8. Control light:dark sample 1 (20 µg protein)
9. Control light:dark sample 1 (20 µg protein)
10. Precision Plus Protein Unstained Standards (2 µl)

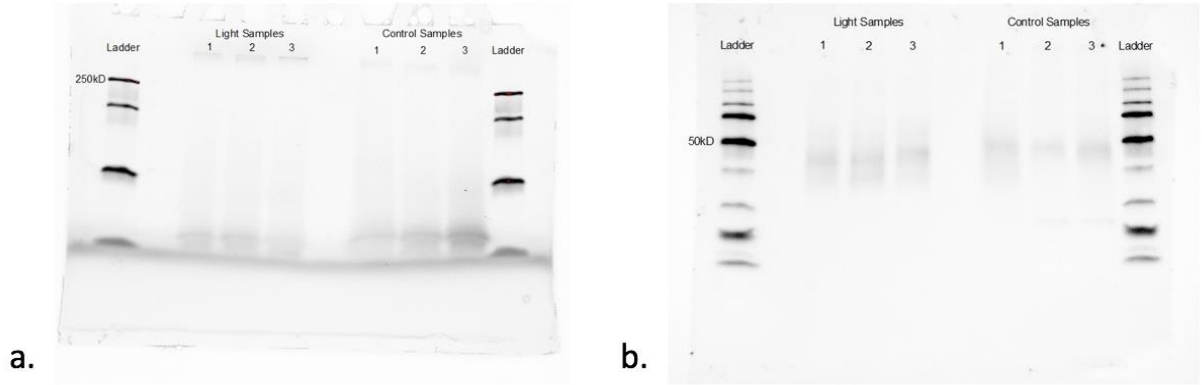

Figure 17: The first and seventh wells on the mini-PRTOEAN TGX Stain-Free gel (Bio-rad) were loaded with 2µL of the Precision Plus Protein Unstained Standards (Bio-rad). The other wells were loaded with 20 µg of protein from each protein sample. Following the loading of the wells the gel was run at a constant voltage (200V) for 35 minutes. Then the proteins were transferred onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad). **a.** Chronic light exposure and control protein samples run on reducing SDS-PAGE. **b.** Western blot displaying chronic light exposure samples and control samples and expression of AOX

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Chronic light sample 1 (20 µg protein)
4. Chronic light sample 2 (20 µg protein)
5. Chronic light sample 3 (20 µg protein)
6.
7. Control light:dark sample 1 (20 µg protein)
8. Control light:dark sample 1 (20 µg protein)
9. Control light:dark sample 1 (20 µg protein)
10. Precision Plus Protein Unstained Standards (2 µl)

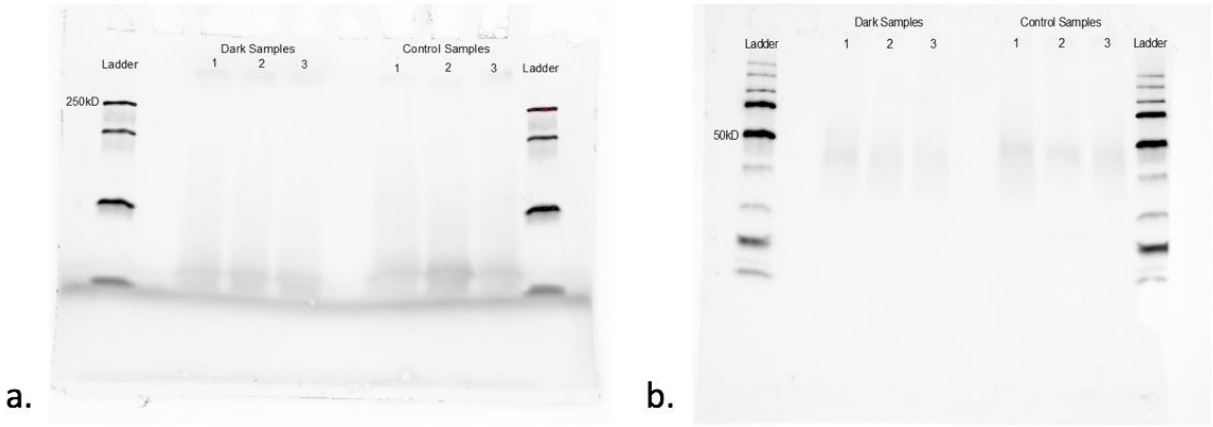

Figure 18: The first and seventh wells on the mini-PRTOEAN TGX Stain-Free gel (Bio-rad) were loaded with 2µL of the Precision Plus Protein Unstained Standards (Bio-rad). The other wells were loaded with 20 µg of protein from each protein sample. Following the loading of the wells the gel was run at a constant voltage (200V) for 35 minutes. Then the proteins were transferred onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad). **a.** Chronic dark exposure and control protein samples run on reducing SDS-PAGE. **b.** Western blot displaying chronic dark exposure samples and control samples and expression of AOX

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Chronic dark sample 1 (20 µg protein)
4. Chronic dark sample 2 (20 µg protein)
5. Chronic dark sample 3 (20 µg protein)
6.
7. Control light:dark sample 1 (20 µg protein)
8. Control light:dark sample 1 (20 µg protein)
9. Control light:dark sample 1 (20 µg protein)
10. Precision Plus Protein Unstained Standards (2 µl)

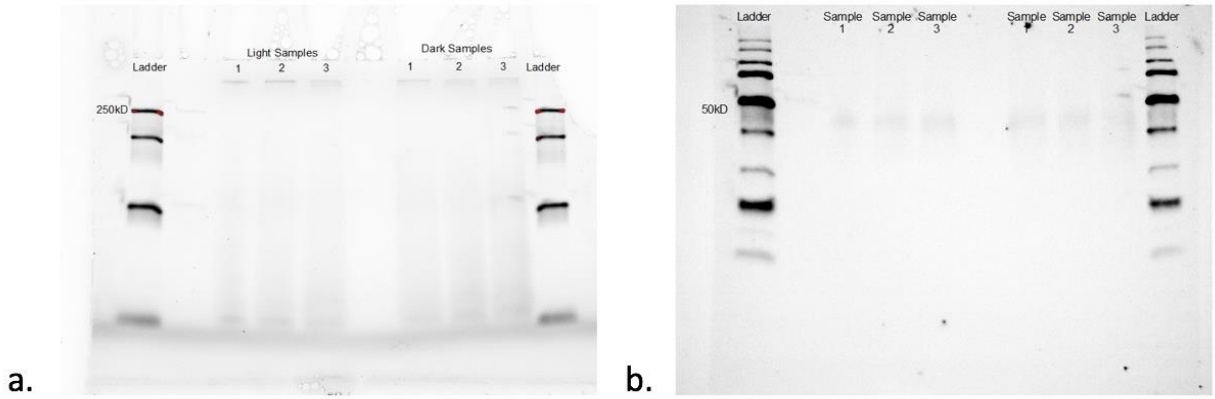

Figure 19: The first and seventh wells on the mini-PRTOEAN TGX Stain-Free gel (Bio-rad) were loaded with 2µL of the Precision Plus Protein Unstained Standards (Bio-rad). The other wells were loaded with 20 µg of protein from each protein sample. Following the loading of the wells the gel was run at a constant voltage (200V) for 35 minutes. Then the proteins were transferred onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad). **a.** Acute light and dark exposure protein samples run on reducing SDS-PAGE. **b.** Western blot displaying both acute light and dark exposure samples and expression of AOX

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Acute light sample 1 (20 µg protein)
4. Acute light sample 2 (20 µg protein)
5. Acute light sample 3 (20 µg protein)
6.
7. Acute dark sample 1 (20 µg protein)
8. Acute dark sample 2 (20 µg protein)
9. Acute dark sample 3 (20 µg protein)
10. Precision Plus Protein Unstained Standards (2 µl)

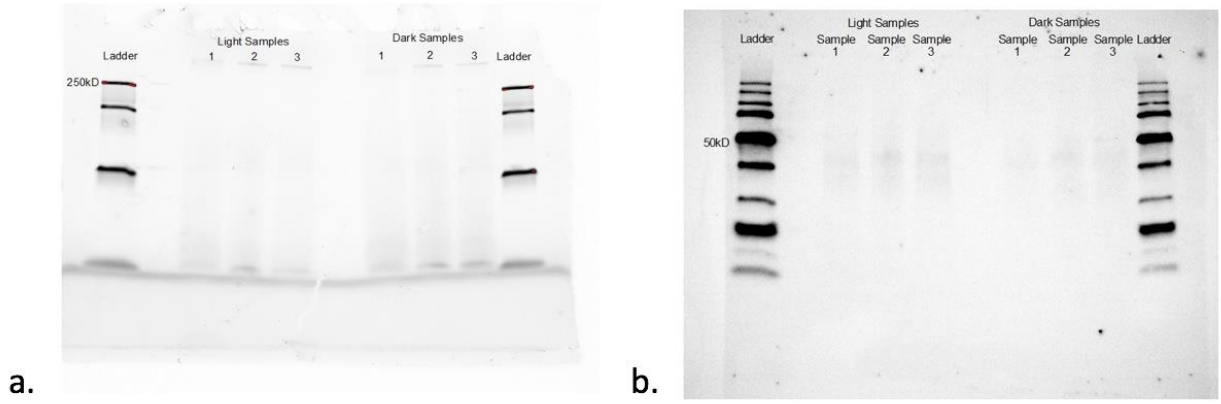

Figure 20: The first and tenth wells on the mini-PRTOEAN TGX Stain-Free gel (Bio-rad) were loaded with 2µL of the Precision Plus Protein Unstained Standards (Bio-rad). The other wells were loaded with 20 µg of protein from each protein sample. Following the loading of the wells the gel was run at a constant voltage (200V) for 35 minutes. Then the proteins were transferred onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad). **a.** Chronic light and dark exposure protein samples run on reducing SDS-PAGE. **b.** Western blot displaying both chronic light and dark exposure samples and expression of AOX

Legend

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Chronic light sample 1 (20 µg protein)
4. Chronic light sample 2 (20 µg protein)
5. Chronic light sample 3 (20 µg protein)
6.
7. Chronic dark sample 1 (20 µg protein)
8. Chronic dark sample 2 (20 µg protein)
9. Chronic dark sample 3 (20 µg protein)
10. Precision Plus Protein Unstained Standards (2 µl)

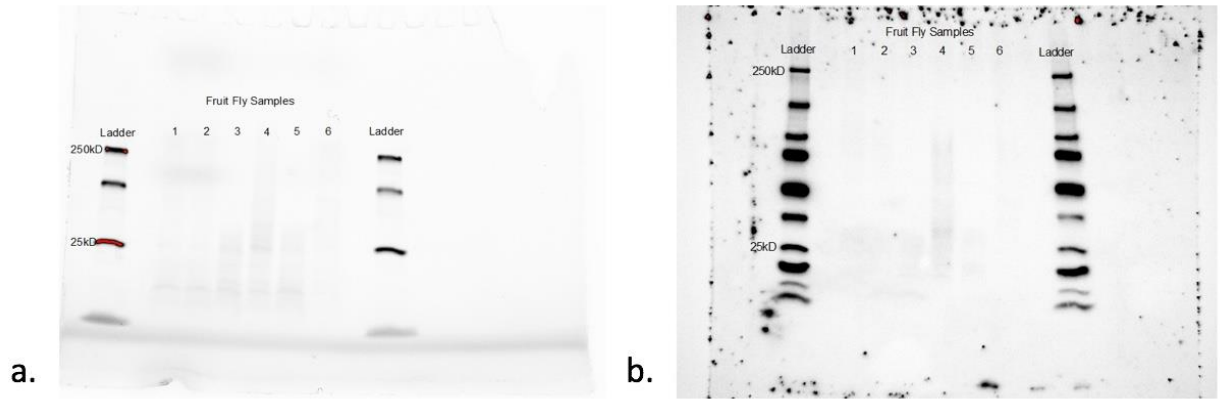

Figure 21: The first and tenth wells on the mini-PRTOEAN TGX Stain-Free gel (Bio-rad) were loaded with 2µL of the Precision Plus Protein Unstained Standards (Bio-rad). The other wells were loaded with 20 µg of protein from each protein sample. Following the loading of the wells the gel was run at a constant voltage (200V) for 35 minutes. Then the proteins were transferred onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad). **a.** *Drosophila melanogaster* protein samples run on reducing SDS-PAGE. **b.** Western blot displaying *Drosophila melanogaster* protein samples and expression of tubulin.

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Fruit Fly sample 1 (20 µg protein)
4. Fruit Fly sample 2 (20 µg protein)
5. Fruit Fly sample 3 (20 µg protein)
6. Fruit Fly sample 4 (20 µg protein)
7. Fruit Fly sample 5 (20 µg protein)
8. Fruit Fly sample 6 (20 µg protein)
9.
10. Precision Plus Protein Unstained Standards (2 µl)

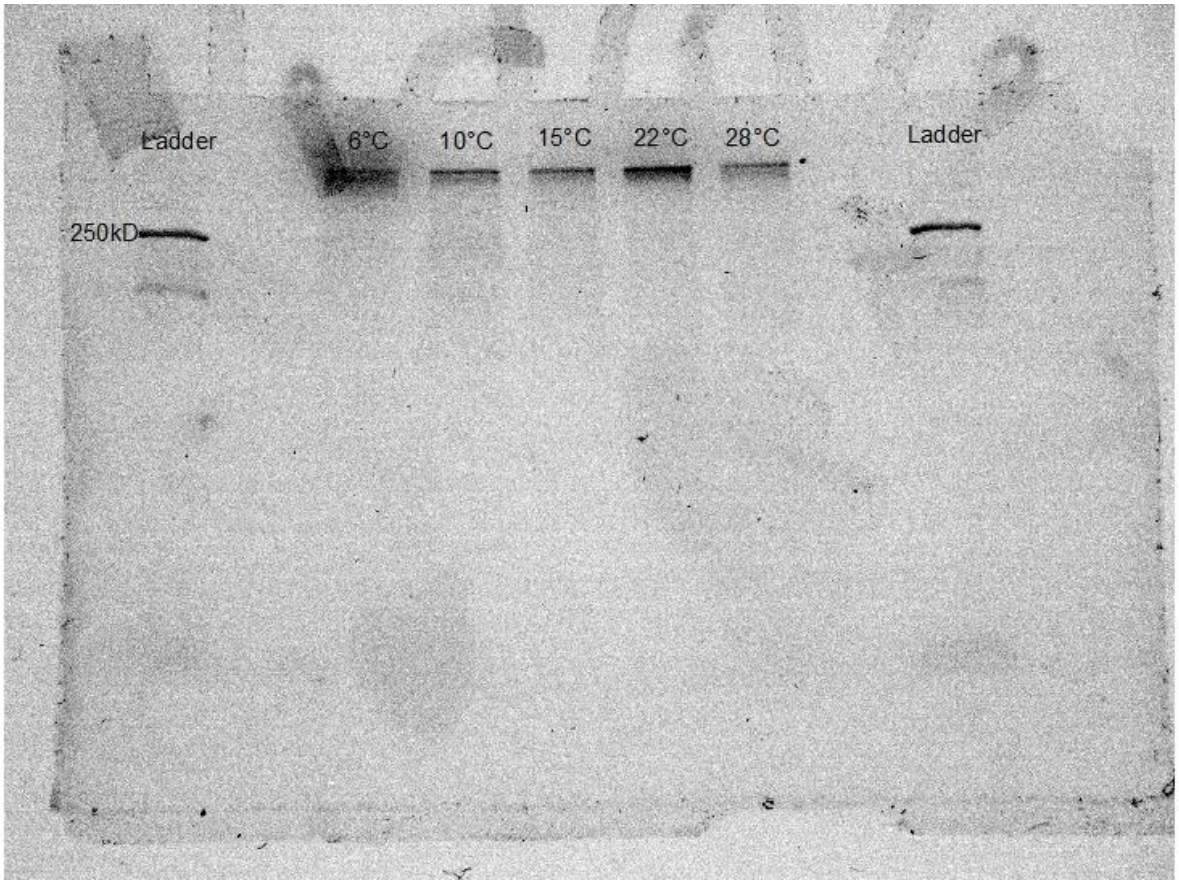

Figure 22: 2nd acute temperature protein samples run on reducing SDS-PAGE post transfer onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad).

Legend

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. 6°C acute sample 2 (20 µg protein)
4. 10°C acute sample 2 (20 µg protein)
5. 15°C control sample 2 (20 µg protein)
6. 22°C acute sample 2 (20 µg protein)
7. 28°C acute sample 2 (20 µg protein)
8.
9. Precision Plus Protein Unstained Standards (2 µl)

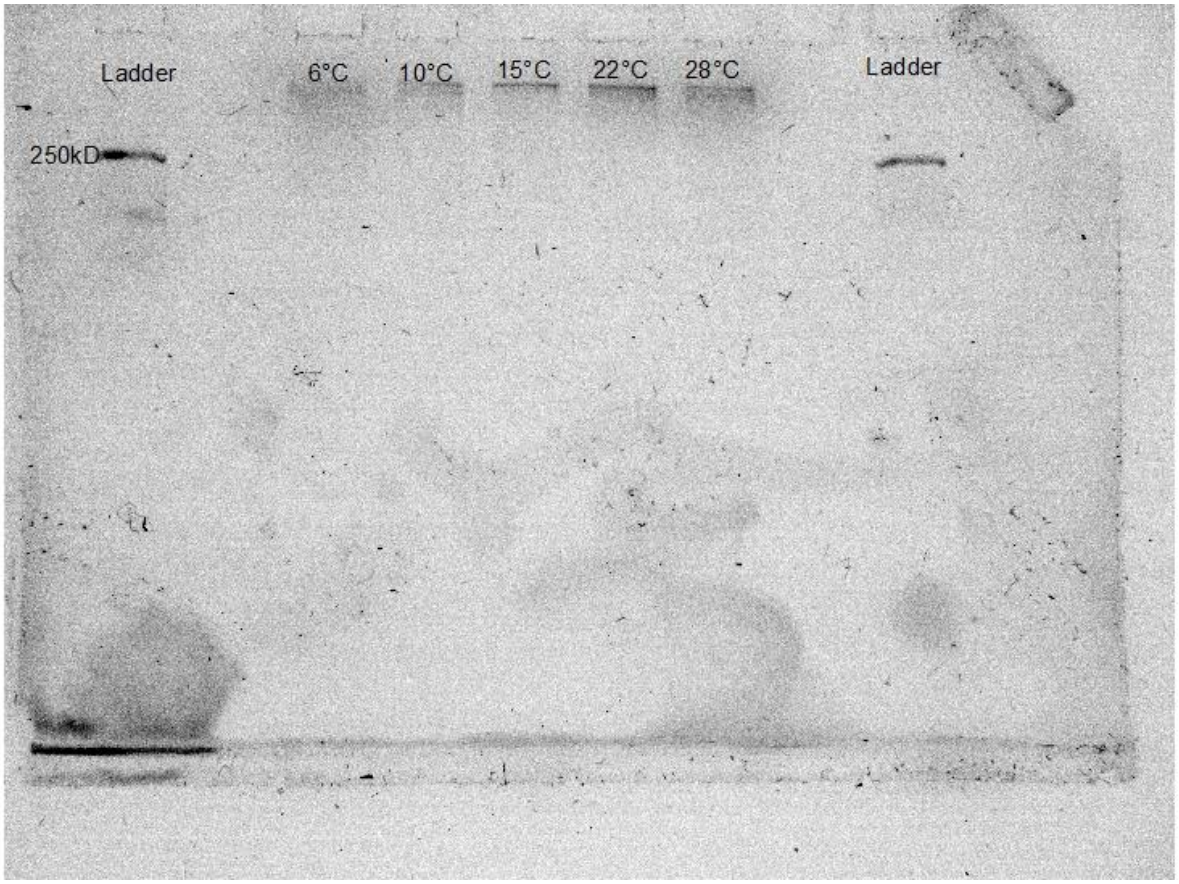

Figure 23: 3rd acute temperature protein samples run on reducing SDS-PAGE post transfer onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad).

Legend

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. 6°C acute sample 2 (20 µg protein)
4. 10°C acute sample 2 (20 µg protein)
5. 15°C control sample 2 (20 µg protein)
6. 22°C acute sample 2 (20 µg protein)
7. 28°C acute sample 2 (20 µg protein)
8.
9. Precision Plus Protein Unstained Standards (2 µl)

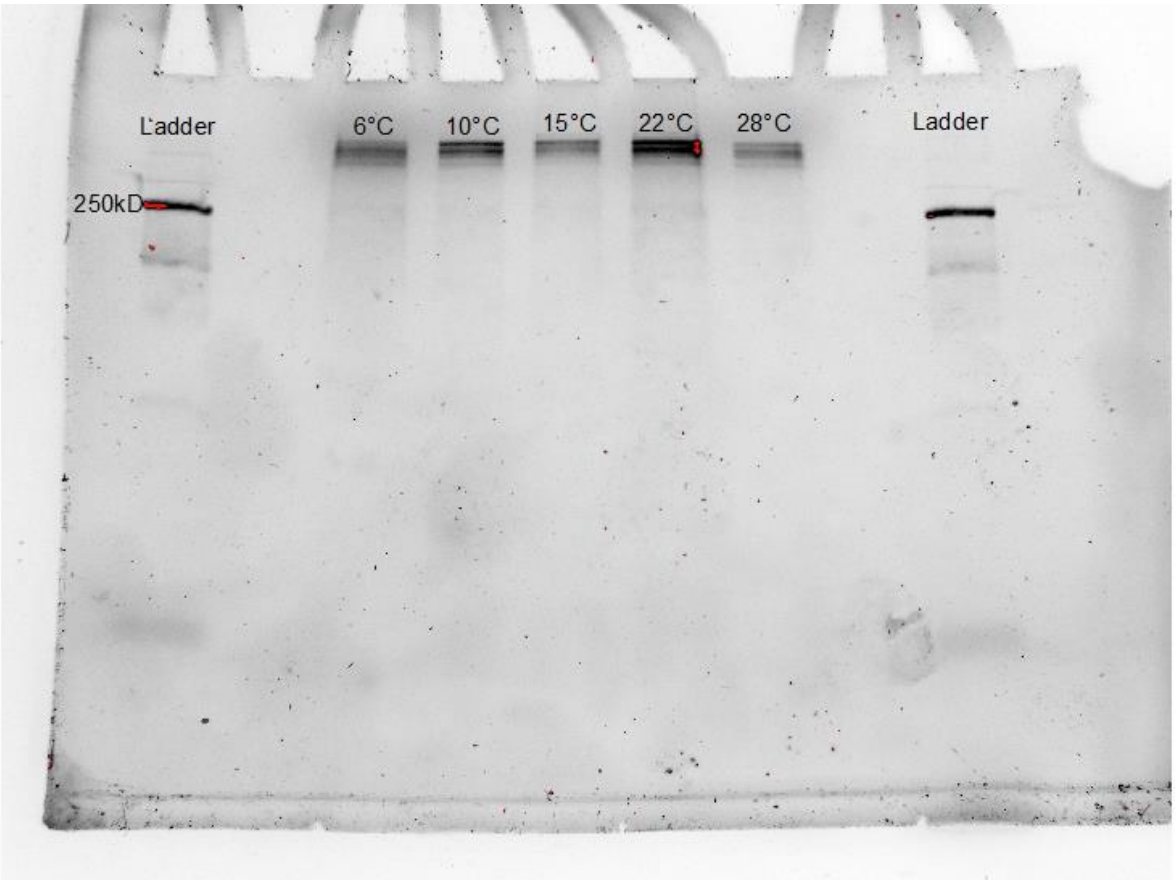

Figure 24: 2nd chronic temperature protein samples run on reducing SDS-PAGE post transfer onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad).

Legend

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. 6°C acute sample 2 (20 µg protein)
4. 10°C acute sample 2 (20 µg protein)
5. 15°C control sample 2 (20 µg protein)
6. 22°C acute sample 2 (20 µg protein)
7. 28°C acute sample 2 (20 µg protein)
8.
9. Precision Plus Protein Unstained Standards (2 µl)

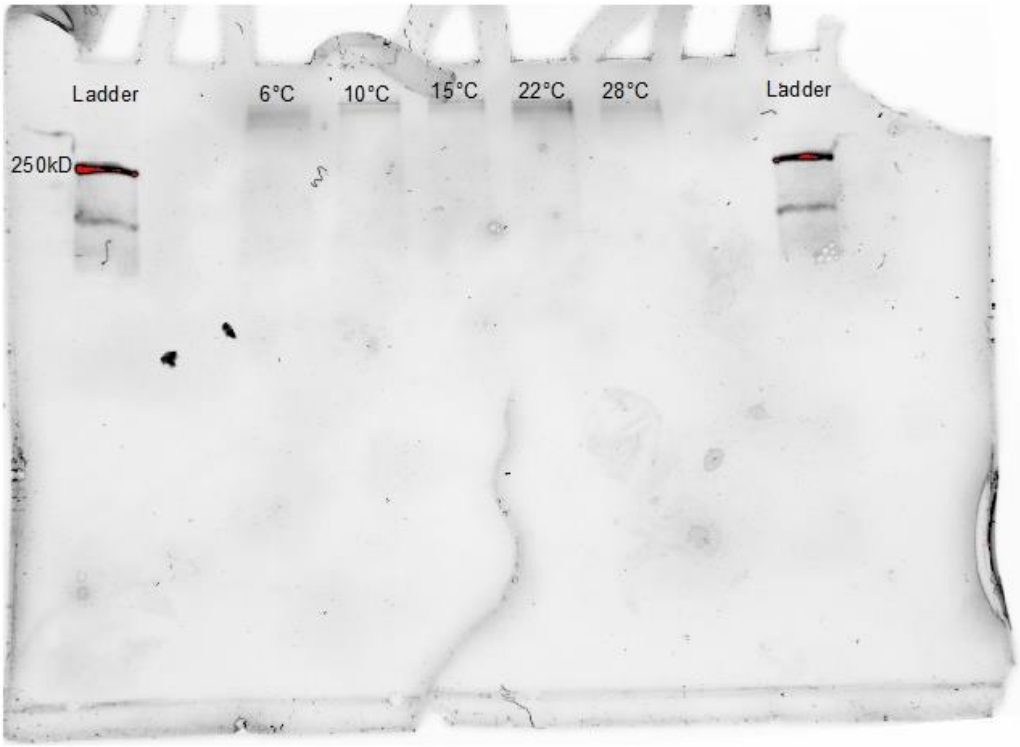

Figure 25: 3rd chronic temperature protein samples run on reducing SDS-PAGE post transfer onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad).

Legend

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. 6°C acute sample 2 (20 µg protein)
4. 10°C acute sample 2 (20 µg protein)
5. 15°C control sample 2 (20 µg protein)
6. 22°C acute sample 2 (20 µg protein)
7. 28°C acute sample 2 (20 µg protein)
8.
9. Precision Plus Protein Unstained Standards (2 µl)

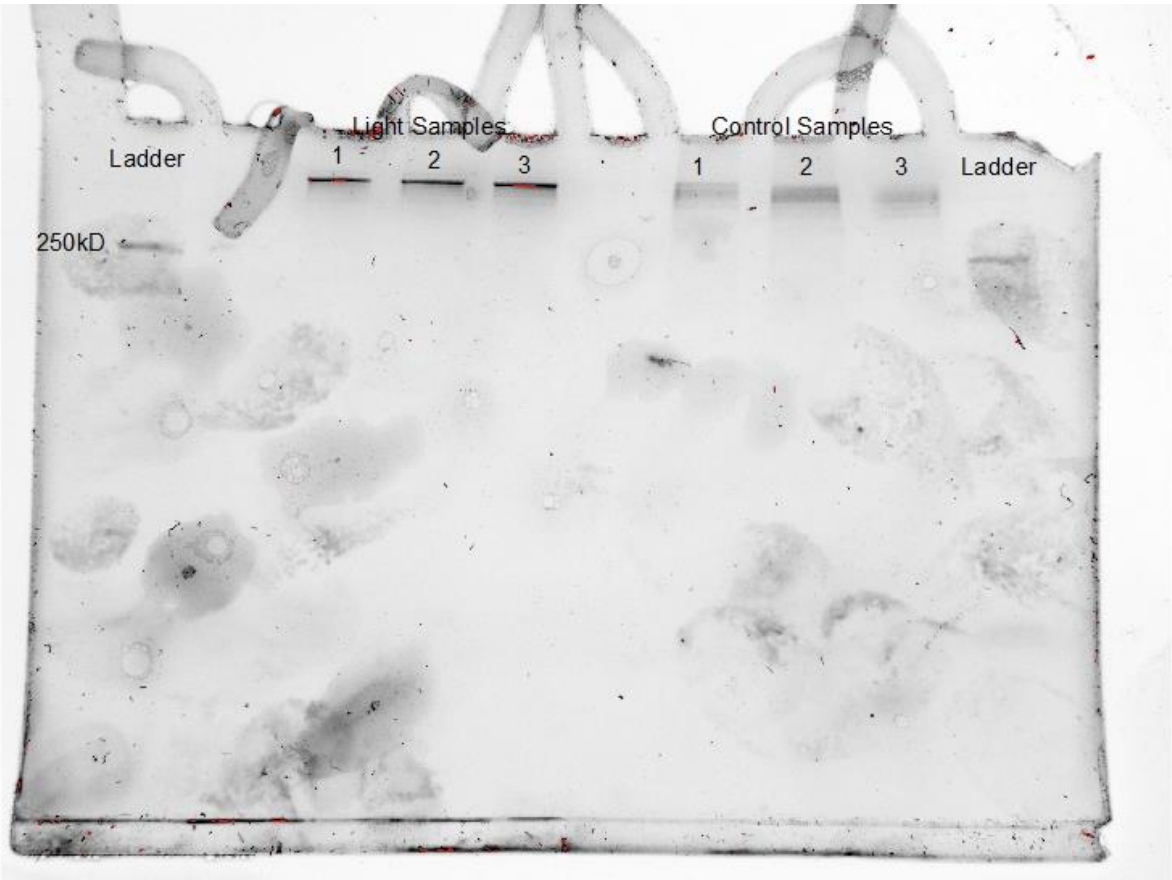

Figure 26: Acute light exposure and control protein samples run on reducing SDS-PAGE post transfer onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad).

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Acute light sample 1 (20 µg protein)
4. Acute light sample 2 (20 µg protein)
5. Acute light sample 3 (20 µg protein)
6.
7. Control light:dark sample 1 (20 µg protein)
8. Control light:dark sample 1 (20 µg protein)
9. Control light:dark sample 1 (20 µg protein)
10. Precision Plus Protein Unstained Standards (2 µl)

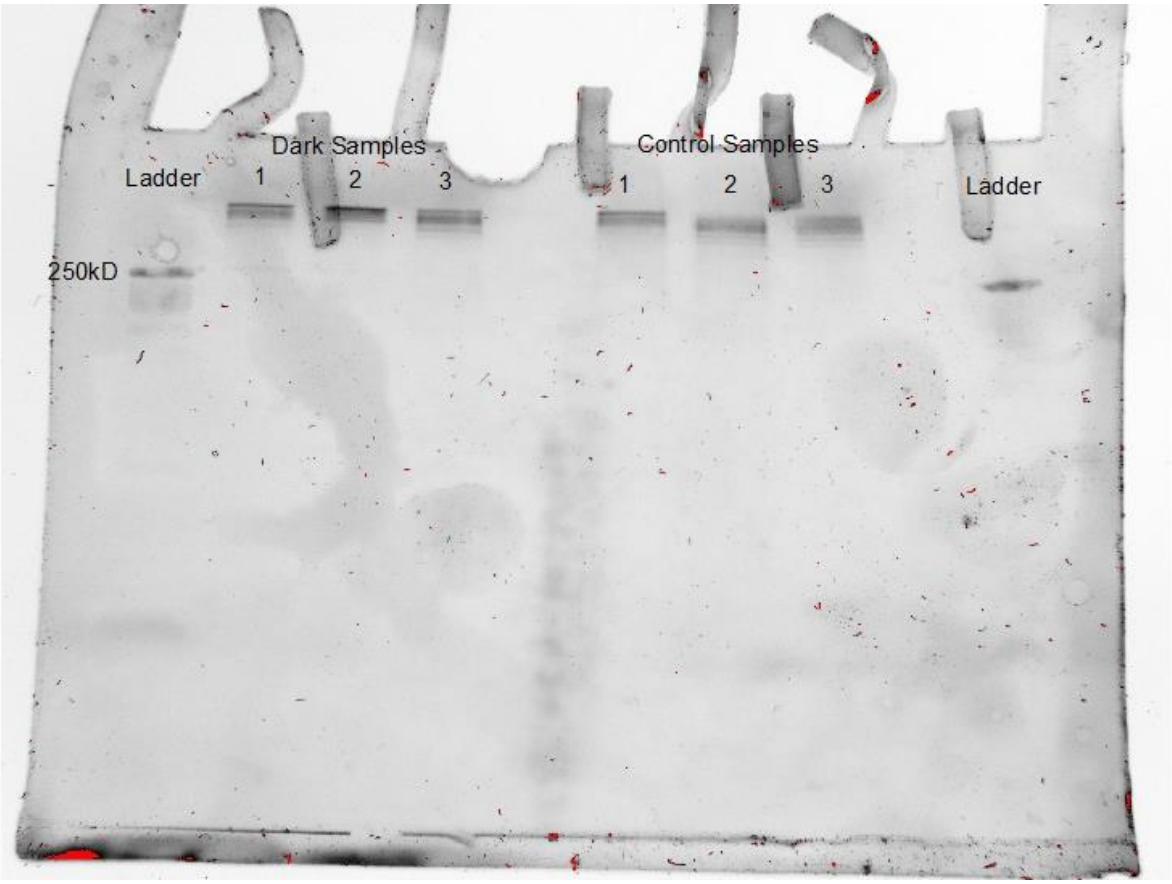

Figure 27: Acute dark exposure and control protein samples run on reducing SDS-PAGE post transfer onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad).

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Acute dark sample 1 (20 µg protein)
4. Acute dark sample 2 (20 µg protein)
5. Acute dark sample 3 (20 µg protein)
6.
7. Control light:dark sample 1 (20 µg protein)
8. Control light:dark sample 1 (20 µg protein)
9. Control light:dark sample 1 (20 µg protein)
10. Precision Plus Protein Unstained Standards (2 µl)

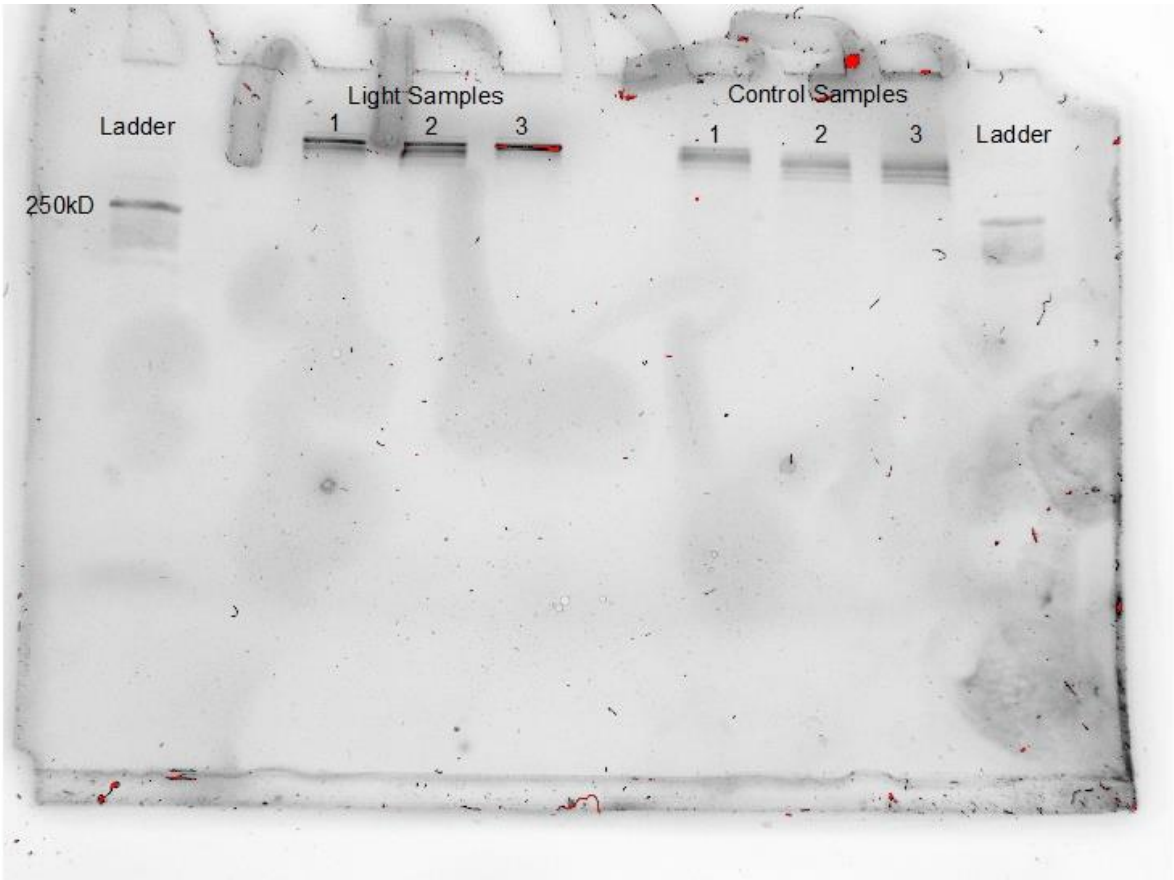

Figure 28: Chronic light exposure and control protein samples run on reducing SDS-PAGE post transfer onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad).

Legend

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Chronic light sample 1 (20 µg protein)
4. Chronic light sample 2 (20 µg protein)
5. Chronic light sample 3 (20 µg protein)
6.
7. Control light:dark sample 1 (20 µg protein)
8. Control light:dark sample 1 (20 µg protein)
9. Control light:dark sample 1 (20 µg protein)
10. Precision Plus Protein Unstained Standards (2 µl)

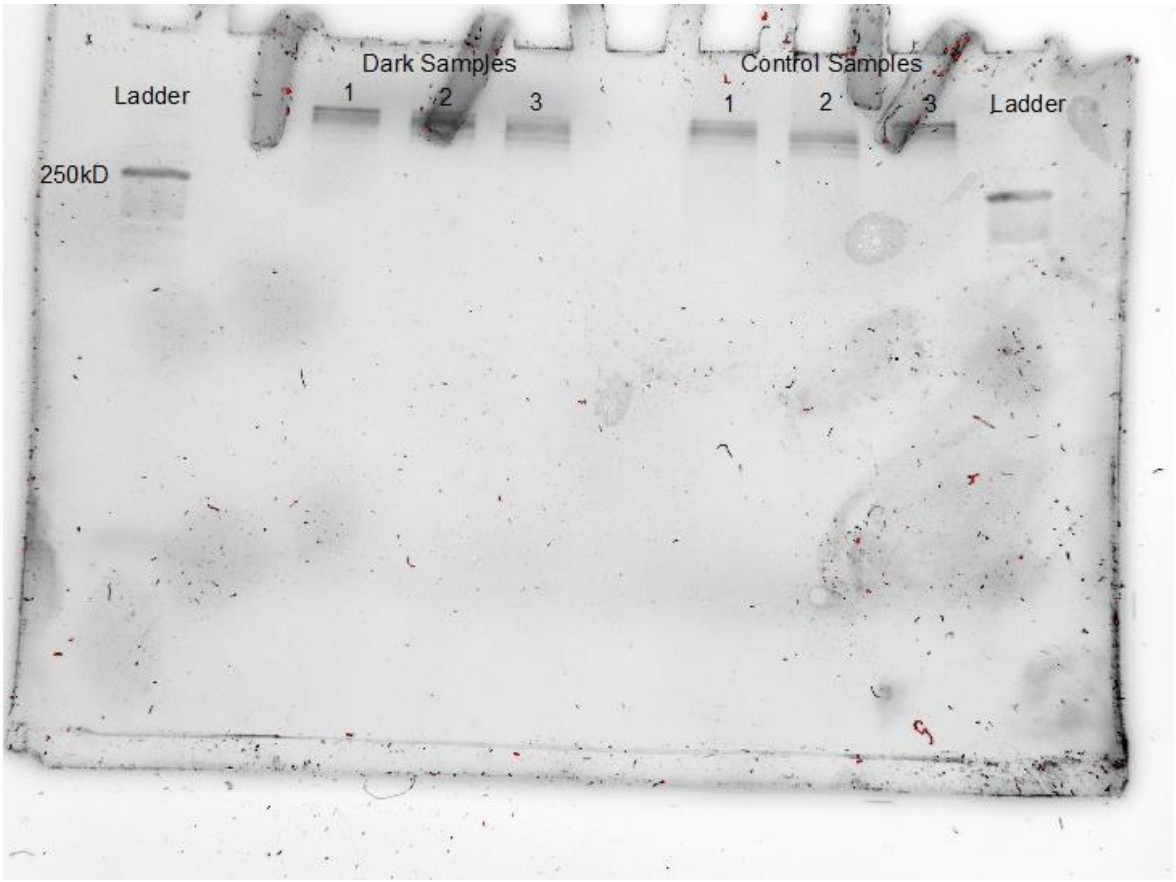

Figure 29: Chronic dark exposure and control protein samples run on reducing SDS-PAGE post transfer onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad).

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Chronic dark sample 1 (20 µg protein)
4. Chronic dark sample 2 (20 µg protein)
5. Chronic dark sample 3 (20 µg protein)
6.
7. Control light:dark sample 1 (20 µg protein)
8. Control light:dark sample 1 (20 µg protein)
9. Control light:dark sample 1 (20 µg protein)
10. Precision Plus Protein Unstained Standards (2 µl)

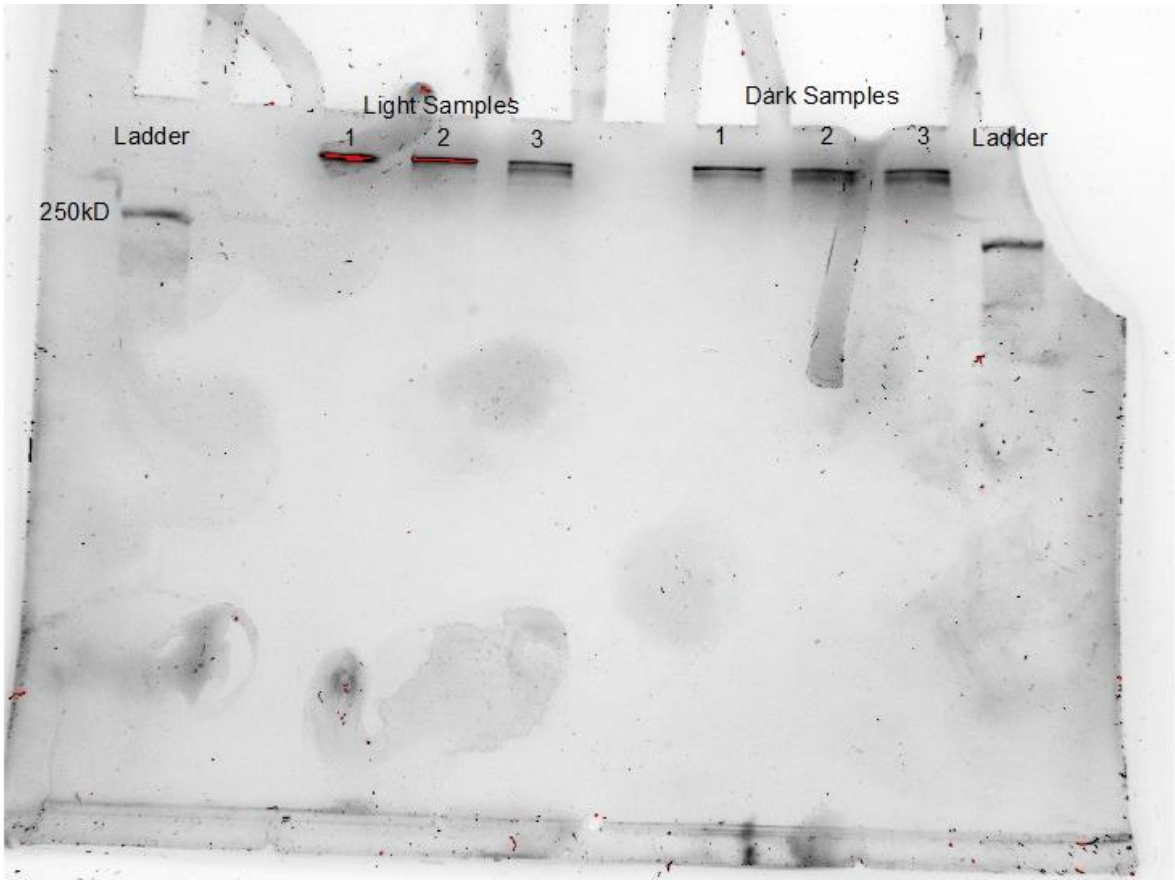

Figure 30: Acute light and dark exposure protein samples run on reducing SDS-PAGE post transfer onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad).

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Acute light sample 1 (20 µg protein)
4. Acute light sample 2 (20 µg protein)
5. Acute light sample 3 (20 µg protein)
6.
7. Acute dark sample 1 (20 µg protein)
8. Acute dark sample 2 (20 µg protein)
9. Acute dark sample 3 (20 µg protein)
10. Precision Plus Protein Unstained Standards (2 µl)

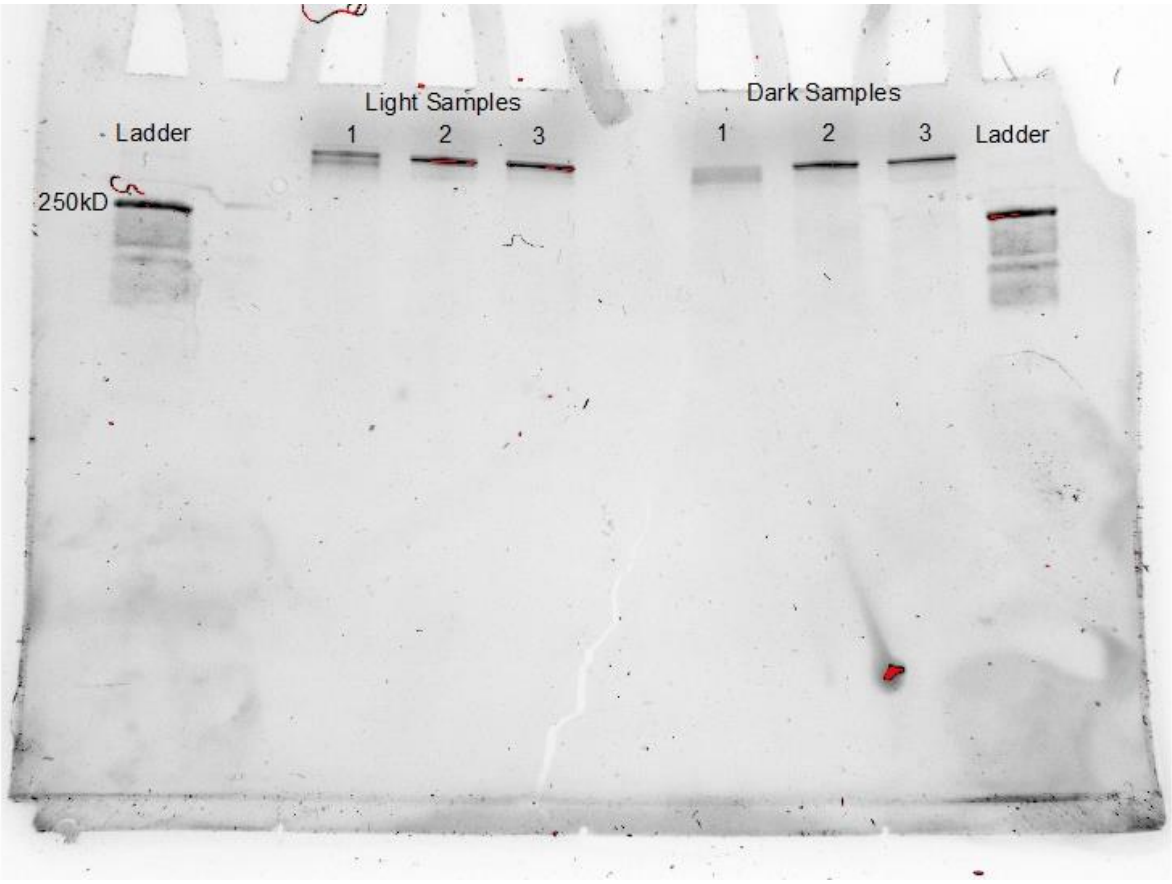

Figure 31: Chronic light and dark exposure protein samples run on reducing SDS-PAGE post transfer onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad).

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Chronic light sample 1 (20 µg protein)
4. Chronic light sample 2 (20 µg protein)
5. Chronic light sample 3 (20 µg protein)
6.
7. Chronic dark sample 1 (20 µg protein)
8. Chronic dark sample 2 (20 µg protein)
9. Chronic dark sample 3 (20 µg protein)
10. Precision Plus Protein Unstained Standards (2 µl)

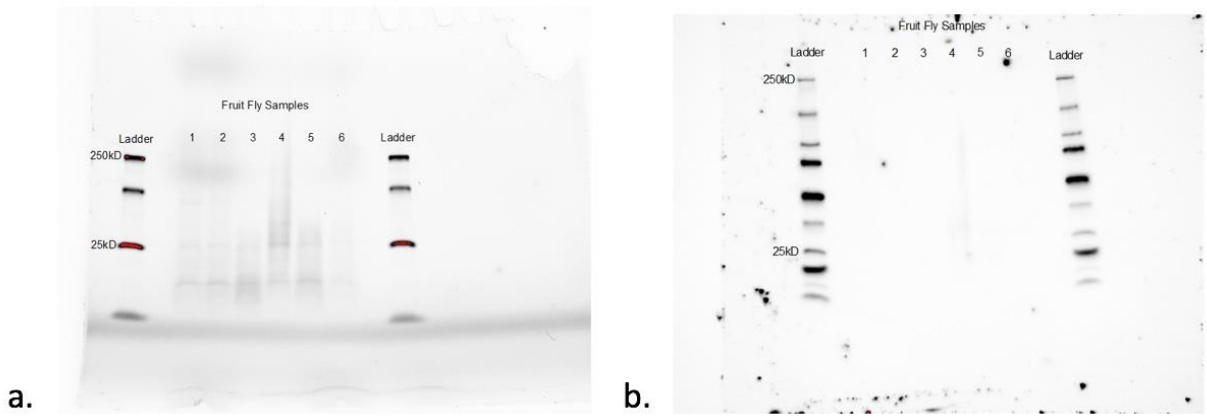

Figure 32: The first and tenth wells on the mini-PRTOEAN TGX Stain-Free gel (Bio-rad) were loaded with 2µL of the Precision Plus Protein Unstained Standards (Bio-rad). The other wells were loaded with 20 µg of protein from each protein sample. Following the loading of the wells the gel was run at a constant voltage (200V) for 35 minutes. Then the proteins were transferred onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad). **a.** *Drosophila melanogaster* protein samples run on reducing SDS-PAGE. **b.** Western blot displaying *Drosophila melanogaster* protein samples and expression of tubulin

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Fruit Fly sample 1 (20 µg protein)
4. Fruit Fly sample 2 (20 µg protein)
5. Fruit Fly sample 3 (20 µg protein)
6. Fruit Fly sample 4 (20 µg protein)
7. Fruit Fly sample 5 (20 µg protein)
8. Fruit Fly sample 6 (20 µg protein)
9.
10. Precision Plus Protein Unstained Standards (2 µl)

Figure 33: *Drosophila melanogaster* protein samples run on reducing SDS-PAGE post transfer onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad).

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Fruit Fly sample 1 (20 µg protein)
4. Fruit Fly sample 2 (20 µg protein)
5. Fruit Fly sample 3 (20 µg protein)
6. Fruit Fly sample 4 (20 µg protein)
7. Fruit Fly sample 5 (20 µg protein)
8. Fruit Fly sample 6 (20 µg protein)
9.
10. Precision Plus Protein Unstained Standards (2 µl)

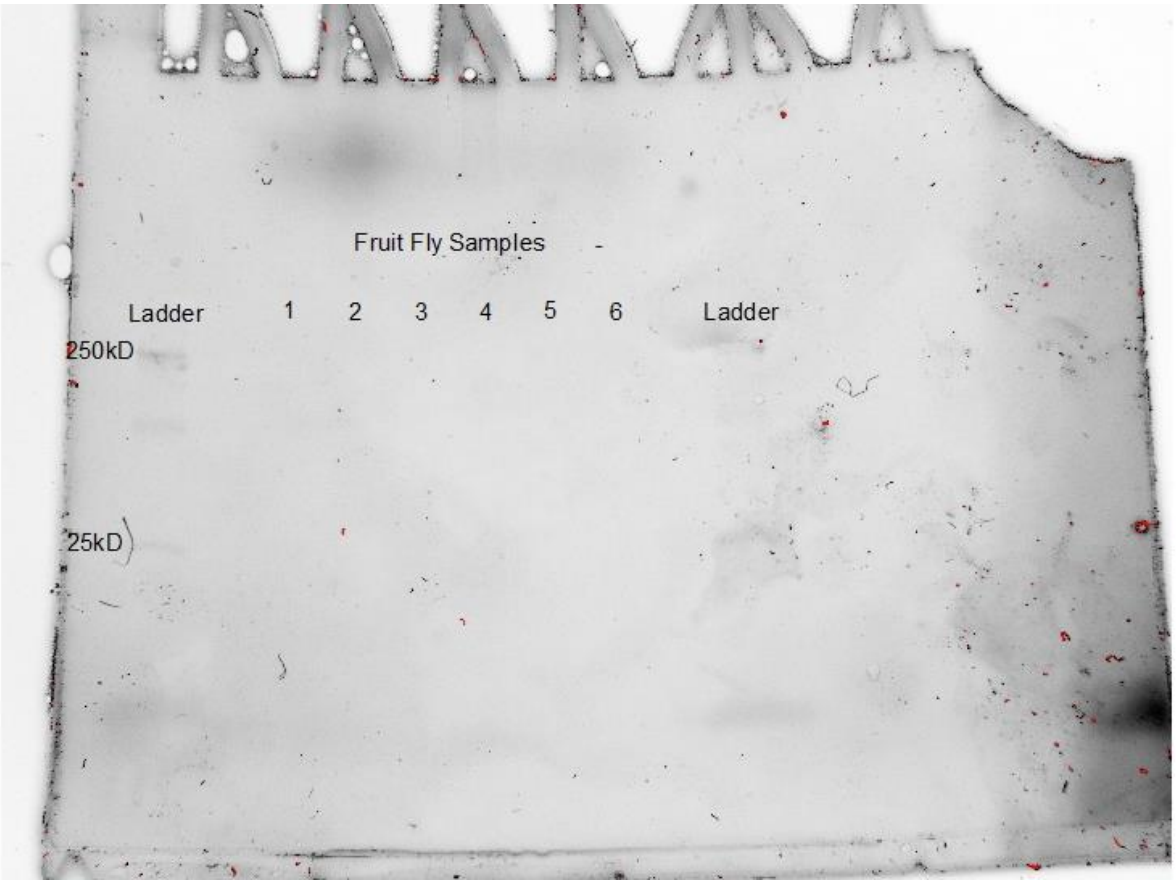

Figure 34: *Drosophila melanogaster* protein samples run on reducing SDS-PAGE post transfer onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad).

Legend

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Fruit Fly sample 1 (20 µg protein)
4. Fruit Fly sample 2 (20 µg protein)
5. Fruit Fly sample 3 (20 µg protein)
6. Fruit Fly sample 4 (20 µg protein)
7. Fruit Fly sample 5 (20 µg protein)
8. Fruit Fly sample 6 (20 µg protein)
9.
10. Precision Plus Protein Unstained Standards (2 µl)

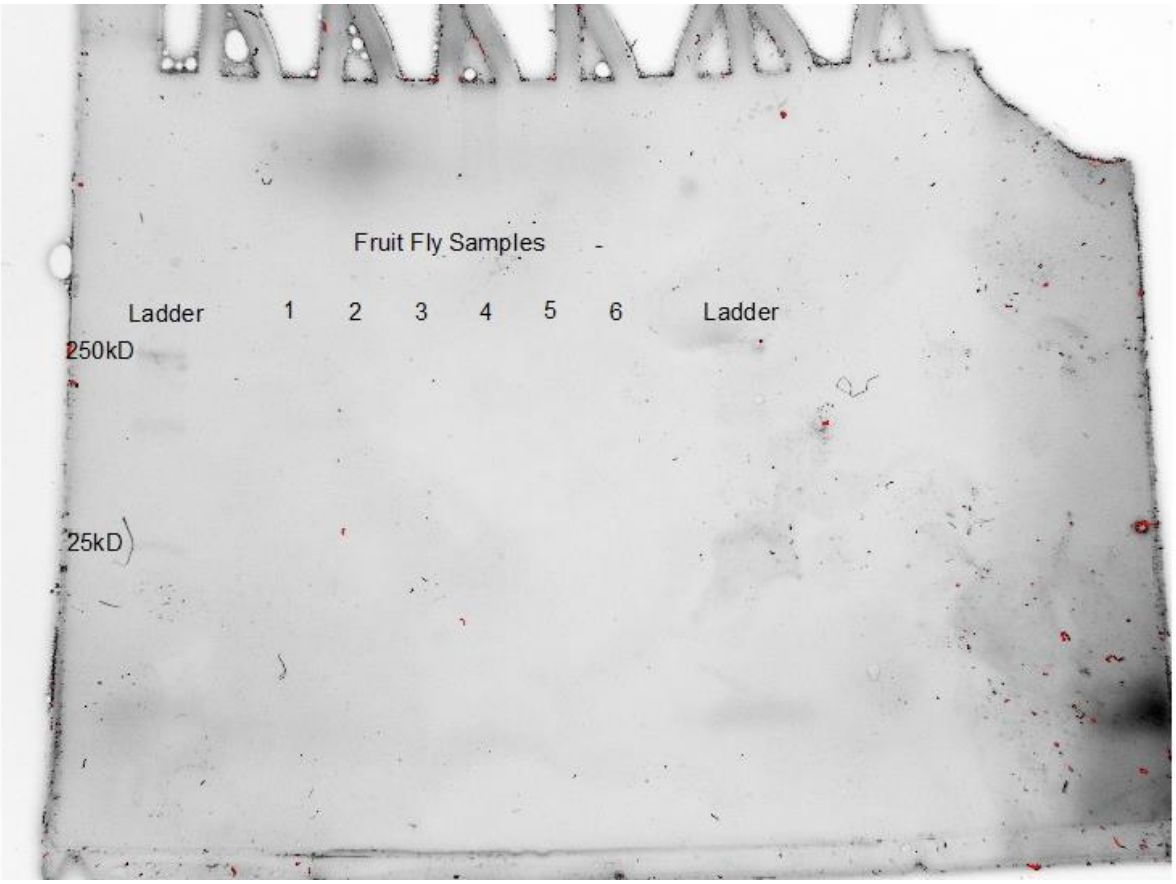

Figure 35: *Drosophila melanogaster* protein samples run on reducing SDS-PAGE post transfer onto a TransBlot Turbo Mini-size Nitrocellulose (Bio-rad) membrane using the Trans-Blot Turbo Blotting System (Bio-rad).

<u>Legend</u>

1. Precision Plus Protein Unstained Standards (2 µl)
2.
3. Fruit Fly sample 1 (20 µg protein)
4. Fruit Fly sample 2 (20 µg protein)
5. Fruit Fly sample 3 (20 µg protein)
6. Fruit Fly sample 4 (20 µg protein)
7. Fruit Fly sample 5 (20 µg protein)
8. Fruit Fly sample 6 (20 µg protein)
9.
10. Precision Plus Protein Unstained Standards (2 µl)

Tables

Table 1: Volume of copepods in Eppendorf tubes after 24 hours (acute) and one week (chronic) of exposure to specified temperatures

Sample	Initial Mass (g)	Final Mass (g)	Total Volume (g)
15°C Sample 1a	0.999	1.047	0.048
15°C Sample 2a	0.993	1.047	0.054
15°C Sample 3a	0.984	1.054	0.070
15°C Sample 1	0.954	1.004	0.050
15°C Sample 2	0.968	1.018	0.050
15°C Sample 3	0.963	1.013	0.050
6°C Acute Sample 1	0.987	1.039	0.052
6°C Acute Sample 2	0.996	1.046	0.050
6°C Acute Sample 3	0.978	1.053	0.075
6°C Chronic Sample 1	0.980	1.032	0.052
6°C Chronic Sample 2	0.997	1.053	0.056
6°C Chronic Sample 3	0.994	1.054	0.060
10°C Acute Sample 1	0.957	1.010	0.053
10°C Acute Sample 2	0.964	1.024	0.060
10°C Acute Sample 3	0.957	1.015	0.058
10°C Chronic Sample 1	0.957	1.015	0.058
10°C Chronic Sample 2	0.960	1.034	0.074
10°C Chronic Sample 3	0.953	1.017	0.064
22°C Acute Sample 1	0.959	1.020	0.061
22°C Acute Sample 2	0.950	1.023	0.073
22°C Acute Sample 3	0.978	1.066	0.088
22°C Chronic Sample 1	0.962	1.026	0.064
22°C Chronic Sample 2	0.966	1.025	0.059
22°C Chronic Sample 3	0.955	1.034	0.079
28°C Acute Sample 1	0.996	1.051	0.055
28°C Acute Sample 2	0.982	1.048	0.066
28°C Acute Sample 3	0.984	1.050	0.066
28°C Chronic Sample 1	0.953	1.003	0.050
28°C Chronic Sample 2	0.962	1.042	0.080
28°C Chronic Sample 3	0.973	1.042	0.069

Table 2: Absorbance at 595 nm of Bradford Standard, and 6 and 28°C samples

Concentration (mg/mL)	1st Absorbance Reading	2nd Absorbance Reading	Average Absorbance	Solved Concentration (mg/mL)
0	0	0	0	
0.125	0.186	0.180	0.183	
0.25	0.342	0.358	0.350	
0.5	0.658	0.636	0.647	
0.75	0.915	0.884	0.899	
1	1.059	1.107	1.083	
1.5	1.532	1.548	1.540	
2	1.687	1.698	1.693	
6°C Acute Sample 1	0.480	0.464	0.472	0.409
6°C Acute Sample 2	0.669	0.648	0.659	0.624
6°C Acute Sample 3	0.616	0.576	0.596	0.552
6°C Chronic Sample 1	0.458	0.460	0.459	0.394
6°C Chronic Sample 2	0.566	0.592	0.579	0.532
6°C Chronic Sample 3	0.850	0.838	0.844	0.838
28°C Acute Sample 1	1.276	1.190	1.233	1.287
28°C Acute Sample 2	1.009	1.041	1.025	1.047
28°C Acute Sample 3	1.555	1.519	1.537	1.639
28°C Chronic Sample 1	1.125	1.183	1.154	1.196
28°C Chronic Sample 2	1.003	1.034	1.019	1.039
28°C Chronic Sample 3	1.275	1.252	1.264	1.322

Table 3: Absorbance at 595 nm of Bradford Standard, and 10, 15 and 22°C samples

Concentration (mg/mL)	1st Absorbance Reading	2nd Absorbance Reading	Average Absorbance	Solved Concentration (mg/mL)
0	0	0	0	
0.125	0.183	0.178	0.181	
0.25	0.365	0.364	0.365	
0.5	0.686	0.643	0.665	
0.75	0.914	0.870	0.892	
1	1.152	1.151	1.152	
1.5	1.389	1.509	1.449	
2	1.675	1.669	1.672	
10°C Acute Sample 1	0.740	0.716	0.728	0.684
10°C Acute Sample 2	0.585	0.563	0.574	0.501
10°C Acute Sample 3	0.922	1.188	1.055	1.073
10°C Chronic Sample 1	1.137	1.302	1.220	1.268
10°C Chronic Sample 2	1.099	1.145	1.122	1.152
10°C Chronic Sample 3	0.630	0.627	0.629	0.566
22°C Acute Sample 1	1.123	1.099	1.111	1.139
22°C Acute Sample 2	1.144	1.204	1.174	1.214
22°C Acute Sample 3	1.214	1.382	1.298	1.361
22°C Chronic Sample 1	0.768	0.764	0.766	0.729
22°C Chronic Sample 2	0.771	0.759	0.765	0.728
22°C Chronic Sample 3	1.122	1.110	1.116	1.145
15°C Sample 1	0.872	0.831	0.852	0.831
15°C Sample 2	0.853	0.853	0.853	0.833
15°C Sample 3	0.989	0.977	0.983	0.987

Table 4: Absorbance at 595 nm of Bradford Standard, fruit fly samples and light and dark exposure samples

Concentration (mg/mL)	1st Absorbance Reading	2nd Absorbance Reading	Average Absorbance	Solved Concentration (mg/mL)
0	0	0	0	
0.125	0.144	0.142	0.143	
0.25	0.296	0.303	0.2995	
0.5	0.574	0.519	0.5465	
0.75	0.542	0.818	0.68	
1	0.957	1.026	0.9915	
1.5	1.271	1.075	1.173	
2	1.417	1.385	1.401	
Fruit Fly Sample 1	2.187	2.153	2.17	3.447
Fruit Fly Sample 2	2.21	2.25	2.23	3.542
Fruit Fly Sample 3	2.121	1.96	2.0405	3.242
Fruit Fly Sample 4	2.051	2.077	2.064	3.279
Fruit Fly Sample 5	2.077	1.382	1.7295	2.749
Fruit Fly Sample 6	1.889	2.159	2.024	3.215
24 Hour Light Sample 1	1.457	1.93	1.6935	2.692
24 Hour Light Sample 2	1.341	1.409	1.375	2.187
24 Hour Light Sample 3	1.243	1.273	1.258	2.001
1 Week Light Sample 1	1.579	1.337	1.458	2.318
1 Week Light Sample 2	1.578	1.64	1.609	2.558
1 Week Light Sample 3	1.643	1.656	1.6495	2.622
24 Hour Dark Sample 1	0.852	0.894	0.873	1.391
24 Hour Dark Sample 2	1.141	1.162	1.1515	1.832
24 Hour Dark Sample 3	1.113	1.205	1.159	1.844
1 Week Dark Sample 1	0.844	0.786	0.815	1.299
1 Week Dark Sample 2	1.265	1.248	1.2565	1.999
1 Week Dark Sample 3	1.133	1.117	1.125	1.790

Table 5: Volume of *D. melanogaster* in Eppendorf tubes

Sample	Initial Mass (g)	Final Mass (g)	Total Volume (g)
F1	0.970	1.075	0.105
F2	0.977	1.110	0.133
F3	0.984	1.219	0.235
F4	0.995	1.079	0.084
F5	0.989	1.068	0.079
F6	0.981	1.013	0.032

CHAPTER 5

General Discussion

1.1 Conclusions and Future Directions

The goal of this project was to analyze a native AOX sequence in an organism that produces it and to gain insights into its physiological function. The use of bioinformatics tools allowed us to determine that the copepod *T. californicus* contains the AOX gene. Comparisons were made to identify similarities and differences between AOX in our organism of study and other plants and animals. Given the confirmation of the presence of the AOX pathway in this copepod, our work then examined AOX protein expression levels when subjected to certain environmental stressors. This is a necessary first step towards exploring the genetic and post-translational regulation of AOX in *T. californicus*. *T. californicus* therefore represents an emerging model organism for the study of AOX in animals because of its abundance, wide geographic distribution, ease of manipulation in laboratory settings, and its ecological relevance (Raisuddin *et al*., 2007).

One of the goals of this research project was to examine the AOX protein sequence in *T. californicus* to see if it possesses the necessary amino acids required for the protein to function. AOX sequences in plants, animals, and more specifically Maxillopoda, were compared to *T. californicus'* AOX sequence, in order to see if the necessary amino acids were conserved in a similar fashion across different organisms. To accomplish the first goal of this research, an effective and efficient DNA isolation technique needed to be identified. Due to *T. californicus'* miniscule body size and hard exoskeleton, it made it difficult to find a DNA isolation technique that did not require a multitude of animals. Previous research has been able to isolate DNA from a single resting egg, which is much smaller in size then a copepod and as well possesses a hard exterior. With some modifications, I was able to develop a technique that is both an efficient and effective method to isolate DNA from our organism of interest, *T. californicus*.

The first major discovery of this thesis is that the AOX protein sequence in *T. californicus* possesses conserved AOX residues, which are required in order for the protein to function. Due to the AOX sequence obtained only being a partial sequence, we were only able to focus on the latter end of the gene sequence (C-terminal region). Based on previous research done by Moore *et al*. (2013), there are 29 universally conserved residues in the AOX protein sequence. In our *T. californicus* AOX, only 23 residues are available for analysis as the other six are not present in the protein region that was being analyzed (Chapter 3, Table 12). Based on the partial AOX sequence that was analyzed, *T. californicus* has all 23 of the universally conserved residues in order to make its AOX gene physiologically functional. Next, to ensure that the AOX sequence recovered from the arthropod *T. californicus* was from an animal species and not from contamination (e.g. from a microbial symbiont or pathogen), that could have taken place throughout the experiment, the sequence was translated to its predicted protein sequence and a C-terminal motif was identified (Chapter 3, Figure 9). All animal AOX protein sequences possess a specific C-terminal motif (N-P-[YF]-X-P-G-[KQE]), which is highly conserved (McDonald *et al*., 2009). To confirm this finding, when comparing *T. californicus'* AOX sequence to plant AOX sequences (Chapter 3, Figure 7), it can be seen that none of the plants possess the same C-terminal motif.

In this thesis, we have added to the evidence that AOX has a widespread taxonomic distribution, as it is present throughout many kingdoms and phyla (Chapter 3, Table 11). We anticipate that as more sequencing data becomes available, it will be revealed that AOX is ubiquitous in the plant kingdom and present in certain taxonomic orders in the animal kingdom. *T. californicus'* AOX protein sequence was compared to AOX sequences from other organisms that are more closely and distantly related based on taxonomic ranking. When comparing AOX

protein sequences amino acid by amino acid, it can be seen that *T. californicus'* sequence was similar to sequences from other Maxillopoda sequences (Chapter 3, Table 13). When comparing the *T. californicus* sequence to both plant and other animal AOX protein sequences, there were comparable percent similarities (Chapter 3, Table 13). Future research should focus on isolating the complete AOX sequence from *T. californicus* to determine if it possesses the other 6 amino acid residues that are imperative for its protein structure and function. With *T. californicus* further investigation of the AOX gene structure is warranted, in particular focusing on the N-terminal region of the protein as well as the intron/exon structure of the gene to determine if they are comparable to other animals and plants. Future research in this area can explore the possibility of alternative splicing of mRNA and regulatory region within introns. A recent research paper demonstrates that alternative splicing of the AOX gene occurs in oysters (Liu and Guo, 2017). Real-time PCR, also known as quantitative pCR (qPCR), should be performed in order to get accurate quantitation of RNA levels and analyze AOX gene expression in an animal model. qPCR will permit the accurate measurement and analysis of the quantitative and qualitative expression of the AOX gene under different sets of experimental conditions. This will allow for more definitive statements regarding the physiological function of AOX in copepods to be made.

The second major finding of this research is that AOX protein levels were detected in an animal that naturally produces the enzyme. This is only the second report of the presence of native AOX protein in an animal (Tward *et al*., 2019). Before we could do this a protein isolation protocol, which permits quantification of proteins, had to be developed. This protein isolation protocol does not only work for copepods, but as well other organisms that are small and have hard exteriors (e.g. Fruit flies, Chapter 4, Table 5).

The third significant finding from this research project is observing that AOX protein expression may help copepods to deal with environmental stressors such as temperature fluctuations. This work marks the first time that AOX protein levels were observed to change in response to environmental stress in any animal. This was determined by conducting temperature and light exposure experiments and analysis. *T. californicus* was exposed to varying environmental temperatures (6, 10, 22, and 28°C), which is particularly stressful to the animal, in comparison to its usual habitat temperature of 15°C. Furthermore, the copepods were exposed to acute (24 hours) and chronic (1week) levels of light and dark exposure.

For the first time, we have identified one of the possible physiological functions of AOX in the copepod *T. californicus*. We demonstrated that when copepods are subjected to changing environmental temperatures (6-28°C), for both acute (24 hours) and chronic (1 week) amounts of time, fluctuations in the expression of AOX protein occurs (Chapter 4, Figures 8-11). Previous research on AOX in plants demonstrated that AOX expression increases when plants experience biotic and abiotic stresses, such as temperature change (Finnegan *et al*., 2004, Giraud *et al*., 2008). Recent work has demonstrated that when AOX is active it will heat the plant during thermogenesis, thereby allowing it to survive at low temperatures (Watling *et al*., 2006).

Our last significant finding is that AOX protein expression does not respond to short (24 hours) or extended (1 week) periods of light and dark stress (Chapter 4, Figures 12-17). This is contradictory to the finding that AOX expression in plants increases when plants experience light stress (Zhang *et al*., 2010). Future research in this area could focus on looking at other environmental stressors such as salinity stress, fluctuations in oxygen levels, and predation.

Overall, this thesis has contributed significant information regarding a native AOX

sequence in an organism that naturally produces it and has identified new areas for AOX research. Since previous AOX work in animals has primarily focused on identifying organisms with the gene and sequencing it from of the organism, there are many opportunities that exist for comparative studies and studies regarding the physiological function of the AOX pathway in animals. This thesis has identified that the amino acids required for the physiological function of AOX are conserved in *T. californicus*. As more AOX data becomes available from animals, it could provide insight as to why such an extensive group of organisms have retained this seemingly wasteful pathway and may help determine further the physiological role(s) of AOX in animals. The resilience of copepods as a model organism and the identification of the AOX gene in copepods offers an advantage over other systems as future knockout experiments can examine the physiological effects that AOX has in these animals.

1.2 Real World Application

This project has led to the advancement of knowledge in many fields of biology. The first requirement of this research was to develop a DNA isolation method for copepods. Previous methodologies for this undertaking required a large quantity of copepods which is not feasible for the space constraints most labs possess. The DNA isolation method that was developed is extremely versatile and can be used to extract DNA from numerous different types of zooplankton and small organisms with hard exteriors. Furthermore, it will provide the necessary amount of DNA in order to accomplish PCR and isolation of a nucleotide sequence. It could be of use to look at the genomes of other species because it can reveal insights into biodiversity and utility for humans. Cataloging biodiversity through projects on species inventory is the first step towards developing an understanding of how various organisms interact with their environments. This is a key fact in understanding an organisms' role in their ecosystem and their potential

utility to humankind. Furthermore, being able to isolate single genes from different organisms allows for the study of their function and helps to establish relationships between species in a comparative genomics way. By being able to situate a species on an evolutionary tree it allows for predictions regarding the structure and function of neighboring organisms on the evolutionary tree. For example, in this research project we identified that *T. californicus*' AOX sequence possessed the C-terminal motif which was determined to be diagnostic for animal AOX proteins. Furthermore, the AOX sequence of *T. japonicas* is almost identical (97.7%) to *T. californicus* as they both come from the same genus *Tigriopus* (Chapter 3, Table 13 and Figure 9).

From transcribed DNA sequences that have been isolated from organisms, the translated protein sequences can be ascertained. Proteins are the most versatile macromolecules in living systems and serve a critical role in essentially all biological processes. The function of a protein is directly dependent on its three-dimensional structure. In order to make inferences regarding the function of a protein, it is often useful to determine what amino acid residues in the protein's primary structure have been conserved. The majority of research regarding the structure of AOX has been done in plants. This project looks at the important amino acids that are required in order for the AOX protein to form the correct secondary and tertiary structure as well optimally function. By comparing *T. californicus'* AOX sequence to that of other plants, and animals that are closely and distantly related, it demonstrates that these pertinent amino acids are conserved between different taxonomic kingdoms. Moreover, it allows for findings that have been made regarding plant AOX function to be highly transferrable to other organisms. For example, one of the functions of AOX in plants is to help plants deal with various kinds of environmental stress. This allows for a similar hypothesis to be formulated regarding the function of AOX in animal

models, such as *T. californicus*. This hypothesis led to the finding that AOX protein expression in *T. californicus* is influenced by exposure to temperature stress.

Another isolation technique, which was optimized for this research project, was for the purpose of extracting proteins from *T. californicus*. This protein isolation process was time effective and only required ~0.05 g of copepods. This technique can also be applied to numerous other zooplankton and small organisms to allow for protein levels to be quantified and analyzed through SDS-PAGE and Western Blots.

By understanding a native AOX's function in an animal model that naturally produces it and why they possess it, we can better understand why some other organisms, including humans, do not express or have lost the AOX gene. The results gathered can be applied to generate possible reasons why AOX has been lost over the course of evolution in some animal species. AOX may have not been conserved in animals because it is seen as an energetically wasteful pathway, as a result of the lower levels of ATP that is biosynthesized. Therefore, in large, multicellular animal species, AOX may be seen as a less efficient pathway for keeping up with the large energy demand required by the active animal.

This can lead to a longer-term goal of making AOX into a potential gene therapy for mitochondrial or electron transport chain disorders in humans. An example of such a disorder is Leigh's Syndrome, which is characterized by movement disorders such as dystonia, which are caused by inability to perform oxidative phosphorylation (Ciafaloni *et al*., 1993). It is genetic in nature, with NADH dehydrogenase and cytochrome c oxidase (complexes I and IV) mutating to become non-functional (Ciafaloni *et al*., 1993). The AOX would act as alternative pathway for cells to reduce oxygen and prevent ETS over-reduction when the COX pathway is impaired or inhibited. In order to achieve the goal of a gene therapy in humans, many more questions about

animal AOXs must be answered. There has been research conducted by Hakkaart and colleagues who have successfully expressed the cyanide-insensitive AOX from *Ciona intestinalis* in cultured human cells. In this study, researchers used an AOX from an animal in the phylum Chordata which was closely related to humans based on the evolutionary tree. Acknowledging the relatedness between the animal species provided a potential route of expression to rescue electron flow and mitigate the deleterious complications involved with respiratory chain dysfunctions (i.e. Parkinson's, Huntington's disease) (Hakkaart *et al*., 2005; Kemppainen *et al*., 2013). The researchers conducted the experiment by obtaining human embryonic kidney cells and transfecting them with a doxycyclin-inducible mammalian vector (Hakkaart *et al*., 2005). The cells that survived the transfections and treatments with antibiotics were then induced to express AOX (Hakkaart *et al*., 2005). The results surprisingly showed the presence of AOX activity within the human cells, regardless of the fact that *C. intestinalis*, is a cold seawater organism (Hakkaart *et al*., 2005). Therefore, the above is just one of the many experiments to come with respect to applying knowledge gathered from AOX to treat possible mitochondrial dysfunctions in humans. Additionally, our bioinformatics results have demonstrated that AOX is present in *T. californicus* and it may serve as a potential target for drug therapy as an alternative treatment method. Therefore, further investigation of AOX regulation and/or inhibitors in animal models is required.

Another long-term application of this research is developing a drug that targets the AOX protein in order to kill copepods that are fish parasites of economically valuable species. Parasitic copepods are common on cultured and wild marine fish and affect the growth, fecundity, and survival of their hosts (Johnson *et al*., 2004). These parasitic copepods feed on host tissues, blood, and mucous (Johnson *et al*., 2004). Their attachment to fishes is responsible

for any primary disease that it may develop and usually ends in death of the host through either direct contact or mortality due to secondary infections (Lin *et al*., 1994). In marine salmon aquaculture, copepods infect the fish, leading to serious diseases and high mortality rates if untreated (Pike and Wadsworth 1999). Presently, outbreaks of diseases caused by sea lice, such as copepods, are rarely reported, although the rates of infection due to sea lice continue to be immense problem as evidenced by the frequent requirement for treatments (Johnson *et al*., 2004). This has led to the reduction in the occurrence of a multitude of the sea lice diseases, and a decrease in economic losses due to fish mortality. Nevertheless, sea lice still pose a significant economic influence due to the reduced growth performance from sea lice and the chemical treatments used to eradicate them from fish aquaculture (Sinnott 1999, Rae 2002). Fish do not possess AOX, but the sea lice that latch on to some of them do. More thorough investigation of AOX in copepods may aid in the development of a drug that can be added to fish aquaculture to exterminate parasitic copepods and prevent the loss of economically valuable fish.

1.3 Integrative Biology

Integrative biology, a fundamentally integrative science, is an essential and effective approach to resolving many of the complex issues researchers face. Taking an integrative approach has allowed me to generate new hypotheses and new questions regarding my organism and protein of interest. For this research project, we took a multipronged approach to understand the sequence and possible function of AOX in the copepod *T. californicus*, a model organism containing a native AOX gene.

The first part of this research project was to analyze the primary sequence of the AOX protein in *T. californicus* by comparing it to AOX protein sequences from other organisms. This step, of my heavily molecular biology focused project, required me to incorporate

bioinformatics. Data-mining exercises exposed me to the common databases and tools that take advantage of the vast repository of biochemical information and elevated my research study. By including a bioinformatics piece in my research, it provided me the tools to allow for me to find and correlate data from different phyla, and enabled connectivity between different types of information (DNA and protein structures). This analysis revealed that AOX protein characteristics are shared not only amongst all animals investigated, but also plants.

The use of bioinformatics also permitted me to design primers to isolate genes that have never been sequenced in *T. californicus*. The use of databases enabled me to develop primers for housekeeping genes and HSPs. This brings researchers one step closer to being able to conduct real-time PCR in the copepod *T. californicus* and understanding the physiological function of a native AOX in an animal model.

The second part of this research project determined that the AOX protein is expressed in the copepod *T. californicus* and that AOX protein levels change in response to temperature but are constitutively expressed during light stress. The cellular stress response is evolutionarily conserved in all living organisms, and a major role is attributed to the induced heat shock proteins (HSPs) and other molecules that confer stress protection (Grover, 2002). Molecular responses elicited by cells dictate whether an organism will adapt, survive, or, if they are injured beyond repair and will undergo death. By understanding the physiological function of AOX in copepods, we can understand why other organisms, such as humans, do not express or have lost the AOX gene. Furthermore, by incorporating our bioinformatics and stress biology results, they may serve as a starting point for developing a potential target for drug therapy as an alternative treatment method to mitochondrial diseases. The study of animal AOX may ultimately lead to the treatment of mitochondrial dysfunction, and disorders in humans using AOX gene therapy.

Therefore, further incorporation of different sectors of biology are needed to investigate the regulation and/or inhibitors of in animal models.

Taking an integrative biology attitude and approach has led to an innovative, progressive, and enlightened research project. Pivotal to any research project is the exchange of ideas between experts in different fields of biology and other sciences. These exchanges, within the lab and across a multitude of institutions, promotes communication and collaboration, resulting in a wider knowledge of techniques, ideas, and literature. The advantage of taking an integrative approach is that it has advanced not only my central discipline but also other biological fields; it has allowed me to generate new hypotheses, techniques, and ideas; and establish myself in an environment that promotes the interaction that facilitates new syntheses and findings.

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