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Identification of the alternative oxidase gene and its expression in the copepod Tigriopus californicus

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Title: Identification of the alternative oxidase gene and its expression in the copepod *Tigriopus californicus*

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Abstract

In addition to the typical electron transport system (ETS) in animal mitochondria responsible for oxidative phosphorylation, in some species there exists an alternative oxidase (AOX) pathway capable of catalyzing the oxidation of ubiquinol and the reduction of oxygen to water. The discovery of AOX in animals is recent and further investigations into its expression, regulation, and physiological role have been hampered by the lack of a tractable experimental model organism. Our recent DNA database searches using bioinformatics revealed an AOX sequence in several marine copepods including *Tigriopus californicus*. This species lives in tidepools along the west coast of North America and is subject to a wide variety of daily environmental stresses. Here we verify the presence of the AOX gene in *T. californicus* and the expression of AOX mRNA and AOX protein in various life stages of the animal. We demonstrate that levels of the AOX protein increase in *T. californicus* in response to cold and heat stress compared to normal rearing temperature. 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.

Key Words: arthropod, electron transport system, environmental stress, mitochondria, protein isolation, respiration, tide-pool

Introduction

The Alternative Oxidase (AOX) Pathway

Cellular respiration in animal cells takes place in the mitochondria via the electron transport system (ETS). The ETS is comprised of multi-subunit complexes and mobile electron carriers in the inner membrane of the mitochondria (Genova, 2014). Four complexes, (I, II, III, and IV), and the mobile carriers ubiquinone/ubiquinol and cytochrome c, shuttle electrons through the system to the final electron acceptor oxygen (Genova, 2014). At the same time, several of these complexes move protons from the matrix to the intermitochondrial membrane space and result in a proton motive force (PMF) across the inner mitochondrial membrane (Genova, 2014). This PMF is used by complex V (ATP synthase), and as protons flow back into the matrix through this enzyme, ATP is synthesized through the process of oxidative phosphorylation (Genova, 2014).

Many animals also contain additional enzymes capable of putting electrons into and/or removing electrons from the ETS (McDonald and Gospodaryov, 2018). One such enzyme is the alternative oxidase (AOX), a terminal quinol oxidase that catalyzes the oxidation of ubiquinol and the reduction of oxygen to water (McDonald and Vanlerberghe, 2004; McDonald, 2008). In contrast to cytochrome c oxidase, which is comprised of nuclear and mitochondria encoded subunits, the AOX is encoded by a single nuclear gene (McDonald, 2008). The AOX protein is composed of four helices and several conserved amino acid residues that are important for its enzymatic function (McDonald, 2008). Use of the AOX pathway causes electrons to bypass complexes III

and IV and results in cyanide-resistant respiration (Rogov et al., 2014). As AOX is non-proton pumping (in contrast to complexes I, III, and IV), it does not contribute directly to the proton motive force (PMF), and the energy associated with electron transport is dissipated as heat (McDonald, 2008). Therefore, AOX is viewed as energetically inefficient.

AOX in Animals

AOX has been identified in several animal species due to the presence of AOX DNA or mRNA sequences in public molecular databases (McDonald and Vanlerberghe, 2004; McDonald et al., 2009). Recent database searches have revealed the presence of AOX DNA or mRNA in the phyla Ctenophora, Platyhelminthes, Arthropoda, Tardigrada, Scalidophora, Brachiopoda, and Rotifera for the first time (McDonald and Gospodaryov, 2018). Experimental evidence for the expression of AOX mRNA exists for the sponge *Ephydatia muelleri*, and the molluscs *Anadara ovalis*, *Crassostrea gigas*, *Crassostrea virginica*, and *Mercenaria mercenaria* (McDonald et al., 2009; Liu and Guo, 2017). Thus far, the only investigation of changes in AOX expression due to environmental conditions has been the use of quantitative PCR (qPCR) to investigate AOX transcript levels in the bivalves *Crassostrea gigas* and *Diplodon chilensis* which increased in response to hypoxia and anoxia (Sussarellu et al., 2012; Yusseppone et al., 2018).

The bulk of information about the AOX of animals exists at the level of genes or transcripts. The only reports of naturally occurring AOX proteins present in animals are from the brine shrimp *Artemia franciscana* and the Pacific oyster *Crassostrea gigas*;

AOX proteins in these species are ~35 kDa in size (Rodriguez-Armenta et al., 2018). Knowledge of other animal AOX proteins is limited to studies that have taken advantage of heterologous expression systems, therefore it is difficult to determine whether the molecular masses of AOX reported reflect reality *in vivo*. For example, the expression of the AOX from the sea squirt *Ciona intestinalis* produced a functional enzyme capable of oxygen consumption of 42 kDa that was targeted to mitochondria in human kidney cells, mice, and fruit flies (Hakkaart et al., 2006; El-Khoury et al., 2013; Kemppainen et al., 2014).). Heterologous expression of the Pacific oyster AOX has also occurred in the yeast *Saccharomyces cerevisiae* (Robertson et al., 2016). It is clear that there is a need for an animal model that naturally expresses the AOX protein. The importance of finding a suitable organism for the study of animal AOX is important as typically used model systems (i.e. mouse and fruit fly) do not contain a naturally occurring AOX and therefore cannot be studied (McDonald et al., 2009). No information exists regarding the post-translational regulation of an animal AOX. In contrast to Complexes I-IV of the ETS, there is no evidence that AOX exists as part of any respiratory supercomplex (Schertl and Braun, 2014). The activation and inhibition kinetics of animal AOX proteins is also unknown. Information on the characteristics of animal AOX proteins is needed, both due to the desire for basic biological knowledge, and the realization that this knowledge is required to assess the efficacy and safety of proposals to use AOX as a gene therapy tool in humans for the treatment of mitochondrial diseases (El-Khoury et al., 2014). This gap in our knowledge exists because to date animals known to contain AOX gene sequences are often expensive to grow and culture in the lab, take up large amounts of laboratory space, and do not have rapid life cycles which makes them impractical and

challenging for scientific study. In addition, research efforts have been hampered by the existence of few genetic, molecular biology, and biochemical protocols and tools that are available for use in these animals. It is clear that a worthy endeavor is to find a useful AOX containing animal that is inexpensive to culture, has a rapid generation time, takes up little space, and for which protocols and tool development is possible. In this paper we assert that the intertidal copepod *Tigriopus californicus* meets all of these criteria and can be used as a model system for the investigation of an animal AOX protein.

Tigriopus californicus

T. californicus is an intertidal species of copepod found on the Pacific coast of North America that inhabits rock pools (Burton and Lee, 1994). Due to *T. californicus'* intertidal habitat, these animals are exposed to ever changing environmental stressors including temperature, salinity, and oxygen levels (Burton and Lee, 1994) and presumably have mechanisms in place to deal with such challenges. *T. californicus* has a short generation time (~2-4 weeks depending on rearing temperature)(Hong and Shurin, 2015), small space needs, and genetically divergent populations that can be cross-bred in the laboratory (Burton & Feldman, 1981). A strong argument for using *T. californicus* as a model organism in ecotoxicology and environmental genomics has been made based on the above characteristics (Raisuddin *et al*., 2007). In addition, a protocol and tool exists for the suppression of gene transcription using RNA interference in this species (Barreto et al., 2015b).

T. californicus has been used to investigate the mechanisms of speciation and incompatibilities in hybrid animals derived from mating different geographical populations (Barreto *et al*., 2015a). Hybrid incompatibilities have been demonstrated to have a negative impact on oxidative phosphorylation and the interaction between the nuclear and mitochondrial genome encoded processes (Barreto *et al*., 2015a). This incompatible hybridization causes mitochondrial dysfunction caused including inefficient OXPHOS, a decrease in ATP biosynthesis, and increased ROS production (Barreto *et al*., 2015a). An investigation into the bioenergetics of *T. californicus* is therefore warranted at the level of the ETS by examining complexes I-IV and AOX.

In this study we confirm the presence of the AOX gene, the expression of AOX mRNA, and demonstrate that the AOX protein is present in the copepod *T. californicus*. We believe this is the first step in establishing this copepod as a model species for animal AOX research. By demonstrating that *T. californicus* is an effective model species with experimental tools that are available, future research can examine the physiological role of AOX in *T. californicus* and will explore questions about the expression and regulation of AOX proteins in an animal system.

Materials and Methods

In silico identification of copepod AOX sequences

Molecular database searches using various animal AOX sequences in BLAST (Basic Local Alignment Search Tool) using default settings at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) (this was an iterative process that used novel AOX sequences as they were discovered; full procedure as

described in McDonald et al., 2009) identified several putative copepod AOX sequences. DNA sequences were translated into protein sequences using the ExPASy Translate Tool (http://web.expasy.org/translate/).

Laboratory Culturing Conditions of *T. californicus*

Animals were obtained from J&L Aquatics Canada, Vancouver, British Columbia. The animals arrived in a ~150mL bottle containing ~1000-2000 individuals at various life stages. Animals used for DNA and RNA isolation were maintained in cultures of 20-200 individuals in 600 mL beakers containing 400mL of seawater (37.5 g/L) with a specific gravity of 0.026 and grown at 21°C (room temperature) (Edmands and Deimler, 2004). The salt water was made with Instant Ocean Salt and the specific gravity was tested with the Instant Ocean Saltwater Aquarium Hydrometer (Instant Ocean, Blacksburg, VA, USA). Each beaker was covered with the top plate of a petri dish to slow the rate of evaporation. Beakers were placed in an incubator programmed to run on a 12 hour light and 12 hour dark cycle. The animals were fed 4 drops of Reef Nutrition Phyto-Feast Live Premium Phytoplankton (J&L Aquatics Canada, Vancouver, British Columbia) every other day for the entire culturing period. Preliminary experiments demonstrated that our animals completed their life cycle faster at 15.0°C, so a change in rearing protocol occurred about 2 years into culturing *T. californicus* in the lab. Animals used for protein isolations were cultured in 400mL beakers filled with 250mL of salt water (41.7 g of Instant Ocean salt per 1L of MilliQ filtered water) at 15°C using a 12 hour light and 12 hour dark cycle. The copepods were fed once a week with 0.01 g of Tetramin fish food (Nutrafin Basix) and 0.005 g of *Spirulina* powder (EarthRise) in 50mL of salt water added to each habitat. The water in each habitat was changed weekly in order to

prevent the build-up of moulted exosketetons and waste in the beakers. Animals used in temperature experiments were acclimated to 15°C (normal growth temperature) for 2-4 weeks before being exposed to 6°C (cold) or 28°C (hot) for 24 hours (acute treatment) or 1 week (chronic treatment) before collection for protein isolation.

DNA and RNA isolation and AOX Gene and Transcript analysis

Primer Design

Gene specific primers for the *T. californicus* 551 bp AOX cDNA fragment (JW502496) identified in the NCBI database were designed using the Primer3 program (http://frodo.wi.mit.edu/primer3). Custom primers were then synthesized (Invitrogen Life Technologies, Carlsbad, CA, USA) for use in PCR and RT-PCR protocols (Table 1). Three different forward and reverse primer sets were created in order to generate cDNA products ranging from 200- 500bp; multiple primer sets were designed in case one or more sets proved unsuccessful in producing a product.

Collection and preparation of copepods

In order to collect the copepods from the cultures for the isolation of DNA and RNA the animals were strained out from the water using Miracloth (pore size: 22-25µm) (Merck KGaA, Darmstadt, Germany). Once the water was completely drained from the beaker, the copepods were washed several times with clean salt water and quickly transferred to a pre-weighed Eppendorf tube, the mass of the animals was determined (~200 mg), and the tube was frozen in liquid nitrogen. The frozen copepods were then removed from the Eppendorf tube and ground into a fine powder using a previously autoclaved and chilled pestle and mortar. The powdered sample was kept cold by grinding under liquid nitrogen.

Isolation of nucleic acids and amplification parameters

For the extraction of DNA and RNA from *T. californicus* Trizol reagent proved most effective (Invitrogen Life Technologies, Carlsbad, CA, USA) and the protocol was conducted as per the manufacturer's instructions. Once the nucleic acids were successfully isolated from *T. californicus* and quantified using spectrophotometry, PCR and reverse transcriptase (RT)-PCR were conducted in order to amplify the targeted AOX sequence. PCR was conducted with 5 µg of *T. californicus* DNA using 0.5 µL iTaq DNA polymerase (Bio-Rad Laboratories Ltd., Mississauga, ON, CA), 2 µL of each 10 µM forward and reverse primer, 2 µL of 10x PCR buffer, 2 µL of dNTP mix, and 1.5 µL of nuclease-free water in 20 µL total volume. The thermal cycler was set for 1 cycle of 2 minutes at 94°C for initial denaturation, 40 cycles consisting of: denaturation for 20 seconds at 94°C, annealing for 10 seconds at 58°C, and extension for 30 seconds at 68°C, followed by 1 cycle of final extension for 10 minutes at 68°C and the final soak cycle at 4°C held indefinitely, until the sample was removed from the thermocycler (Bio-Rad Laboratories Ltd., Mississauga, ON, CA).

RT-PCR was conducted using the Access RT-PCR Introductory System (Promega Corporation, Madison, WI, USA) with 2 µg of total DNase treated RNA in 50 µL reactions containing 10 µL AMV/Tfl 5x reaction buffer, 1 µL dNTP mix, 2 µL of each 10 µM forward and reverse primer, 2 µL of 25 mM $MgSO_4$, 1 µL AMV RTase, 1 µL Tfl DNA polymerase, and 29.5 µL nuclease-free water. The thermal cycler was set for first strand cDNA synthesis consisting of 45 minutes at 45°C for reverse transcription and 2 minutes at 94°C for AMV RT inactivation. This was followed by 40 cycles of denaturation for 30 seconds at 94°C, annealing for 1 minute at 60°C, and extension for

2 minutes at 68°C, followed by 1 cycle of final extension for 7 minutes at 68°C and the final soak cycle held at 4°C, until the sample was removed from the thermocycler (Bio-Rad Laboratories Ltd., Mississauga, ON, CA).

The amplification products were separated using DNA gel electrophoresis using a 1.2% agarose gel containing 1mL 50x Tris-acetate-EDTA (TAE) buffer, 49 mL nuclease-free water (dH_2O), and 0.60 g agarose powder run in a tank with 1x TAE running buffer. 5 µL 1 kb or 100 bp DNA ladder (GeneDirex, USA) was loaded in the first lane of the gel, followed by 5-10 µL of each PCR or RT-PCR reaction combined with 6X DNA loading buffer in subsequent lanes. Gels were run at 80-120V for 45 minutes and stained with ethidium bromide in order to visualize the DNA under UV light (VersaDoc 400) (Bio-Rad Laboratories Ltd., Mississauga, ON, CA).

Isolation of Proteins from Copepods

For each protein extraction, approximately 0.05-0.10 g of copepods were isolated from their habitats by filtration using a 100 µm filter (Fisherbrand) and placed in an Eppendorf tube. Various combinations of different volumes of SDS-PAGE sample buffer (350-500 µL) and 20 µL of β-mercaptoethanol solution were pipetted into each Eppendorf tube. Each sample was sonicated (Omni International Inc.) for 10 seconds at a frequency of 6 kHz and/ or homogenized to extract the proteins from the copepods. The Precellys 24 lysis and homogenization machine (Bertin Technologies) was used to perform the homogenization of copepod tissue. The homogenization processes consisted of adding beads into each sample, loading the samples into the machine, and 3D bead-beating for 30 seconds at 5000rpm. These various combinations were used in

243 order to identify the optimal conditions for protein isolation from our laboratory grown
244 copepods.

245 Protein Analysis: Gel Electrophoresis

246 Protein samples were analyzed using reducing SDS-PAGE and Western blotting.
247 For gel electrophoresis, a 15 well mini-PROTEAN TGX Stain-Free gel (Bio-rad), was
248 loaded with 15 µL of each copepod sample. In order to determine the molecular weight
249 of the proteins in our samples, two wells were loaded with 5 µL of the Precision Plus
250 Protein WesternC Standards (Bio-rad). A yeast sample expressing the Pacific oyster
251 alternative oxidase (Robertson et al., 2006) served as a positive control in order to verify
252 the cross-reactivity of the AOX antibody with our copepod samples. A negative control
253 blot was run using proteins isolated from the algal food source to rule out the possibility
254 of an algal AOX being detected in our copepod samples. Following the loading of the
255 wells the gel was run at a constant voltage (200V) for 35 minutes. After the run was
256 complete, the gel was visualized using a VersaDoc (Bio-rad, USA) so an image could
257 be taken to visualize protein loading. For protein gels using our temperature experiment
258 samples, proteins in each sample were quantified using Quick Start Bradford Protein
259 Assay using bovine serum albumin standards and a spectrophotometre. Each sample
260 consisted of 20 µg of protein added to each lane.

261 Protein Analysis: Western Blot

262 The Trans-Blot Turbo Transfer System (Bio-rad) was used to transfer the
263 proteins onto a nitrocellulose membrane in 3 minutes using Trans-Blot Transfer Medium
264 (Bio-rad). Following protein transfer, the membrane was placed in 5% dry milk in 10 mL

of 1X TBS-T on a shaking platform for 1 hour. The membrane was then subsequently washed three times in TBS-T. 4µL of the primary AOX antibody (Plant AOX1/2, Cat. # ABIN3197483, Agrisera Antibodies) was then added to 20 mL of 1X TBS-T for 1 hour (dilution 1: 10,000). Following this, the membrane was again washed three times (15 minutes, 10 minutes, and 10 minutes) with TBS-T. The membrane was then incubated in 2 µL secondary antibody, a goat anti rabbit IgG (Cat. # ABIN101988, Agrisera Antibodies, dilution 1:25,000), and 2 µL of the Precision Protein StrepTactin HRP Conjugate (Bio-rad) in 20mL of 1x TBS-T for 1 hour followed by three washes in 1X TBS-T. It was 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 placed in the VersaDoc, and the chemiluminescent signal was detected and imaged.

Results

Identification of putative AOX sequences in copepods

A molecular database search using a BLAST (Basic Local Alignment Search Tool) at the National Center for Biotechnology Information (NCBI) revealed the presence of putative AOX sequences in 14 different copepod species (Table 2). The copepod species are representatives of 3 different copepod orders and 12 different families (Table 2). The predicted AOX proteins all contained at least 2 or more of the 4 iron-binding motifs (Table 2) containing conserved glutamate and histidine residues required for the enzyme to be functional (McDonald et al., 2009). AOX DNA and mRNA

286 in copepods are present in multiple developmental stages, both sexes, and various
287 habitats around the world (Table 2).

288 *In silico* analyses of the *Tigriopus californicus* AOX sequence

289 A molecular database search revealed a putative *T. californicus* AOX DNA
290 sequence of 551 bp (Figure A.1). The predicted protein from *T. californicus* was a
291 partial sequence, however 3 out of the 4 iron binding motifs containing conserved
292 glutamate and histidine residues required for the enzyme to be functional (McDonald et
293 al., 2009) were present (Figure A.1). From previous studies, the C-terminal motif N-P-
294 [YF]-X-P-G-[KQE], was determined to be diagnostic for animal AOX proteins (McDonald
295 et al., 2009). *T. californicus* demonstrates a high level of motif conservation in the region
296 of the protein mentioned above (i.e. N- P-F-E-K-G-K) (Figure A.1).

297 Molecular analyses of the *T. californicus* AOX

298 Trizol reagent proved to be effective in isolating RNA from the copepods and
299 allowed us to proceed with RT-PCR. All three AOX primer sets yielded cDNA fragments
300 of the expected sizes (Figure 1). Proteins were successfully isolated from *T. californicus*
301 using 0.05 g of copepods and 350 or 500 µL of SDS-PAGE sample buffer containing β-
302 mercaptoethanol and using various combinations of sonication and homogenization
303 (Figure A.2). The *T. californicus* AOX was recognized by a plant AOX antibody and was
304 ~ 50 kDa in size (Figure 2). The antibody failed to cross-react with the AOX protein
305 likely present in the algae provided to the copepods as food (Figure A.3). Previous
306 studies have demonstrated that algal AOXs contain features that differ from those of
307 other organisms (e.g. extended N-terminus and other insertion/deletions) (Nemanis et

al., 2013). We therefore expected that a plant AOX antibody would not cross-react with the algal AOX.

Response of AOX Protein Levels to Temperature Treatments

Copepods were acclimated to 15°C for 2-4 weeks and then subjected to one of three different temperatures (6, 15, or 28°C) for 24 hours or 1 week and then sampled. Protein gels demonstrated that equal protein loading had occurred (Figure A.4 and A.5). Western blots indicate that AOX protein levels are low under control conditions (24 hours at 15°C), but AOX levels are higher after 24 hours of exposure to 6°C and 28°C (Figure 3). Similarly, AOX protein levels are low under control conditions (1 week at 15°C), but AOX levels are higher after 1 week of exposure to 6°C and 28°C (Figure 4).

Discussion

Discovery of AOX in Copepods and the Phylum Arthropoda

We have shown that AOX is in the genomes of multiple species of copepods from around the world that inhabit a wide variety of ecological niches (Table 2.) The first reports of AOX from arthropods were in the brine shrimp *Artemia franciscana* and putative sequences from other members of the Chelicerata, Hexapoda, and Crustacea (Rodriguez-Armenta et al., 2018; McDonald and Gospodaryov, 2018). It was previously hypothesized that AOX was not present in arthropods due to a gene loss event (McDonald et al., 2009), however, the above data refute this hypothesis.

Based on information gathered using bioinformatics tools, the copepod *T. californicus* contains an AOX sequence (Figure A.1, Table 2). Our next goal was to

329 determine whether the AOX gene of *T. californicus* is expressed in the animal. RT-PCR
330 using RNA isolated from pooled developmental stages of the organism verified that
331 AOX mRNA is expressed in the copepod (Figure 1). This represents the first AOX
332 mRNA detected experimentally in any arthropod using gene specific primers for AOX.

333 In order to verify that the AOX sequence recovered from the arthropod *T.*
334 *californicus* was from this animal and not a contaminant (e.g. from a microbial symbiont
335 or pathogen) the animal's 551 bp cDNA sequence was translated to its predicted protein
336 sequence and was found to possess the C-terminal region N-P-F-E-K-G-K (Figure A.1).
337 This C-terminal motif is of particular interest because it is characteristic of only animal
338 AOXs and is different from the C-terminal regions observed in plants and fungal species
339 (McDonald et al., 2009).

340 We wished to confirm that AOX protein was detectable in *T. californicus*. Reducing
341 SDS-PAGE followed by Western blotting with a commercial plant AOX1/2 antibody
342 detected a protein of ~ 50 kDa in our copepod samples (Figure 2). Heterologously
343 expressed AOX proteins from *Ciona intestinalis* and *Crassostrea gigas* are 35-42 kDa in
344 size (Hakkaart et al., 2006; Robertson et al., 2016). The AOX protein of *Artemia*
345 *franciscana* is between 25 and 37 kDa on Western blots using the anti-AOX
346 *Sauromatum guttatum* monoclonal antibody (Rodriguez-Armenta et al., 2018). It is
347 expected that the food source (*Spirulina*) would contain AOX as it is an algae, however,
348 the AOX antibody did not recognize a protein in this sample (Figure A.3).

349 Finally, we wished to determine if AOX protein levels would change in response to
350 an environmental stressor. The copepods are normally reared at 15°C and have been

cultured at this temperature for several years and have likely adapted to this growth temperature over many generations in our lab. We therefore selected 15°C as our control temperature and exposed treatment groups to either 6°C (cold) or 28°C (hot) for an acute time period of 24 hours or a chronic time period of 1 week prior to sampling the animals. Animals grown at 15°C exhibited very low levels of detectable AOX protein (Figures 3 and 4). In contrast, animals grown at 6°C or 28°C exhibited higher levels of AOX protein after 24 hours of exposure (Figure 3) or 1 week of exposure (Figure 4). Similar to what we report here, an increase in AOX protein levels in response to cold stress has been reported in a variety of plants (McDonald, 2008). In addition, AOX1 protein levels sharply increase in response to heat stress in the green alga *Chlamydomonas reinhardtii* (Zalutskaya et al., 2015). Unfortunately, no other studies have examined the effects of biotic or abiotic stressors on AOX protein levels in any animal that naturally possesses the enzyme. The only other investigation of a native AOX from an animal is a Western blot confirming its presence in the brine shrimp *Artemia franciscana* (Rodriquez-Armenta et al., 2018). Our finding is therefore the first to demonstrate that the levels of a native AOX protein in an animal change in response to an environmental stressor.

The Power of *T. californicus* as an Experimental System to Examine AOX

T. californicus represents an emerging model organism for research because of its abundance, wide geographic distribution, ease of manipulation in laboratory settings, and its ecological relevance due to its position in marine food webs (Raisuddin et al., 2007). Lab grown cultures of *T. californicus* can be maintained for many generations and are relatively inexpensive to house. We were able to isolate copepod DNA and

RNA using Trizol reagent and present a protocol here for successfully isolating proteins from whole animals. Coupled with newer techniques such as RNA interference (RNAi) (Barreto et al., 2015), it is clear that many experimental tools can be used to answer questions about AOX in this organism.

Based on our results, we can begin to ask questions about the genes involved in regulatory pathways that control transcript expression levels in response to environmental changes, developmental stages, and gender. Analysis of AOX expression levels can be conducted by subjecting *T. californicus* to a variety of environmental stressors such as alterations in salinity and temperature (Lauritano et al., 2012; Zhang et al., 2013) in order to observe patterns of AOX gene expression under varying environmental conditions. The environmental stress of fluctuating salinity levels is a key stressor to *T. californicus* and has been noted to affect the expression of a variety of genes in the copepod (Burton and Lee, 1994; Van Aken et al., 2009). We have demonstrated that AOX protein levels increase in *T. californicus* in response to temperature stress, however, at this time it is not known why *T. californicus* has the gene for and produces AOX protein. It is thought that the AOX pathway provides metabolic flexibility and gives the organism the ability to survive under a multitude of environmental stressors (Vanlerberghe, 2013). In addition, this system also represents an opportunity to study the post-translational regulation of the AOX protein in *T. californicus*.

Future Direction and Applications

Knowledge about animal AOX also has applications in human and animal medicine. Comparative research may aid in the treatment of diseases caused by parasitic protists, where AOX is a current target of drug design (May et al., 2017). AOX research could lead to the development of anti-parasitic drugs that can be used to kill parasitic copepods that live on the skin of economically valuable fish species. Humans do 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 (El-Khoury et al., 2013; Fernandez-Ayala et al., 2009; Hakkaart et al., 2006; Kemppainen et al., 2014). Heterologous expression systems provide a potential route of expression to rescue electron flow and test hypotheses in order to mitigate the deleterious complications involved with respiratory chain dysfunctions (such as Parkinson's, diabetes mellitus, and deafness) (Hakkaart et al, 2006; Kemppainen et al., 2014), but this requires extensive knowledge about the regulation of AOX gene expression and the post-translational regulation of the AOX protein.

We believe that *T. californicus* is an excellent model system in which to study AOX. These animals can be used to study the physiological role of AOX and to test functional hypotheses about the enzyme. This will lead to an increase in our knowledge of AOX in general and contribute to the assessment its future applications in healthcare and aquaculture.

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418 Association (FOSSA) of Wilfrid Laurier University.

419 The authors report no conflict of interest.

420 **Figure Captions**

421 Figure 1. Reverse-transcriptase PCR products using *T. californicus* AOX gene specific
422 primers with DNase treated RNA. Lane 1, 5 µL 100bp DNA ladder; lane 2, 10 µL AOX1
423 primer set product (232bp product size); lane 3, 10 µL AOX2 primer set product (344bp
424 product size); lane 4, 10 µL AOX3 primer set product (503bp product size); lane 5, 10
425 µL positive control from kit (323bp product size).

426 Figure 2. Detection of *T. californicus* AOX protein by reducing SDS-PAGE and Western
427 blotting using a plant AOX antibody. All *T. californicus* samples per well are 14µL of
428 protein samples derived from 0.05 g of copepods of mixed developmental stages
429 subjected to either sonication (S), homogenization (H), or a combination of the two (H &
430 S). The protein sample buffer volume used (in microliters) is indicated by the number
431 below the isolation method. Ladder lanes contain 5µL of the Precision Plus Protein
432 Western C Standards. The positive control is 14 µL of a protein sample from isolated
433 mitochondria from *Saccharomyces cerevisiae* overexpressing the *Crassostrea gigas*
434 AOX.

435 Figure 3. Detection of *T. californicus* AOX protein by reducing SDS-PAGE and Western
436 blotting using a plant AOX antibody under 24 hours exposure to different temperatures.
437 All *T. californicus* samples per well are 20 µg of protein sample derived from 0.05-0.10 g
438 of copepods of mixed developmental stages. Ladder lanes contain 5µL of the Precision

439 Plus Protein Western C Standards. Temperature treatments are indicated above each
440 sample well.

441 Figure 4. Detection of *T. californicus* AOX protein by reducing SDS-PAGE and Western
442 blotting using a plant AOX antibody under 1 week exposure to different temperatures.
443 All *T. californicus* samples per well are 20 µg of protein sample derived from 0.05-0.10 g
444 of copepods of mixed developmental stages. Ladder lanes contain 5µL of the Precision
445 Plus Protein Western C Standards. Temperature treatments are indicated above each
446 sample well.

447 Figure A1. cDNA and predicted protein sequences of the AOX from *Tigriopus*
448 *californicus*. The highlighted region contains the C-terminal motif that is highly
449 conserved in animal AOX proteins.

450 Figure A2. Protein samples run on reducing SDS-PAGE. Ladder lanes contain 5µL of
451 the Precision Plus Protein Western C Standards. The positive control is 14 µL of a
452 protein sample from isolated mitochondria from *Saccharomyces cerevisiae*
453 overexpressing the *Crassostrea gigas* AOX. All *T. californicus* samples per well are
454 14µL of protein samples derived from 0.05 g of copepods of mixed developmental
455 stages subjected to either sonication (S), homogenization (H), or a combination of the
456 two (H & S). The protein sample buffer volume used (in microliters) is indicated by the
457 number below the isolation method.

458 Figure A3. Protein samples run on reducing SDS-PAGE and Western blotted using a
459 plant AOX antibody. The ladder lane contains 5µL of the Precision Plus Protein Western
460 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 14µL were loaded into the well.

Figure A4. Protein samples run on reducing SDS-PAGE. Ladder lanes contain 5µL of the Precision Plus Protein Western C Standards. All *T. californicus* samples per well are 20 µg of protein samples derived from 0.05-0.010 g of copepods of mixed developmental stages subjected to 24 hours at the temperatures indicated above the wells of the gel.

Figure A5. Protein samples run on reducing SDS-PAGE. Ladder lanes contain 5µL of the Precision Plus Protein Western C Standards. All *T. californicus* samples per well are 20 µg of protein samples derived from 0.05-0.010 g of copepods of mixed developmental stages subjected to 1 week at the temperatures indicated above the wells of the gel.

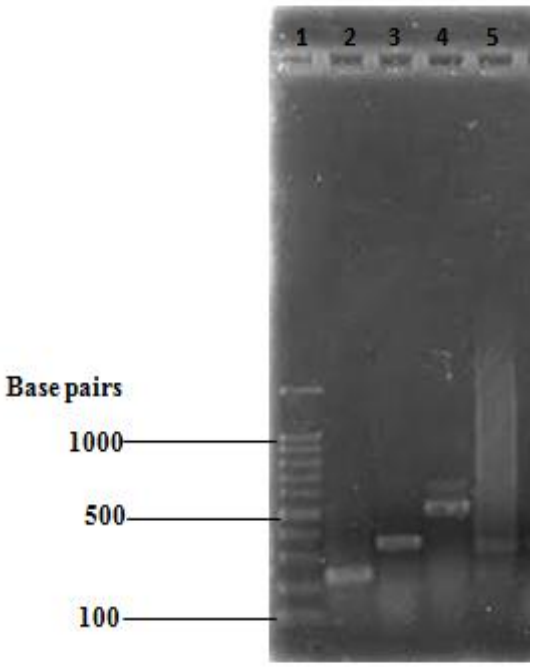
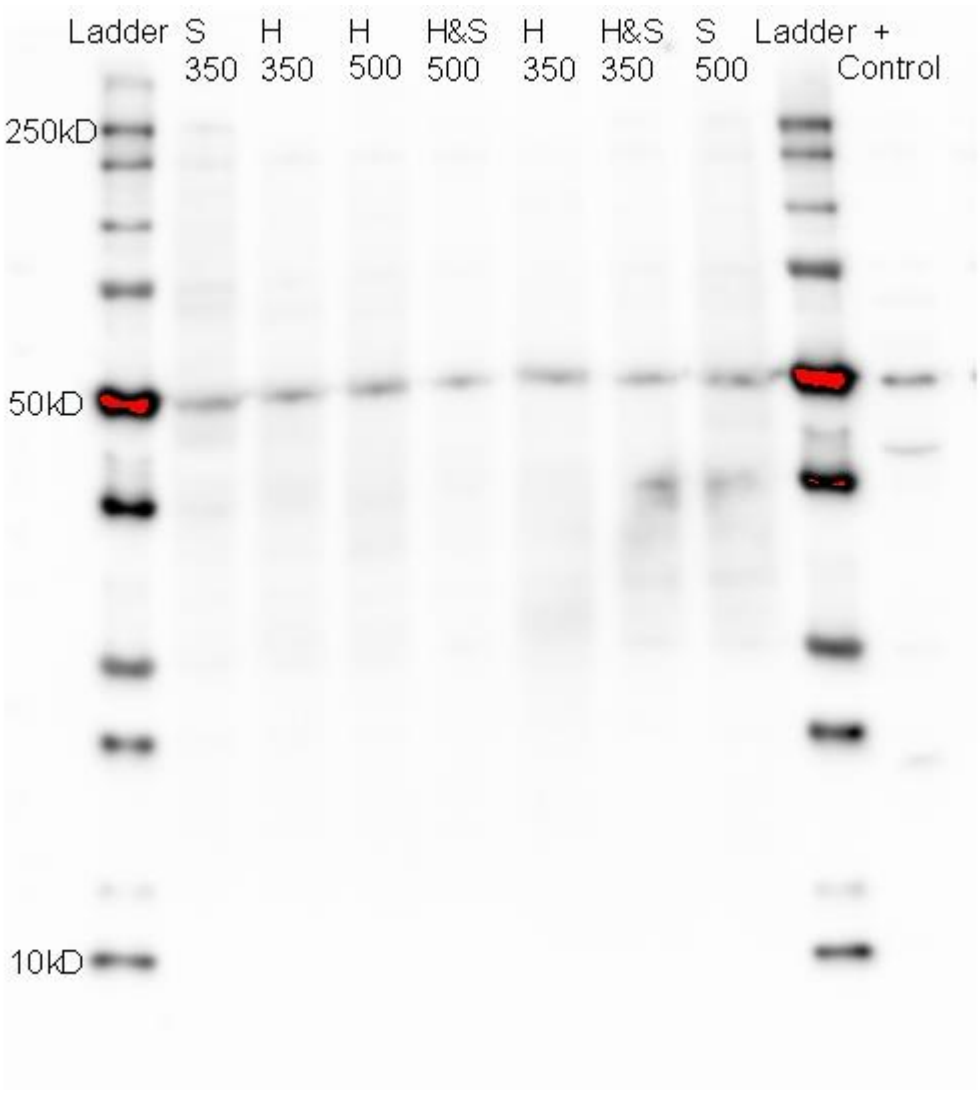


Figure 1.



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502 Figure 2.

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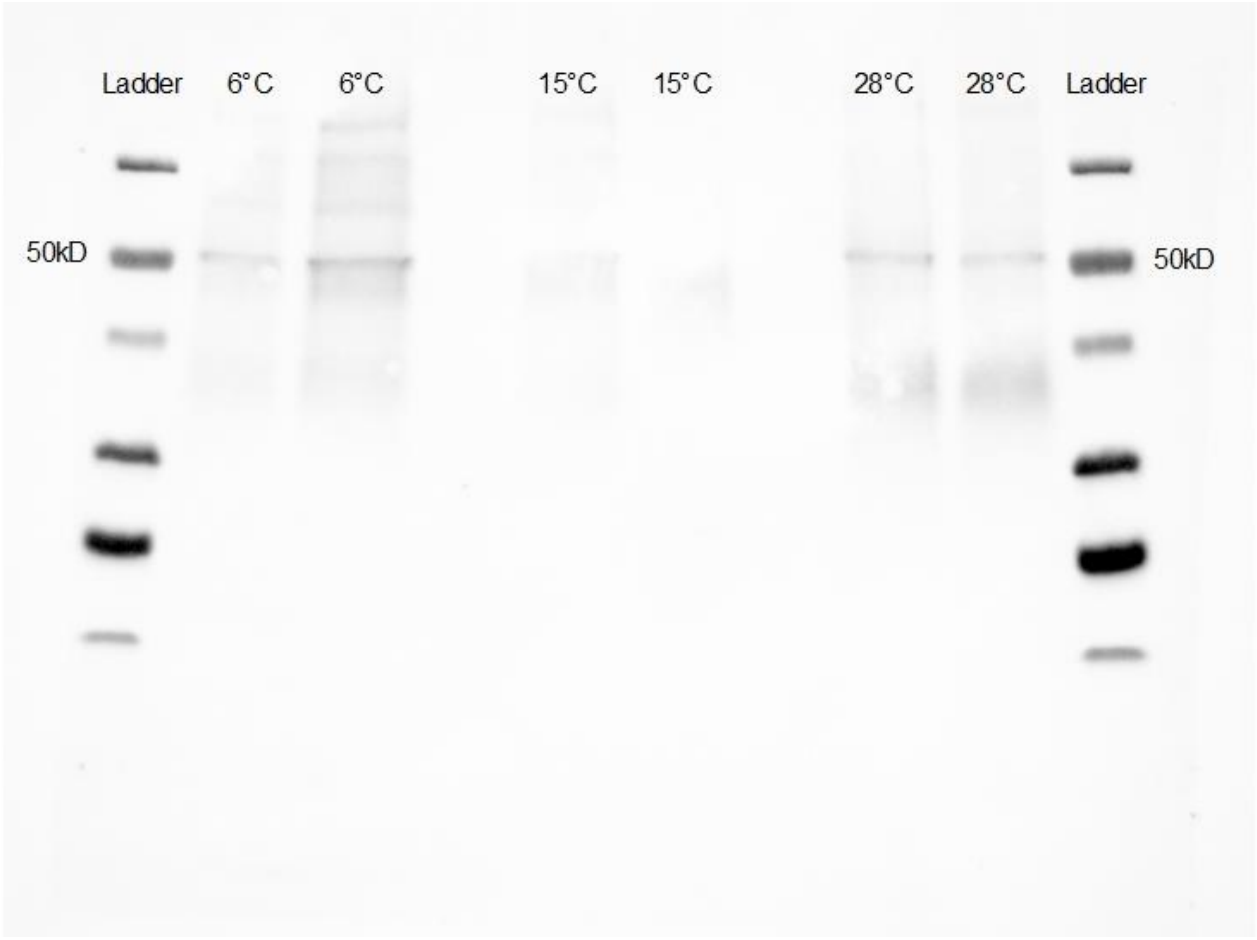


Figure 3.

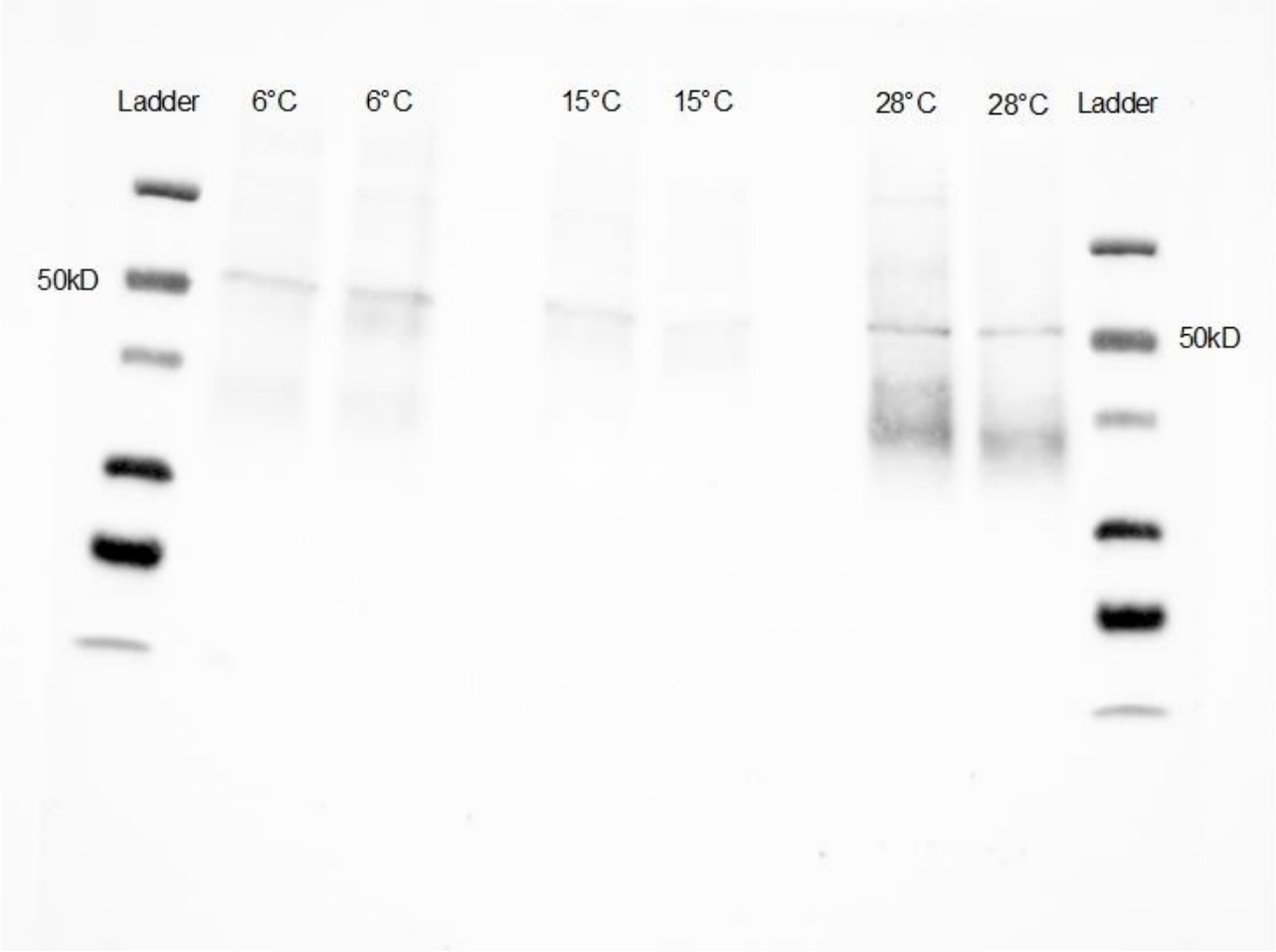


Figure 4.

Tigriopus californicus 551bp cDNA sequence

GAATGCACGTGATCATGGATGGATCCACACTCTCCTGGAAGAAGCGGAGAATGAA

AGGATGCATCTGATGACCTTCATGCGACTCCGAAGACCCGGGCCCATTTTCCGAG

GCACCGTGATCCTGACTCAATGGCTGTTCACATTTACGTTCTCATTCGCTTACATAC

TGTCGCCCAATTTTTGCCACAGATTTGTTGGGTATTTGGAAGAGCAAGCAGTGGTC

ACTTACACTCACATCCTGGAAGAAATCGACGCAGGACGATTGCCCATGTGGAAGA

CCTTGCCAGCTCCGGAATTGGCCATCAAGTATTGGAGATTGCCCGAAGACGCCAA

GATGCGGGAAGTCATTTTGGCAATCCGAGCCGATGAAGCTCATCATCGGCTTGTG

AATCACACCCTTGGATCGATGGACCTCAAATCAGACAATCCTTTTGAGAAAGGGAA

ATAACTTTTGTCTTCGGCTCAGATACAAATTTAATGGTCAATAACAAGTATTCCATG

GTAAAAACGAACGGAGGTTGAGAAATATAAATAAATTGTCGAGTCTTTTC

Tigriopus californicus predicted protein sequence

NERMHLMTFMRLRRPGPIFRGTVILTQWLFTFTFSFAYILSPNFCHRFVGYLEEQAVVT

YTHILEEIDAGRLPMWKTLPAPELAIKYWRLPEDAKMREVILAIRADEAHHRLVNHTLGS

MDLKDNPFEKGK

Figure A1.

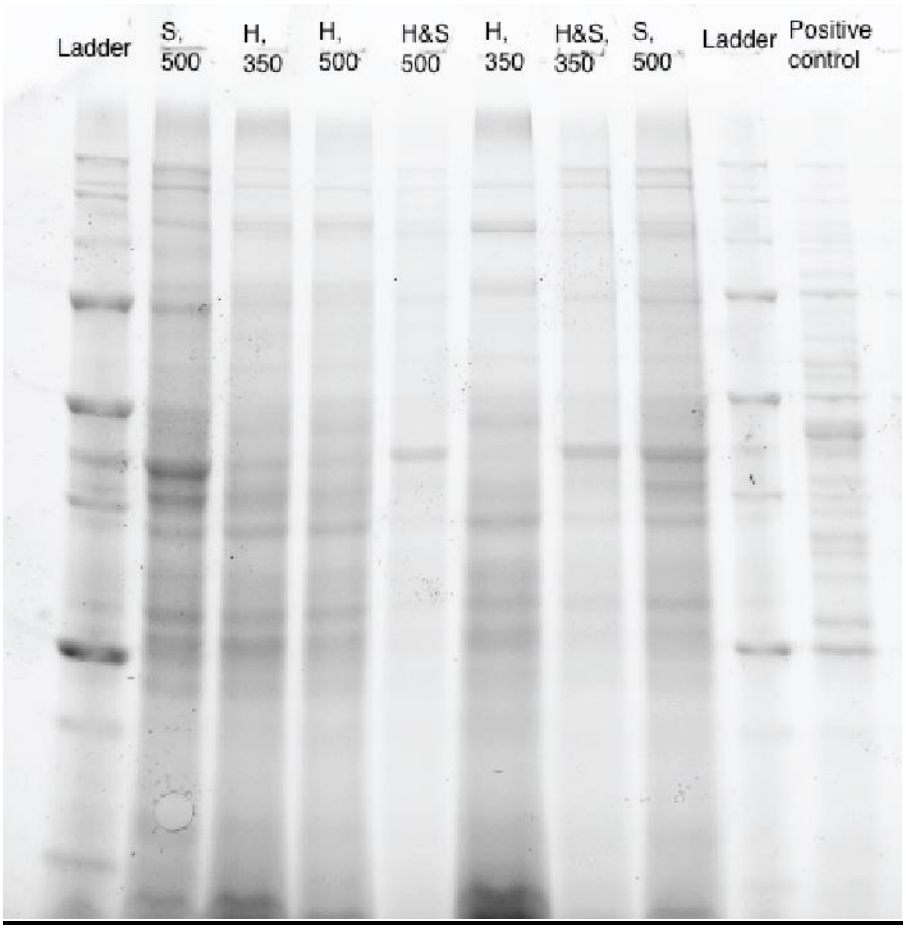

Ladder
S, 500
H, 350
H, 500
H&S 500
H, 350
H&S, 350
S, 500
Ladder
Positive control

Figure A2.

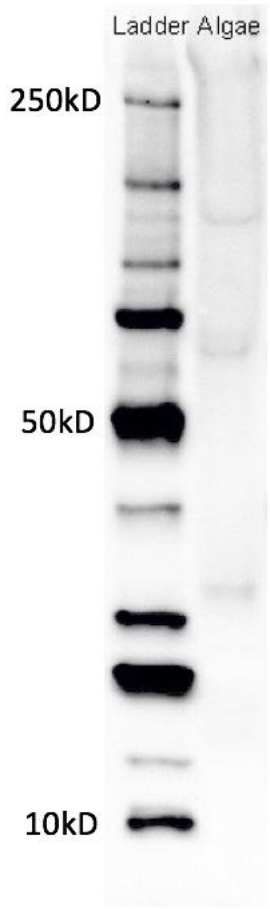
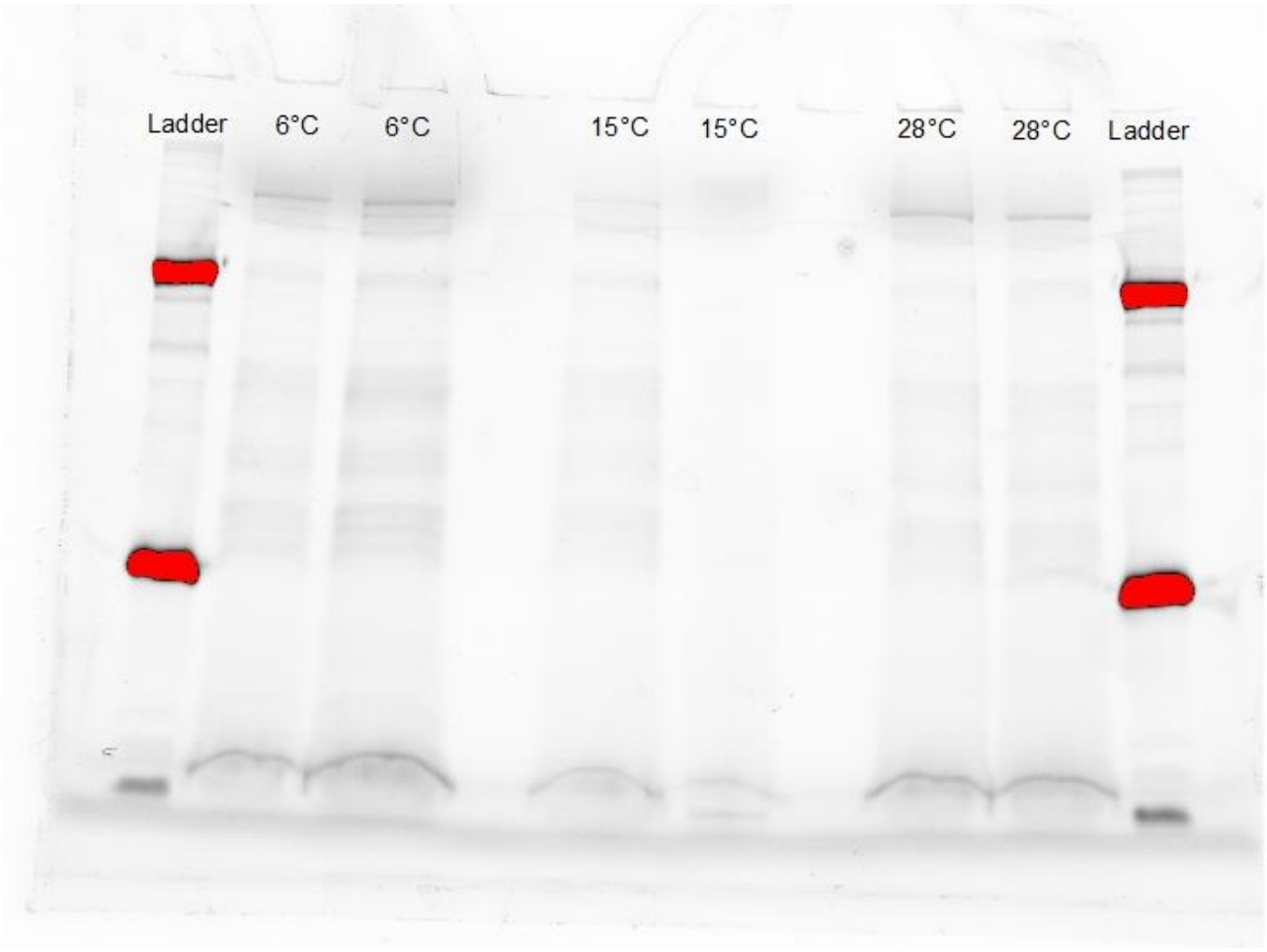


Figure A3.



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570 Figure A4.

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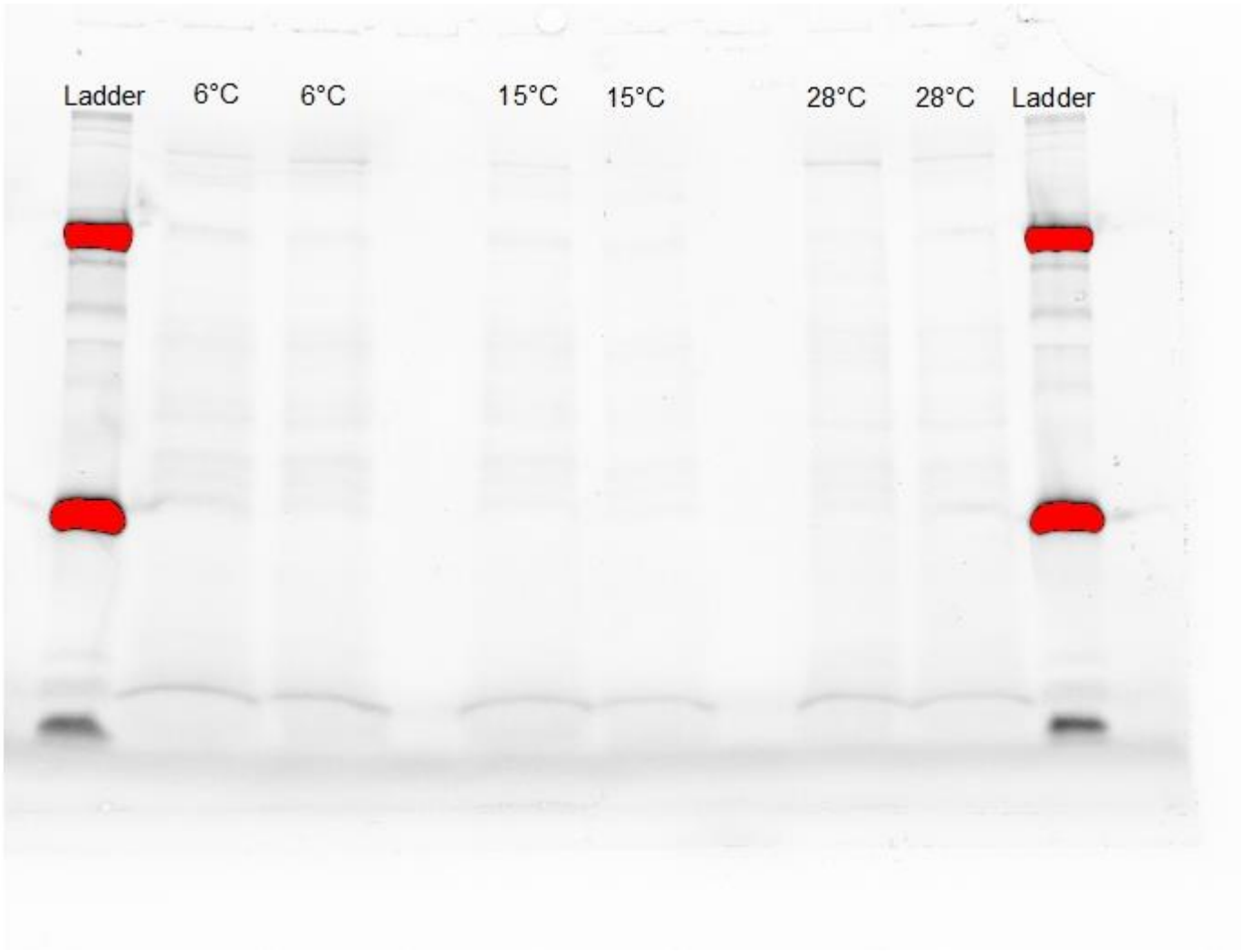

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579 Figure A5.

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Table 1. Gene-specific primers designed for *Tigriopus californicus* AOX amplification.

Primer Name	**Forward Primer Sequence (5' → 3')**	**Reverse Primer Sequence (5' → 3')**	**Expected cDNA Product Size (bp)**
AOX1	CCTGACTCAATGGCTGTTCA	CAAAATGACTTCCCGCATCT	232
AOX2	TGCCACAGATTTGTTGGGTA	TTCTCAACCTCCGTTCGTTT	344
AOX3	GGATCCACACTCTCCTGGAA	CTCAACCTCCGTTCGTTTTT	503

600 Table 2. Putative AOX sequences in copepods recovered using bioinformatics searches
601 of public databases.

Order	Family	Species	Representative Accession Number	Iron-binding sites present in translated AOX protein	Information on Source Material
Calanoida	Acartiidae	*Acartia tonsa*	HAGX01001020	All 4	All developmental stages
	Calanidae	*Calanus finmarchicus*	GAXK01135432	All 4	C5 copepodite, Trondheim, Norway
		Calanus glacialis	HACJ01021827	IBS 1, 2, & 3	Whole organism, Norway
		Neocalanus flemingeri	GRUD01065088	All 4	Adult female
	Caligidae	*Caligus rogercresseyi*	GAZX01011857	IBS 1, 2, & 3	Mixed developmental stages and sexes
	Metridinidae	*Pleuromamma xiphias*	GFCI01346245	All 4	Whole adults, both sexes, BATS, Atlantic Ocean
	Pontellidae	*Labidocera madurae*	GFWO01190644	All 4	
	Temoridae	*Eurytemora affinis*	GBGO01037152	All 4	Whole females
Cyclopoida	Cyclopettidae	*Paracyclopina nana*	GCJT01014574	All 4	Whole animals, Gangneung, South Korea
	Cyclopidae	*Eucyclops serrulatus*	GARW01011119	All 4	
	Oithonidae	*Oithona nana*	FTRT01002078	IBS 1 & 2	

Harpacticoida	Harpacticidae	*Tigriopus californicus*	JW502496	All 4	San Diego population
	Harpacticidae	*Tigriopus japonicas*	GCHA01002206	All 4	Whole animals, Gangneung, South Korea
	Harpacticidae	*Tigriopus sp. 1 SL-2012*	GDFW01016749	All 4	Tidal pool, Maxwell Bay, Antarctica

[1] Trace sequence archive database at NCBI

[2] Nucleotide database at NCBI

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