HOW DOES THE GREEN EELGRASS ISOPOD *PENTIDOTEA RESECATA*
PROTECT ITS TISSUE AGAINST HIGHLY FLUCTUATING OXYGEN
CONDITIONS?

by

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A THESIS

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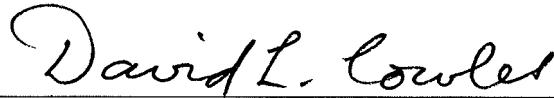WALLA WALLA UNIVERSITY

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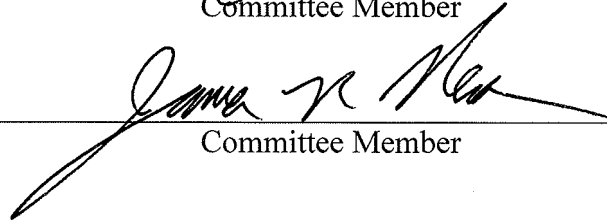
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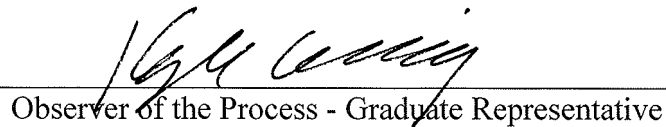
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ABSTRACT

Organisms that live in hyperoxic or in intermittent hypoxic conditions are likely to be exposed to high levels of reactive oxygen species (ROS). ROS can result in oxidative stress, causing damage to proteins, lipids, and DNA. The green eelgrass isopod *Pentidotea resecata* lives among *Zostera marina* eelgrass beds. Because *Z. marina* lives in shallow water and produces oxygen during photosynthesis, *P. resecata* lives in a hyperoxic and highly fluctuating oxygen environment, with a range from below 20% to over 200% air saturation. They likely protect themselves against potential damaging ROS with antioxidants. The enzymatic antioxidant capacity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in the tissue of *Pentidotea resecata* was investigated. The activities of these enzymes were compared to that of *Ligia pallasii* tissues, a marine isopod that lives terrestrially, occasionally being exposed to the water, and therefore probably only encounters normoxic conditions. Because *P. resecata* experiences hyperoxic and highly fluctuating oxygen conditions and therefore potentially higher levels of ROS, higher enzymatic antioxidant levels were expected in *P. resecata* compared to *L. pallasii*. Instead, the activity of SOD, CAT, and GPx in *P. resecata* tissues was significantly lower than that of *L. pallasii* tissues. Perhaps *P. resecata* have other preventive mechanisms and physical defenses to protect against ROS from highly fluctuating oxygen conditions.

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INTRODUCTION

Reactive oxygen species

Organisms that live in environments with high oxygen levels are prone to oxidative stress and oxygen toxicity because of the formation of reactive oxygen species. Reactive oxygen species (ROS) are unstable molecules which are a result of only partial reduction of oxygen and include free radicals such as superoxide radical anion (O_2^-), singlet oxygen ($^1O_2$), hydroxyl radical (HO·), and hydrogen peroxide (H_2O_2), which forms a radical after reacting. Free radicals are atoms or molecules with an unpaired electron and can damage cells by altering proteins, lipids, and DNA (Lobo et al. 2010).

ROS damage proteins by causing site-specific amino acid modifications, fragmentation of the peptide chain, and protein carbonylation (Lesser 2006). ROS can also cause lipid peroxidation which causes membrane damage and alters membrane fluidity. Products of lipid peroxidation include malondialdehyde (MDA), thiobarbituric acid-reactive substances (TBARS), and hydrocarbons such as ethane and ethylene (Lesser 2006). ROS cause lesions in DNA bases that cause deletions and mutations (Lesser 2006).

ROS are produced endogenously in chloroplasts, mitochondria, the endoplasmic reticulum, and microbodies such as peroxisomes by photosynthetic and respiring cells (Lesser 2006, Suzuki & Mittler 2006). All aerobic organisms must deal with the continuous production of ROS (Storey 1996). ROS can be important signal transduction molecules, in that they regulate a number of physiological processes such as growth

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factor stimulation and the generation of the inflammatory response (Finkel 2011). ROS also activate stress response pathways and therefore defense mechanisms if present in the cell, but if present in larger than normal amounts during oxidative stress, they may do so much damage that they trigger apoptosis and cell necrosis in marine organisms (Lesser 2006, Livingstone 2003).

Coping with oxidative stress

Most organisms that live in aerobic conditions use special components such as antioxidants to deal with oxygen radicals and other dangerous oxygen products. Oxidative stress occurs in the presence of an imbalance of ROS and antioxidants, in that antioxidants are unable to quench ROS fast enough to prevent the damage mentioned above (Lesser 2006). Detoxification of these excess and dangerous ROS is essential for survival.

Some of the most common enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). These enzymes are important for breaking down and neutralizing dangerous oxygen free radicals. Upregulation of antioxidant defenses is an important preparation against oxidative stress. There are also some non-enzymatic antioxidants such as ascorbic acid, glutathione, tocopherol, carotenoids, and some small-molecule antioxidants (Lesser 2006).

Superoxide dismutase (SOD, EC 1.15.1.1) catalyzes the dismutation or disproportionation of the superoxide radical anion (O_2-) into both O_2 and H_2O_2. Metal cofactors such as copper (Cu), zinc (Zn), manganese (Mn) and iron (Fe) are associated

with the three different types of SOD: Cu/Zn SOD, Mn SOD, and Fe SOD. The Cu/Zn SOD form is usually found in eukaryotes in the cytosol, but is also found in bacteria, chloroplasts, and peroxisomes. The Mn SOD form is mostly found in mitochondria and bacteria. The third type, Fe SOD, is found in bacteria and chloroplasts (Lesser 2006). Some organisms may exhibit one, two or all three types of SOD. Marine arthropods lack the cytosolic Cu/Zn SOD that is seen in most other eukaryotes. They have an unusual cytosolic Mn SOD form which lacks the signal transit peptide which would normally direct it to the mitochondria. All Crustaceans that use a copper-dependent hemocyanin for oxygen transport use this form of Mn SOD instead of the Cu/Zn SOD. This is believed to be linked to the high demand for copper in hemocyanin synthesis (Lesser 2006). Marine arthropods also have a normal mitochondrial Mn SOD as do other eukaryotes (Brouwer et al. 1997) to quench ROS produced in the mitochondria.

Catalase (CAT, EC 1.11.1.6) is an antioxidant enzyme which protects the cell from oxidative damage by catalyzing the decomposition of hydrogen peroxide (H_2O_2) into H_2O and O_2. Because CAT is inactivated by light in the presence of oxygen, however, (Feierabend et al. 1996), the ability of photoautotrophs to cope with oxidative stress may be affected (Lesser 2006). CAT has a high K_m for H_2O_2, which makes it functionally effective primarily at high concentrations of H_2O_2 (Lesser 2006).

Gluthathione peroxidase (GPx, EC 1.11.1.9) catalyzes the reduction of free H_2O_2 to H_2O using the reduced form of glutathione (GSH). Oxidized glutathione (GSSG) is produced when hydrogen peroxide is reduced by GPx and is then recycled to its reduced state (GSH) by glutathione reductase and NADPH (Forstrom et al. 1978).

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Temperature and ROS production

In addition to endogenous sources, ROS can also be produced exogenously as a result of environmental stressors. Temperature stress can be caused either by fluctuations in or by increased temperature. Among other abiotic stresses, heat stress from increased temperature causes increased accumulation of toxic ROS in cells by disrupting cellular homeostasis and uncoupling metabolic processes (Potters et al. 2007, Suzuki & Mittler 2006). Elevated ROS production is usually observed under heat or increased temperature stress (Davidson & Schiestl 2001). ROS generation along with antioxidant response in bivalves and limpets increases in the presence of heat stress (Heise et al. 2003, Abele et al. 2002, Abele et al. 1998).

Complications with fluctuations in oxygen conditions

Organisms that experience hypoxic conditions or intermittent hypoxia frequently encounter ROS. When organisms return from hypoxic to normal atmospheric conditions, these animals' tissues are perfused with large amounts of oxygen, and therefore ROS. Reoxygenation after hypoxia causes lipid peroxidation, which is caused by damage from overproduced ROS following ischemia and reperfusion (Lushchak et al. 2001). During anoxia or reoxygenation from hypoxia, organisms will encounter oxidative stress which causes increased activity of antioxidants (Parrilla-Taylor & Zenteno-Savín 2011) in preparation against harmful ROS.

Increases in environmental oxygen levels can also lead to increased ROS production and therefore increased damage from ROS (Lushchak et al. 2005).

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Antioxidant levels will increase in response to high ROS production under elevated oxygen levels (Welker et al. 2013). As oxygen concentration increases from normoxia to hyperoxia, ROS production in the mitochondria increases proportionally (Welker et al. 2013). Antioxidant defense is also upregulated when the organism returns to normoxia from hyperoxia, probably in preparation or anticipation for other rounds of hyperoxia (Lushchak et al. 2005). Extended hyperoxia causes increase an in ROS production which leads to oxidative injuries such as lipid peroxidation (Welker et al. 2013). Most organisms live in environments that have oxygen pressures close to atmospheric conditions; many experience oxygen pressures lower than atmospheric conditions, but few experience oxygen pressures higher than atmospheric conditions for any extended period of time.

Habitats which exhibit low oxygen conditions

Hypoxia is common in marine environments where dissolved oxygen (DO) diffusion through the water is limited. Oxygen depletion is most widely observed in hydrothermal vents, the arctic (under ice sheets), productive habitats and tidepools at night, and anoxic and fluctuating oxygen conditions are even present within the sediment in marine environments (Sand-Jensen et al. 2005). The lethal limit of aquatic hypoxia for some organisms is considered to be 2 mg/L, or only 20% of air saturation (Diaz 2001), where 100% DO air saturation is approximately 6-8 mg/L, depending on salinity and temperature. This value however, is on the extreme end of hypoxia tolerance among aquatic organisms, where most aquatic organisms experience physiological deficiencies

and issues below 70% air saturation (Chabot & Claireaux 2008). In Atlantic cod, swimming and digestion of food is significantly reduced below 70% air saturation (Chabot & Claireaux 2008). High hypoxia tolerance is common in fish but may not be as prevalent in crustaceans. The DO minimum value for crustaceans ranges from 0.9 to 5.3 mg/L at temperatures from below 10°C to a little over 20°C (Froehlich et al. 2016), however this value is the point at which most crustaceans would die.

<u>Habitats which exhibit elevated oxygen conditions</u>

What qualifies as a high oxygen environment is relative to the organism. The Royal Navy declared it dangerous for humans to breathe 100% oxygen underwater at depths lower than 7.6 m (25 feet), or at 1.76 atmospheres absolute (ata) oxygen partial pressure (Harabin & Survanshi 1993), where normal oxygen saturation in air is about 0.21 ata. Since oxygen diffuses more rapidly through air compared to water, elevated oxygen levels (above the normal 157 to 158 mmHg O_2 found in air at atmospheric pressure; 158 mmHg = 1 atmospheric pressure of O_2) are encountered only in very limited conditions in the terrestrial environment. However, the limited capacity for oxygen diffusion through water may allow oxygen to build up to high levels if near an oxygen source such as photosynthesis. Plankton blooms, for example, often exhibit highly oxygenated conditions and release ROS, causing fish to experience oxidative stress (Kim et al. 1999). One such environment that exhibits high oxygen conditions is eelgrass beds.

Any fluctuations in oxygen levels such as reperfusion from hypoxia to normal atmospheric conditions, transition from normal to hyperoxia, or extended hyperoxia can

result in oxidative stress from ROS damage. In this study, I examined oxygen characteristics within a bed of eelgrass, *Zostera marina*, and studied its effects on one of its common inhabitants, the isopod *Pentidotea resecata*.

<u>Background information on the eelgrass *Zostera marina*</u>

Sea grass communities are widely distributed across the world and are among the most productive marine ecosystems (Kharlamenko et al. 2001). *Zostera marina* is a species of sea grass called common eelgrass. It is found along the western coast of North America from Alaska down to Baja California and along the eastern coast from North Carolina to Labrador, and is also widespread elsewhere in the North Atlantic Ocean (Short et al. 2007). In the Puget Sound, WA, *Z. marina* blades can reach up to 2 m in length (Hylarides 2015). *Z. marina* relies on the water column as a sole source of oxygen because the sediments it lives in are usually anoxic or nearly so (Sand-Jensen et al. 2005). *Z. marina* is an important habitat for marine invertebrates and many fish because they use it to hide from predators and lay their eggs (Gotceitas 1997).

Since *Z. marina* is autotrophic, it regularly produces oxygen through photosynthesis. Light levels remain high in the spring and summer, however, providing *Z. marina* with ample light for photosynthesis (Hylarides 2015). During late summer and fall productivity increases to a maximum but declines again during the winter. Though low during the winter, light levels remain sufficient for eelgrass beds to survive and grow (Hylarides 2015).

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Tidal height may also play an important role on productivity of eelgrass beds. Penhale (1977) observed low photosynthetic productivity in the eelgrass during the spring and early summer, probably because of environmental factors associated with tidal height. Photosynthesis may be limited at high tide due to diminished light penetration into the deeper water column, while low tide brings eelgrass populations closer to the surface of the water, increasing light penetration (Hylarides 2015) and therefore increased photosynthesis. These extremes may limit *Z. marina* beds in the Puget Sound during the summer (Hylarides 2015).

According to data collected from the NOAA National Estuarine Research Reserve System, DO levels fluctuate rapidly in the water column among *Z. marina* eelgrass beds in Padilla Bay, WA, from below 20% to above 200% oxygen saturation (NOAA National Estuarine Research Reserve System, Figure 1). Even amidst the unstable conditions which are often hyperoxic but then may plunge deep into hypoxia, some organisms manage to survive. One marine organism in particular, *Pentidotea resecata*, spends most of its life living among shallow *Z. marina* beds, within the most intense of these fluctuating oxygen conditions.
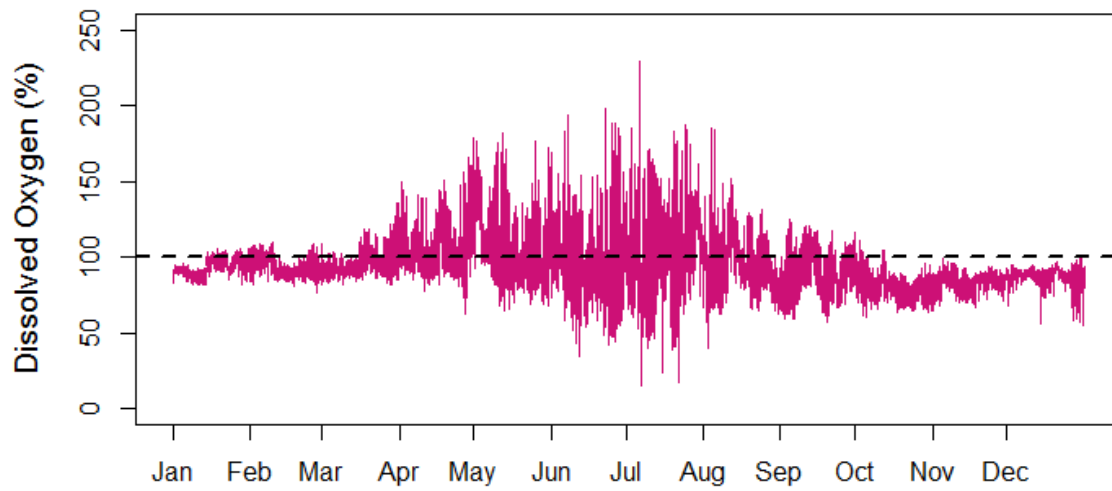
Figure 1. Percent saturation of dissolved oxygen in Padilla Bay, WA obtained from NOAA National Estuarine Research Reserve System from January to December 2016. See Figure 5, site B for location where the data were collected.

Background information on *Pentidotea resecata*

Pentidotea resecata Stimpson, 1857 (Crustacea: Isopoda: Valvifera) is found along the western coast of North America from southeastern Alaska to Baja California (Lee & Gilchrist 1972), and prominently in the Puget Sound. This coastal marine isopod exists in two color morphs, green (Figure 2) and brown. The green morph is usually found clasped onto the blades of the eelgrass *Zostera*, while the brown morph is found on the stipes of the giant kelp, *Macrocystis.* The endocuticle of the brown morph has a green pigment, whereas the exocuticle has a red pigment, giving the animal an overall brownish color (Lee & Gilchrist 1972). Earlier studies concluded that the green color of the green morph was also due to proteins in the cuticle (Lee & Gilchrist 1972). However, more recent studies suggest that the bright green color of the green morph is mainly due to a green hemolymph and from whole plant cells containing chloroplasts in the hindgut (McLarty 2015).

Isopods can be marine, freshwater, or terrestrial, and range from 0.05 to 50 cm in length (Brusca and Brusca 2003). *P. resecata* is a marine isopod found from the intertidal zone to 18 m depth. From observation in the field, adult individuals can be up to 5.5 cm in length, which is rather large for an isopod. *P. resecata* is a prominent inhabitant of *Z. marina* beds during the spring and summer but, like the brown morph in the kelp beds (Lee and Gilchrist 1972), seems to disappear during the fall and winter. The reason for its disappearance is unknown. Winter storms break up the *Macrocystis* kelp that the brown morph lives on, but the *Z. marina* beds are not disturbed as much due to their protected

Figure 2. Ventral view of *Pentidotea resecata*.

location in bays. Nonetheless, the green morph seems to disappear from *Z. marina* in the fall and winter as well (McLarty 2015).

These isopods rely almost completely on their eelgrass substrate for food (Morris et al. 1980), but in some cases, they can be cannibalistic when crowded or in close proximity with one another (McLarty 2015). Analysis of gut contents of *P. resecata* revealed chlorophyll breakdown products such as pheophytin, and respiration experiments suggest that photosynthetic activity may be occurring within the gut of the animal (Cowles 2015, McLarty 2015). This may further raise pO_2 levels around and within the tissues.

Background information on *Ligia pallasii*

Ligia pallasii Brandt, 1833 (Crustacea: Isopoda: Oniscoidea), commonly called the rock louse, is another isopod that lives in the Puget Sound (Figure 3). It is found along the western coast of North America from Alaska to central California (Eberl 2010). Unlike *P. resecata, L. pallasii* lives in cracks in rocks in a high intertidal and supratidal habitat and is constantly exposed to damp air. It rarely spends any substantial amount of time immersed in seawater, though it does dip its pleopod gills in the water to keep them moist (Morris et al. 1980) at dawn and dusk, during which it feeds on algae that washes onto shore. This species would therefore be exposed to normoxic conditions and have little, if any, exposure to either hyperoxic or hypoxic water, and therefore would be expected to encounter less amounts of damaging ROS.

Figure 3. Ventral view of *Ligia pallasii*.

<u>Objectives and hypotheses</u>

The purpose of this study was to first record the DO levels in Padilla Bay among the eelgrass beds, in proximity to where the isopods were collected; then to assess and compare the antioxidant enzymatic capacity of *P. resecata* and *L. pallasii* in order to understand how *P. resecata* protects its tissue against ROS from highly fluctuating oxygen conditions while living among *Z. marina* eelgrass beds. I expected to observe hyperoxic as well as hypoxic conditions in the water column among the eelgrass due to high productivity and the fact that DO does not disperse as readily throughout the water as it does in the air. Therefore, I expected *P. resecata* to be subject to this highly fluctuating oxygen environment. As a result of this, I also expected to observe high levels of principal enzymatic antioxidants such as SOD, CAT, and GPx in the tissues of *P. resecata,* relative to that of *L. pallasii* which rarely immerses itself in the water and is presumed to live at normoxic conditions.

METHODS

Oxygen and temperature data collection

Dissolved oxygen profiles were measured among the *Z. marina* eelgrass beds in Padilla

Bay, WA (Figures 4 and 5, site A) on several occasions during the summer months of

2016 using a YSI 550A oxygen field electrode at low, moderate, and high tides; during

morning, afternoon, and dawn; and on cloudy and sunny days, which was subjectively

determined by looking at the sky on each of these days. Light intensity profiles were also

recorded using a 360° light sensor (Li-Cor Spherical sensor) connected to a light intensity

data logger (Li-Cor LI-1400®) at high tide.

At low tide, oxygen and temperature data were collected at the main study site

(Figure 5, site A) by wading into the bay with the oxygen electrode. At high tide, data

were collected from a boat. DO and temperature measurements were collected every 10

cm for the first two meters from the surface, after which measurements were taken every

20 cm down to the ocean bed.

Oxygen saturation and temperature data from 2016 were also downloaded from a

YSI 6600 sonde which was located at a depth of 1.1 m below MLLW (mean lower low

water), operated by NOAA National Estuarine Research Reserve in a nearby channel, 3.8

km from the main study site (Figure 5, site B). Temperature data during July and August

were also collected closer to the main study site by attaching a temperature logger

(HOBO U22 Water Temp Pro v2) to a piling in the Padilla Bay channel, at about 6 m

depth at high tide and 0.4 km from the main study site (Figure 5, site C).

Figure 4. The blue balloon marks the location of Padilla Bay on the western coast of USA. GPS coordinates are: 48.50015 N, 122.55374 W. Map taken from usgs.gov.
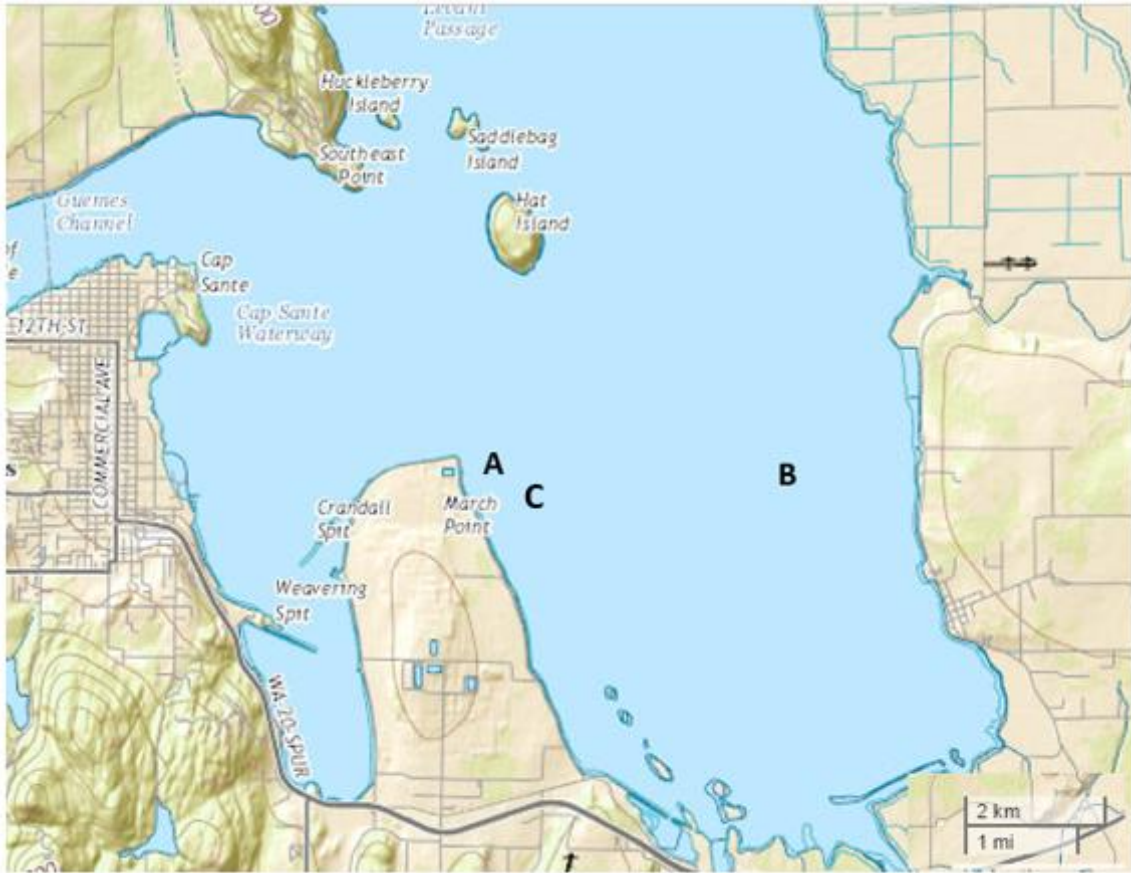
Figure 5. Map showing study locations in Padilla Bay on the western coast of Washington. A represents the approximate location of the collection of *P. resecata* and oxygen and temperature profile measurements. B represents the approximate location of the NOAA National Estuarine Research Reserve buoy, in a channel 3.8 km from the main study site, from which yearlong oxygen saturation and temperature data were downloaded. C represents the location of the summer-season temperature measurements, 0.4 km away from site A. Map taken from usgs.gov.

Animal collection

Pentidotea resecata were collected from the same eelgrass beds in Padilla Bay, WA at

site A. The animals were usually collected at low tide by dragging a coarse-meshed net

through the beds and using smaller hand nets to capture individuals that were dislodged

from the eelgrass and were actively swimming through the water. At high tide, animals

were captured by pulling a plankton net through the eelgrass by boat. Only animals larger

than approximately 4 cm were used in analysis. Sufficient tissue could not be collected

from animals with eggs or under approximately 4 cm, so they were returned to the water

if collected in the nets. The mean length of animals used was 4.7 cm. Data from

Hunnicutt et al. (2016) indicate that *P. resecata* of this size are predominantly if not

exclusively males. Animals were placed in a closed container with sea water, a bubbler,

and eelgrass, then transported to the lab for dissection.

　　　　L. pallasii were collected from cracks in rock faces on Northwest Island in

Rosario Bay, WA. Animals were placed in a container with sufficient oxygen while

transported to the lab for dissection. *L. pallasii* animals were collected during the day and

weather and tide conditions varied. The mean length of animals used was 2.8 cm. All *P.*

resecata and *L. pallasii* animals were collected between early July through the first week

in August of 2016.

Animal dissection

Animals were sacrificed and dissected in the lab approximately one hour after capture. *P.*

resecata animals were first placed in ice-cold sea water to slow their metabolism. This

method calms the animal and is reported in the literature as one of the humane methods for sacrificing crustaceans (Martin 2016; Ng 2017). They were then rinsed in distilled water to remove diatoms and then sacrificed by decapitation just anterior to the mouth (to avoid cutting into the digestive tract). Two longitudinal cuts were made through the exoskeleton on either side of the isopod's body, avoiding the legs. The exoskeleton and gut were peeled away and the tissue was obtained.

Since it was more difficult to dissect *L. pallasii* without contaminating the tissues with gut fluid, a different method of dissection was used. *L. pallasii* animals were placed in a chilled chamber with dry ice to anesthetize them before they were dissected. The animals were dissected by opening the ventral side of the animal and then removing the gonads and gut. The body cavity was bathed in chilled PBS, then blotted to wash away gut fluid. The tissues were then scraped out of the body cavity and prepared for homogenization.

Sample preparation and storage

Tissues from each animal were weighed, then 20 ml of chilled PBS was added for every gram of tissue. Tissues were homogenized using a chilled mortar and pestle, then the samples were placed on dry ice until tissues from all the isopods being dissected that day were prepared. Samples were then centrifuged at 4°C for 15 minutes at 10,000xg. The supernatant was removed and stored at -80°C until further use for assays.

Enzymatic antioxidant activity assays

The enzymatic antioxidant activity of superoxide dismutase, catalase, and glutathione peroxidase in tissues of *P. resecata* and *L. pallasii* were determined by assay kits from a Sigma Aldrich protocol adapted from McCord and Fridovich (1969), OxiSelect ™ Catalase Activity Assay Kit (STA-341) by Cell Biolabs, and Glutathione Peroxidase Assay Kit (Item No. 703102) by Cayman Chemicals, respectively.

In the superoxide dismutase (SOD) assay, the superoxide radical was produced by reacting xanthine with water in the presence of oxygen; a reaction which was catalyzed by xanthine oxidase. Oxidized cytochrome c was then reduced by the superoxide radical. One unit of superoxide dismutase is defined as the amount of SOD which inhibits the rate of reduction of cytochrome c by 50%, by competing for the superoxide radical, which normally reduces cytochrome c. The tissue samples for which SOD activity was to be assayed were added to 50 mM potassium phosphate, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.01 mM cytochrome c, 0.05 mM xanthine, and distilled water. Then 0.05 mM xanthine oxidase was added to initiate the formation of the superoxide radical. Samples and reagents were kept on ice until all reagents were added to the reaction. The rate by which the SOD in the samples inhibited the reduction of cytochrome c by competing for the superoxide radical was followed spectrophotometrically using a Beckman DU-520 by measuring the absorbance at 550 nm every 25 seconds for 5 minutes. Absorbance was read at room temperature and the pH of this assay was 7.8. Sample sizes were n=10 for *Pentidotea resecata* and n=10 for *Ligia pallasii*.

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Catalase (CAT) converts hydrogen peroxide (H_2O_2) into water and oxygen. The rate of decomposition of H_2O_2 is proportional to the concentration of catalase present. Catalase activity was determined by adding 50 µl of 8.82 M H_2O_2 solution diluted with assay diluent to each tissue sample of 20 µl, then stopping the reaction by adding 50 µl of catalase quencher containing sodium azide to the reaction. The remaining H_2O_2 in the reaction then facilitated the coupling reaction of 3,5-dichloro-2-hydroxy-benzenesulfonic acid (DHBS) and 4-aminoantipyrene (AAP) in conjunction with a horseradish peroxidase (HRP) catalyst. A chromogenic solution was added to spectrophotometrically detect the change in absorbance of the quinoneimine dye coupling product. Samples and reagents were kept on ice until all reagents were added to the reaction. Absorbance was read in a temperature-controlled SpectraMax 190 96-well plate reader spectrophotometer at 25 ± 1°C once at the beginning of incubation and once at the end of the 60 minute incubation. A catalase standard was provided in the kit, in which one unit of catalase is the amount of enzyme that will decompose 1.0 µmole of H_2O_2 per minute at 25ºC. The pH for this assay was not specified by the manufacturer. Sample sizes were n=15 for *Pentidotea resecata* and n=15 for *Ligia pallasii*.

Glutathione peroxidase (GPx) activity was determined indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione is produced when hydrogen peroxide is reduced by GPx and is recycled to its reduced state by glutathione reductase and NADPH. The reaction between NADPH, glutathione, and glutathione reductase, along with the glutathione peroxidase in the tissue samples was initiated by adding 20 µl of cumene hydroperoxide for every 20 µl of sample. Samples and reagents were kept on

ice until all reagents were added to the reaction. The oxidation of NADPH was accompanied by a decrease in absorbance which was measured spectrophotometrically at 340 nm once every minute for 10 minutes. The rate of decrease in the absorbance is directly proportional to the glutathione peroxidase activity in the sample. Absorbance was read in a temperature-controlled SpectraMax 190 96-well plate reader spectrophotometer at $25 \pm 1°C$. Bovine erythrocyte glutathione peroxidase was provided in the kit as a standard. The pH of this assay was 7.6. Sample sizes were n=28 for *Pentidotea resecata* and n=25 for *Ligia pallasii*.

Sample sizes were different for each of the assays because of the small size of individuals and the small amounts of tissue mass obtained. All assays were done in duplicate and some samples were done in triplicate. Different individuals were used for each assay. Each assay included individuals of both species collected in July through the first week of August. The smaller number of samples for the SOD assay was due to limitation of tissue mass obtained, as a sample volume of at least 90 µl was needed for this assay. Unequal sample size in the GPx assay reflects error in adding reagents during the assay, therefore those samples that were assayed incorrectly were removed from the analysis.

Analyses

Plots of year-long DO and temperature levels in the channel and of daily oxygen and temperature profiles in the eelgrass bed were created in R. All data analyses were conducted in R (v.3.3.0). All data were checked for normality and homoscedasticity using

Shapiro-Wilks and Bartlett tests, respectively. A confidence interval of 95% was used for all analyses. Data from the SOD assay were tested for statistical significance using a Welch two sample t-test. Data from the CAT assay were not normal, so a Wilcoxon test was used to test for statistical significance. Data from the GPx assay were not normal nor homoscedastic, so a permutation t-test was done to test statistical significance.

RESULTS

Oxygen profiles

The percent saturation of DO levels collected by NOAA in a channel just offshore of the

eelgrass beds in the Padilla Bay area ranged from below 20% to over 200% in 2016

(Figure 1). Organisms were collected 3.8 km from the NOAA site during summer months

(June to August), during which DO portrayed the most variability.

DO data collected from the same location where *P. resecata* animals were

collected (site A) on a sunny afternoon during high tide show approximately 140% to

155% saturation (Figure 6A). Data collected at dawn on a cloudy day at low tide show

approximately 75% to 90% saturation of DO, or mildly hypoxic conditions (Figure 6B).

Data collected on a sunny afternoon at low tide show approximately 90% to 130% DO

saturation (Figure 6C). Data collected on a cloudy morning at low tide show 80% to 90%

DO saturation (Figure 6D).

Oxygen levels closer to the sediment and therefore among the eelgrass blades

were often higher than at the surface of the water (Figure 6). In the profile taken on a

sunny afternoon, a 10% increase in oxygen content is seen close to the sediment (Figure

6A), likely due to the abundant eelgrass at that depth.

Temperature data

Temperature data collected at the same time and location oxygen profiles were collected

at site A show a range from 14 – 19°C (Figure 7). Temperature tends to decrease closer to
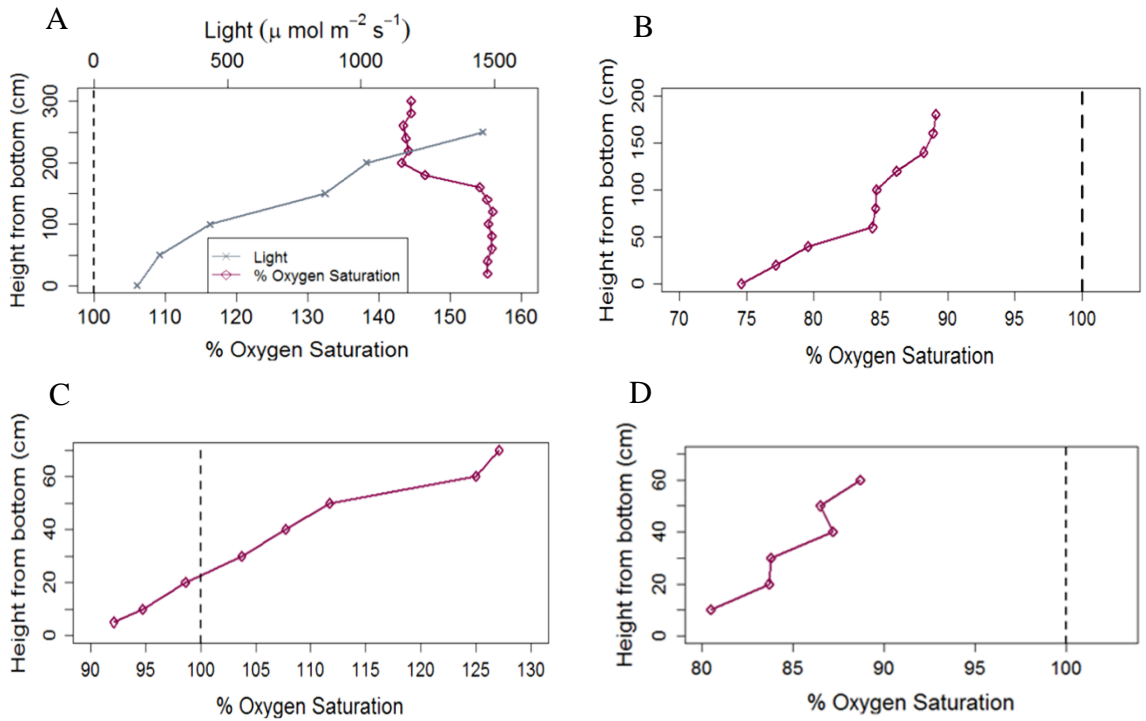
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Figure 6. Percent saturation of dissolved oxygen in Padilla Bay, WA. The dashed line represents 100% air saturation. A. Data collected on a sunny afternoon at high tide, shown along with light level. B. Data collected at dawn on a cloudy day at moderate tide. C. Data collected on a sunny afternoon at low tide. D. Data collected on a cloudy morning at low tide. Eelgrass height extended to 100 – 150 cm from the bottom. See Figure 5, site A for location where the data were collected.
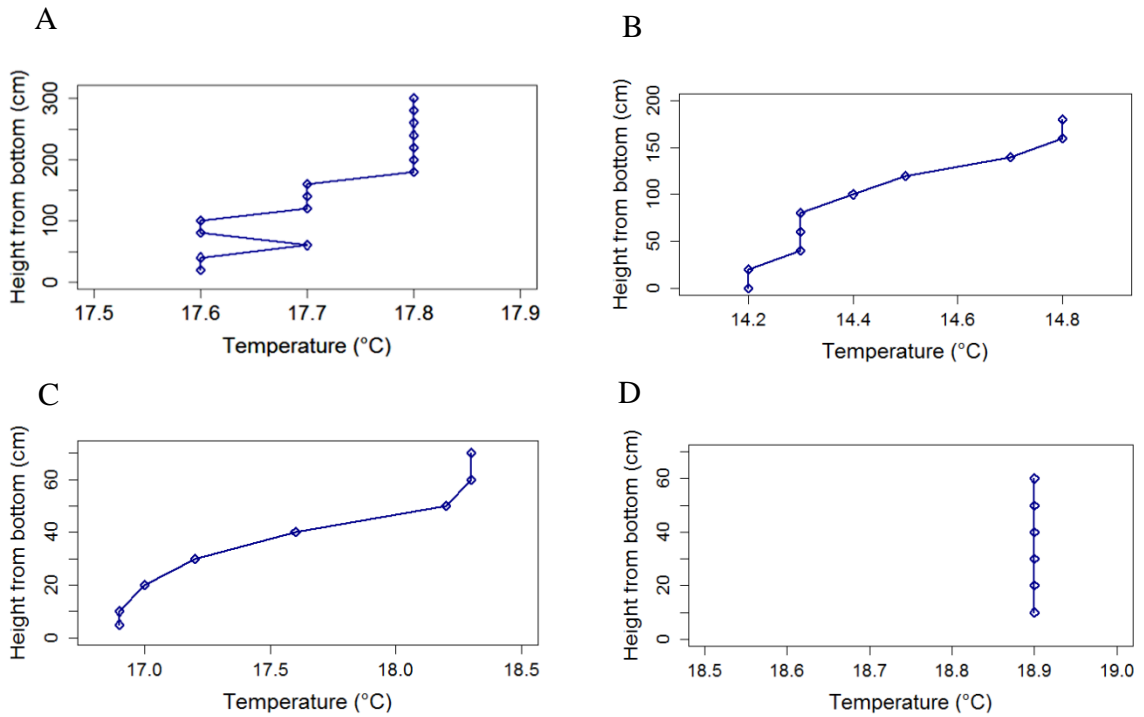
Figure 7. Temperature profiles in Padilla Bay, WA at the same location of oxygen profiles in Figure 6. A. Data collected on a sunny afternoon at high tide. B. Data collected at dawn on a cloudy day at moderate tide. C. Data collected on a sunny afternoon at low tide. D. Data collected on a cloudy morning at low tide. Eelgrass height extended to 100 – 150 cm from the bottom. Resolution of the temperature probe was 0.1 C. See Figure 5, site A for location where the data were collected.

the bottom, with the exception of the constant temperature with depth observed at low

tide on a cloudy morning (Figure 7D).

Yearlong data compiled from NOAA, 3.8 km from the *Pentidotea resecata*

collection site, show fluctuations from below 5°C to over 20°C, with the highest

temperatures and most fluctuation during June through September (Figure 8). Organisms

were collected within this time period for this study. Summer temperature measurements

collected at 6 m depth at high tide, in a channel only 0.4 km from the collection site

(Figure 9) show fluctuations in temperature ranging between 12°C and 20°C, with the

highest reading at approximately 20°C in late July and the greatest daily fluctuation in

temperature is approximately 5°C.

Enzymatic antioxidant activity assays

L. pallasii tissues had approximately 50% higher SOD activity than *P. resecata* tissues

did (Welch's t-test. t= -8.2975, df= 11.592, p= 3.258e-06, Figure 10, Table 1). CAT

activity in *L. pallasii* tissues was approximately 30% higher than in *P. resecata* tissues

(Wilcoxon rank sum test. W= 63, p= 0.04084, Figure 11, Table 1). GPx activity was

approximately 70% higher in *L. pallasii* tissues than in *P. resecata* tissues (Permutation t-

test. W= 63, p≤ 0.00001, Figure 12, Table 1).

Overall, the enzymatic activity of *P. resecata* tissue was significantly less than

that of *L. pallasii* for all the antioxidant enzymes assayed, which is inconsistent with the

initial hypothesis.
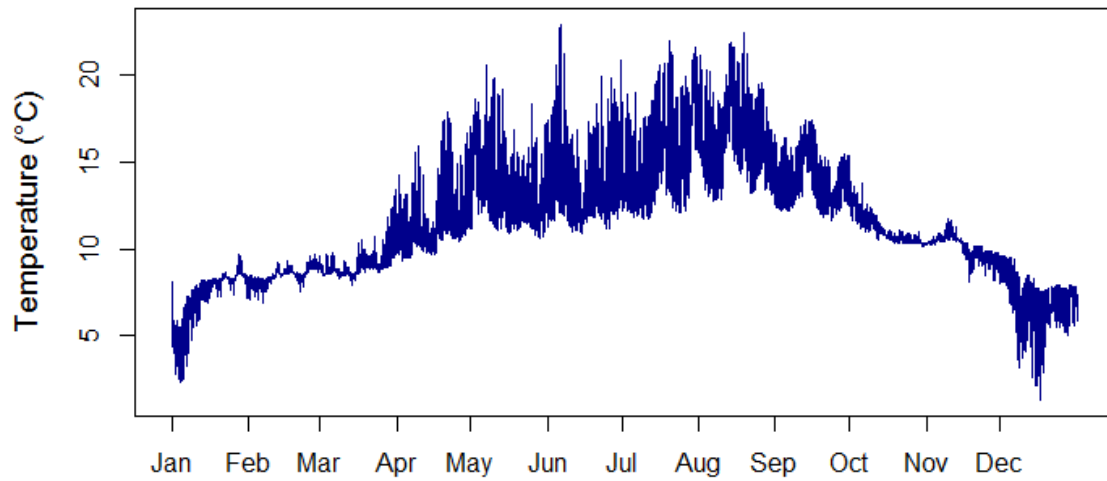
Figure 8. Temperature measurements in Padilla Bay, WA obtained from NOAA
National Estuarine Research Reserve System from January to December 2016. See
Figure 5, site B for location where the data were collected.

Figure 9. Temperature measurements collected in the summer of 2016 in Padilla Bay, at 6 m depth at high tide in a channel approximately 0.4 km from where *Pentidotea resecata* were collected. See Figure 5, site C for location where the data were collected.

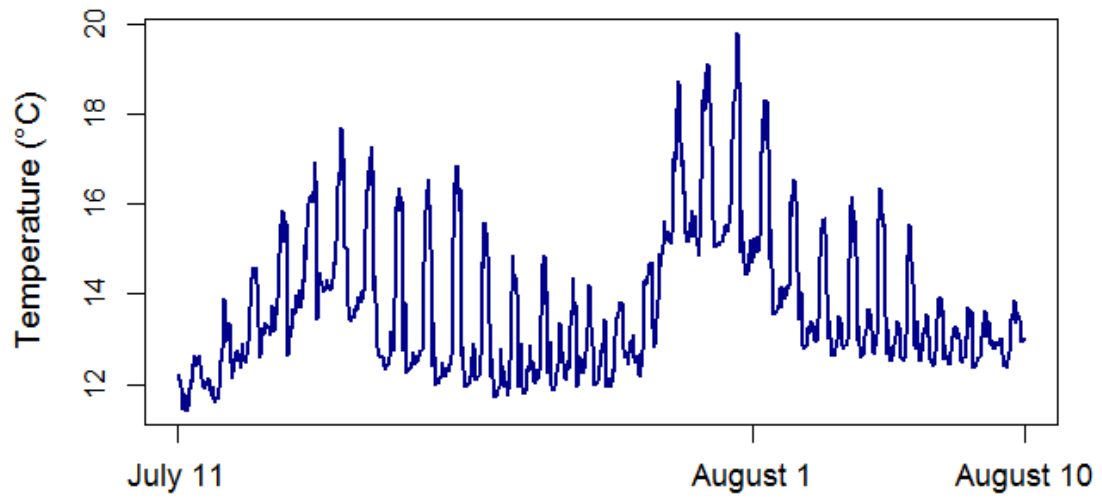**Figure 10. Superoxide dismutase activity in *P. resecata* vs. *L. pallasii* tissues.** SOD activity of *L. pallasii* (n=10) was significantly higher than that of *P. resecata* (n=10). (Welch's test. t= -8.2975, df= 11.592, p= 3.258e-06.) In this box plot, the band inside the box represents the median, the bottom of the box represents the first quartile, the top of the box represents the third quartile, whiskers represent the highest and lowest values, and open circles are outliers.

Figure 11. Catalase activity in *P. resecata* vs. *L. pallasii* tissues. CAT activity of *L. pallasii* (n=15) was significantly higher than that of *P. resecata* (n=15). (Wilcoxon rank sum test. W= 63, p= 0.04084.)

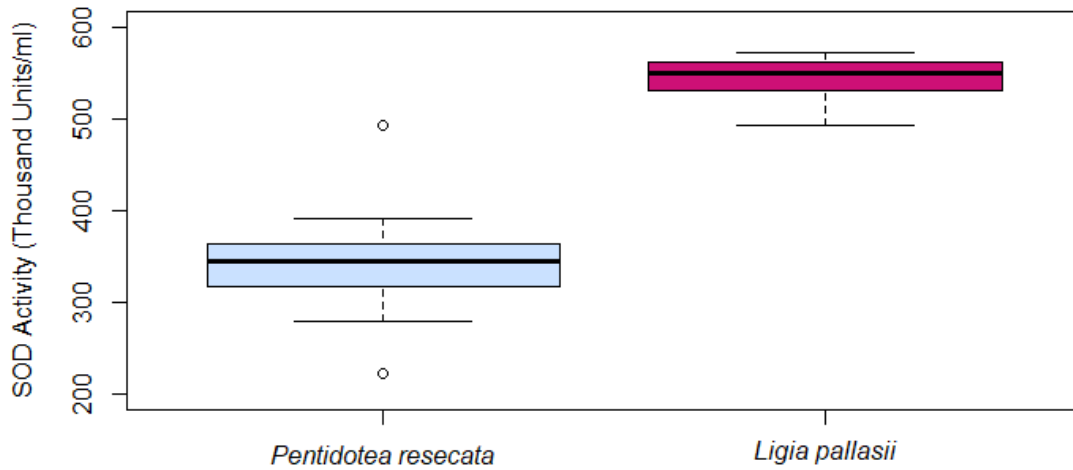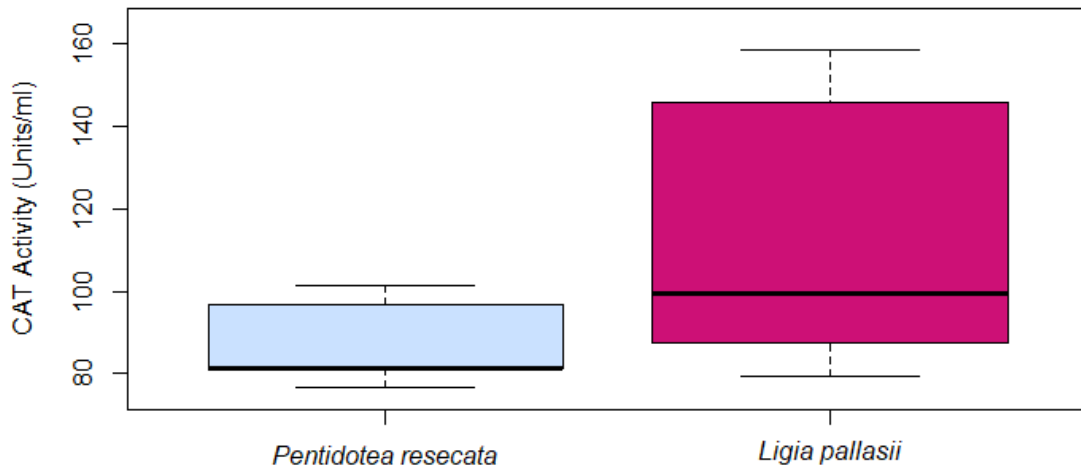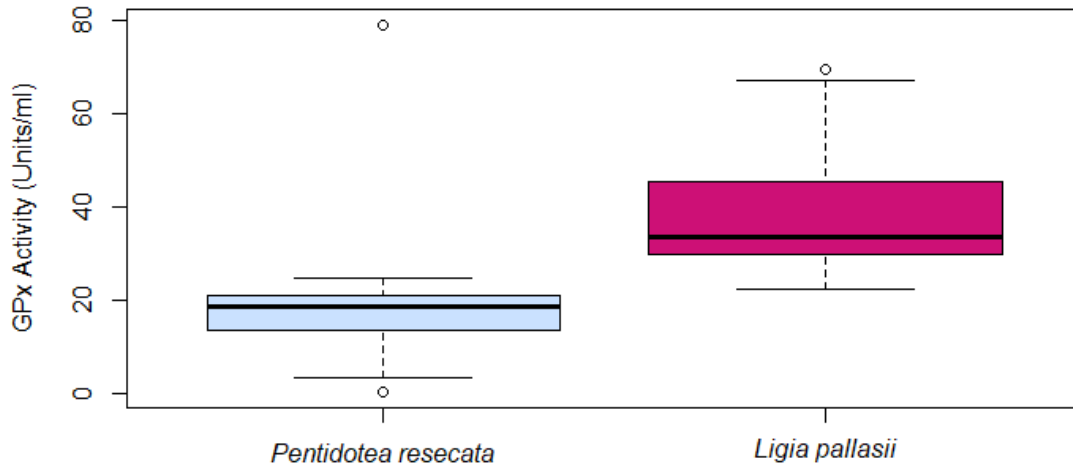**Figure 12. Glutathione peroxidase activity in *P. resecata* vs. *L. pallasii* tissues.** GPx activity of *L. pallasii* (n=25) was significantly higher than that of *P. resecata* (n=28). (Permutation t-test. W= 63, p≤ 0.00001.)

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Table 1. Summary of enzymatic antioxidant capacity in *Pentidotea resecata* and *Ligia pallasii* tissues. Data represent mean ± standard deviation. One unit of enzyme is defined as the amount of enzymatic activity that will catalyze the conversion of 1 µmole of substrate per minute.

	Pentidotea resecata	*Ligia pallasii*
SOD (units/ml)	343369.7 ± 70847.91	542470.7 ± 27171.81
CAT (units/ml)	88.48 ± 9.27	115.88 ± 31.57
GPx (units/ml)	18.48 ± 13.38	38.59 ± 13.65

DISCUSSION

<u>Dissolved oxygen data</u>

Yearlong data of DO levels in the water column in Padilla Bay from 2016 suggest that conditions among *Z. marina* beds can be hypoxic, normoxic, or hyperoxic (Figure 1). The most variability in oxygen levels was recorded during summer months (June to August), suggesting that *P. resecata* experience more fluctuating oxygen conditions during summer months compared to the rest of the year. These highly fluctuating oxygen conditions are due to oxygen production during photosynthesis of *Z. marina*, combined with high oxygen demand by the densely-packed eelgrass community during the night. *P. resecata* animals are exposed to these fluctuating DO levels in the water column, and if their antioxidants are unable to quench dangerous ROS faster than the production of these radicals, they may experience damage to their proteins, lipids, and DNA.

Oxygen profiles taken during summer months at site A also show that DO levels are hypoxic, normoxic, hyperoxic, and highly fluctuating among the eelgrass beds where *P. resecata* live (Figure 6). DO at this location are higher than at the NOAA site, possibly because these oxygen profiles were taken among eelgrass beds where photosynthesis is readily occurring. During high tides, oxygen levels closer to the depth level of the eelgrass (100 – 150 cm from the bottom) itself show higher oxygen levels and daily fluctuation than at the surface of the water, likely because this is the site of continuous photosynthesis.

An oxygen profile taken on a sunny afternoon during high tide shows 140% to 155% saturation of DO, or hyperoxic conditions (Figure 6A), suggesting that *Z. marina* was highly productive that day. Hypoxic conditions were observed on a cloudy morning at low tide (Figure 6D) and at dawn at moderate tide (Figure 6B). DO levels are 70% to 90% oxygen saturation due to the lack of sunlight in the early morning on these days. *Z. marina* in these cases likely had not yet had a chance to photosynthesize and produce large amounts of DO that day; while respiration by the plants and associated community had reduced oxygen levels in the water column. Variation in oxygen levels both spatially and temporally was also observed on a sunny afternoon at low tide (Figure 6C). It is likely that *Z. marina* had sufficient sunlight to photosynthesize from morning until afternoon when the data were collected, and since the water level was low during low tide, DO was unable to disperse effectively in the water column as it would during high tide.

Temperature data

The yearlong temperature data from the nearby channel buoy (Site B in Figure 5), as well as temperature data taken near the eelgrass beds over the summer months from mid-July to mid-August during which organisms were collected, show substantial fluctuations in temperature (Figures 8 and 9). These daily fluctuations in temperature likely cause heat stress to *P. resecata* and therefore may enhance ROS production in their tissues. So it is appropriate to assume that *P. resecata* would have elevated antioxidant capacity to protect against excess ROS in response to heat stress as well as to unstable oxygen levels.

Other potential sources of ROS

High ultraviolet radiation is likely prevalent in exposure to the sun in such shallow water among *Z. marina* communities. UV radiation is known to be a stressor which can cause high ROS levels and insufficient ROS scavenging capacity, leading to oxidative stress and therefore damage to proteins, lipids, and DNA (Hideg, et al. 2013). Therefore *P. resecata* are likely to be encountering harmful ROS as a result of increased UV radiation as well.

Toxins from exposure to pollution or eutrophication as a result of anthropogenic effects can also cause increased ROS production (Lesser 2006), and could possibly be the case in *Z. marina* eelgrass beds in Padilla Bay because of its proximity to an oil refinery and runoff from nearby sloughs. Other environmental stresses such as high salinity, chilling, metal toxicity, and pathogens which disrupt cellular homeostasis can lead to enhanced ROS production, at least in plants (Sharma et al. 2012). However, the lower antioxidant enzyme activities found in *P. resecata* in this experiment do not support the theory that any of these have a substantial effect.

Enzymatic antioxidant activity assays

The results from the enzymatic antioxidant assays of SOD, CAT, and GPx all indicate that *L. pallasii* has significantly higher enzymatic antioxidant capacity of these important enzymes than does *P. resecata* (Figures 10, 11, 12, Table 1). The thousand-fold higher rate of activity of SOD compared to CAT and GPx of both *P. resecata* and *L. pallasii*

may suggest that SOD is the main antioxidant defense utilized by isopods to quench ROS. Still, these results are unexpected, as *L. pallasii* presumably encounter normoxic conditions while living in cracks in rocks, while *P. resecata* encounter highly fluctuating oxygen conditions while living among *Z. marina* beds, as was observed in this study. There is also some evidence that *P. resecata* is photosynthetic (Cowles 2015, McLarty 2015), which could raise the pO_2 levels around the tissues even further.

As a result of these fluctuating DO conditions, I expected *P. resecata* to encounter higher amounts of ROS. The amount of dangerous free radicals and the extent of damage caused by ROS in the tissues was not directly measured, therefore I am uncertain whether *P. resecata* actually does encounter higher amounts of ROS than does *L. pallasii*. It is possible that *P. resecata* does not experience extensive damage from ROS due to other defense mechanisms, which may be the reason it possesses lower enzymatic antioxidant capacity than *L. pallasii*.

If *P. resecata* does experience more ROS than *L. pallasii*, it may possess other mechanisms by which it protects its tissue against damage from ROS. In a study done on a freshwater gammarid amphipod, increased availability of dietary carotenoids reduced the activity of endogenous enzymatic antioxidants such as superoxide dismutase and catalase (Babin et al. 2010). Perhaps *P. resecata* employs carotenoids from its diet instead of enzymatic antioxidants to protect against ROS. Also, *P. resecata* ingests the *Z. marina* eelgrass that it resides on while *L. pallasii* eats algae that washes onto the shore. This difference in diet may have an effect on antioxidant activity because of the presence of different carotenoids and other pigments in the eelgrass and algae.

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Another explanation for these unexpected results is that perhaps exogenous ROS from the highly fluctuating environment are not diffusing into the tissues of *P. resecata*. In a study on arctic amphipods, Camus and Gulliksen (2005) found low levels of antioxidant defenses in a sympagic amphipod living in hypoxic environments and proposed the ability of the amphipod to either prevent diffusion of exogenous H_2O_2 into the gills, or to excrete internal H_2O_2. Perhaps *P. resecata* employ this defense against harmful ROS.

Other explanations for low *P. resecata* antioxidant activity

L. pallasii lives mostly terrestrially, where temperature disperses through the air more readily than it does in aquatic environments. Therefore *L. pallasii* may encounter more fluctuations in temperature, resulting in greater heat stress and causing enhanced ROS production, which therefore may be a possibility for possessing higher enzymatic antioxidant capacity than *P. resecata*. This potentially greater heat stress is likely partially mitigated by the fact that *L. pallasii* spends much of the daytime within cracks away from exposure to direct sunlight.

All assays of SOD, CAT, and GPx were conducted at 25°C, and this may have affected the activity of these enzymes. Evidenced by temperature data from the summer (Figure 7), *P. resecata* usually live at approximately 14 – 19°C, whereas since *L. pallasii* live mostly terrestrially, and is probably usually found at slightly warmer temperatures, at least during the summer when this research took place. Therefore, the antioxidant enzymes of *L. pallasii* likely have better functionality at 25°C than that of *P. resecata*,

hence the experimentally observed significantly higher enzymatic activity of *L. pallasii* compared to that of *P. resecata*.

Although environmental conditions were not recorded in the habitat of *L. pallasii*, it can be assumed that they experience normoxic conditions because the cracks that they live in are wide enough for gas exchange, and they were found usually not more than 6 cm from the opening of the cracks. Although these animals crowd together in these cracks, they probably do not experience hypoxia or oxidative stress because of the nature of the wide cracks.

A given *L. pallasii* individual has a lower respiring mass relative to a *P. resecata* individual, and therefore a higher metabolic rate, presumably leading to increased endogenous ROS production. At dawn and dusk when *L. pallasii* run from the cracks in the rocks to the water to moisten their gills and to feed on algae, this activity may cause bursts of metabolic activity and lead to an increase in ROS production. If ROS production is predominantly endogenous from metabolic processes, these factors may lead to a higher need for antioxidant activity.

One other possibility for higher observed antioxidant capacity in *L. pallasii* may be the presence of gut fluid in the tissue samples. The dissection of *P. resecata* was clean and gut fluid did not seem to contaminate the tissues. However, the extraction of *L. pallasii* tissues without contamination from gut fluid was not as easy. Although the tissues were bathed in PBS and then blotted to remove gut fluid, it is possible that

residual gut fluid contaminated the tissue samples, which may have caused elevated antioxidant activity.

Although the results obtained were unexpected, they are supported by similar results in another study. Dann (2017) also found significantly lower levels of enzymatic and non-enzymatic antioxidants and lower total antioxidant capacity in the hemolymph of *P. resecata* hemolymph than in the hemolymph of *L. pallasii*.

Future directions

Helpful future research may include documentation of the amount of damage done by ROS in the tissues, such as by measuring lipid peroxidation or the accumulation of malondialdehyde (MDA). Or perhaps *P. resecata* simply do not encounter high ROS levels relative to *L. pallasii*; therefore, further research would quantify the amount of ROS in the tissues of these isopods. Perhaps differences in metabolism between *P. resecata* and *L. pallasii* played a role in inconsistent results. Other helpful research may include examining and comparing antioxidant activity of *P. resecata* to a more similar marine isopod species in the same genus such as *Pentidotea wosnesenskii*.

CONCLUSION

Although Padilla Bay exhibits high and fluctuating dissolved oxygen among *Z. marina* eelgrass beds in which *P. resecata* live, these isopods unexpectedly have significantly lower enzymatic antioxidant capacity compared to that of *L. pallasii*, which live in normoxic conditions and therefore presumably do not encounter high ROS. A number of factors may contribute to these unexpected results: differences in diet between *P. resecata* and *L. pallasii*, the possible ability of *P. resecata* to prevent diffusion of harmful ROS into the tissues, the employment of other defense mechanisms, the effect of temperature on enzyme activity, or the contamination from gut fluid in *L. pallasii* tissues. Future experiments may assess the damage done by ROS by measuring lipid peroxidation, quantify the amount of ROS *P. resecata* encounters, or compare antioxidant activity to a more similar marine isopod species.

ACKNOWLEDGEMENTS

LITERATURE CITED

Abele, D., & Puntarulo, S. (2004). Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *138*(4), 405-415.

Abele, D., Burlando, B., Viarengo, A., & Pörtner, H. O. (1998). Exposure to elevated temperatures and hydrogen peroxide elicits oxidative stress and antioxidant response in the Antarctic intertidal limpet *Nacella concinna*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *120*(2), 425-435.

Abele, D., Heise, K., Pörtner, H. O., & Puntarulo, S. (2002). Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. *Journal of Experimental Biology*, *205*(13), 1831-1841.

Anderson, S. J., Hunnicutt, F. E. & Cowles, D. L. (2017). Activity levels of *Pentidotea resecata* support conclusion that the animal is photosynthetic. Poster presented at ASLO Aquatic Sciences Meeting, Honolulu, HI.

Babin et al. (2010). Dietary supplementation with carotenoids improves immunity without increasing its cost in a crustacean. *The American Naturalist*, *176*(2), 234-241.

Belão, T. C., Leite, C. A. C., Florindo, L. H., Kalinin, A. L., & Rantin, F. T. (2011). Cardiorespiratory responses to hypoxia in the African catfish, *Clarias gariepinus* (Burchell 1822), an air-breathing fish. *Journal of Comparative Physiology B*, *181*(7), 905.

Brouwer, M., Brouwer, T. H., Grater, W., Enghild, J. J., & Thogersen, I. B. (1997). The paradigm that all oxygen-respiring eukaryotes have cytosolic CuZn-superoxide dismutase and that Mn-superoxide dismutase is localized to the mitochondria does not apply to a large group of marine arthropods. *Biochemistry*, *36*(43), 13381-13388.

Brusca, R. C., Brusca, G. J., & Haver, N. J. (2003). Invertebrates. 2nd ed. Sinauer Associates, Sunderland, MA. 936 pp.

Camus, L., & Gulliksen, B. (2005). Antioxidant defense properties of Arctic amphipods: comparison between deep-, sublittoral and surface-water species. *Marine biology*, *146*(2), 355-362.

Chabot, D., & Claireaux, G. (2008). Environmental hypoxia as a metabolic constraint on fish: the case of Atlantic cod, *Gadus morhua*. *Marine Pollution Bulletin*, *57*(6), 287-294.

Cowles, J. M. (2015). Does photosynthesis take place in the gut of *Pentidotea resecata*? M.S. thesis, Walla Walla University, College Place, WA.

Dann, L. E. (2017). Antioxidant capacity, pigmentation, and pro-phenoloxidase activity in the hemolymph of the marine isopod *Pentidotea resecata*. M.S. thesis, Walla Walla University, College Place, WA.

Davidson, J. F., & Schiestl, R. H. (2001). Mitochondrial respiratory electron carriers are involved in oxidative stress during heat stress in *Saccharomyces cerevisiae*. *Molecular and cellular biology*, *21*(24), 8483-8489.

Diaz, R. J. (2001). Overview of hypoxia around the world. *Journal of environmental quality*, *30*(2), 275-281.

Eberl, R. (2010). Sea-land transitions in isopods: pattern of symbiont distribution in two species of intertidal isopods *Ligia pallasii* and *Ligia occidentalis* in the Eastern Pacific. *Symbiosis*, *51*(1), 107-116.

Feierabend, J., Streb, P., Schmidt, M., Dehne, S., & Shang, W. (1996). Expression of catalase and its relation to light stress and stress tolerance. In *Physical Stresses in Plants* (pp. 223-234). Springer Berlin Heidelberg.

Finkel, T. (2011). Signal transduction by reactive oxygen species. *The Journal of cell biology*, *194*(1), 7-15.

Forstrom, J. W., Zakowski, J. J., & Tappel, A. L. (1978). Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine. *Biochemistry*, *17*(13), 2639-2644.

Froehlich, H. E., Gentry, R. R., & Halpern, B. S. (2016). Synthesis and comparative analysis of physiological tolerance and life-history growth traits of marine aquaculture species. *Aquaculture*, *460*, 75-82.

Gotceitas, V., Fraser, S., & Brown, J. A. (1997). Use of eelgrass beds (*Zostera marina*) by juvenile Atlantic cod (*Gadus morhua*). *Canadian Journal of Fisheries and Aquatic Sciences*, *54*(6), 1306-1319.

Harabin AL, Survanshi SS. (1993). A statistical analysis of recent Navy Experimental Diving Unit (NEDU) single-depth human exposures to 100-percent oxygen at pressure. Bethesda, M.D. Naval Medical Research Institute Report NMRI 93-59.

Heise, K., Puntarulo, S., Pörtner, H. O., & Abele, D. (2003). Production of reactive oxygen species by isolated mitochondria of the Antarctic bivalve *Laternulaelliptica* (King and Broderip) under heat stress. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *134*(1), 79-90.

Hideg, É., Jansen, M. A., & Strid, Å. (2013). UV-B exposure, ROS, and stress: inseparable companions or loosely linked associates? *Trends in plant science*, *18*(2), 107-115.

Hunnicutt, F. E., Anderson, S. J., & Cowles, D. L. (2016). Where do all the isopods go? A population study of *Pentidotea resecata*. Poster at Murdock Conference, Spokane, WA, November 2016.

Hylarides, M. J. (2015). Light level as a potential limiting factor in the growth of *Zostera marina* eelgrass in the Pacific Northwest. M.S. thesis. Walla Walla University, College Place, WA.

Kharlamenko, V. I., Kiyashko, S. I., Imbs, A. B., & Vyshkvartzev, D. I. (2001). Identification of food sources of invertebrates from the seagrass *Zostera marina* community using carbon and sulfur stable isotope ratio and fatty acid analyses. *Marine Ecology Progress Series*, *220*, 103-117.

Kim, C. S., Lee, S. G., Lee, C. K., Kim, H. G., & Jung, J. (1999). Reactive oxygen species as causative agents in the ichthyotoxicity of the red tide dinoflagellate *Cochlodinium polykrikoides. Journal of Plankton Research*, *21*(11), 2105-2115.

Lee, W. L., & Gilchrist, B. M. (1972). Pigmentation, color change and the ecology of the marine isopod *Idotea resecata* (Stimpson). *Journal of Experimental Marine Biology and Ecology*, *10*(1), 1-27.

Lesser, M. P. (2006). Oxidative stress in marine environments: biochemistry and physiological ecology. *Annu. Rev. Physiol.*, *68*, 253-278.

Livingstone, D. R. (2003). Oxidative stress in aquatic organisms in relation to pollution and aquaculture. *Revue de Medecine Veterinaire*, *154*(6), 427-430.

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy reviews*, *4*(8), 118.

Lushchak, V. I., Bagnyukova, T. V., Husak, V. V., Luzhna, L. I., Lushchak, V., & Storey, K. B. (2005). Hyperoxia results in transient oxidative stress and an adaptive response by antioxidant enzymes in goldfish tissues. *The international journal of biochemistry & cell biology*, *37*(8), 1670-1680.

Lushchak, V. I., Lushchak, L. P., Mota, A. A., & Hermes-Lima, M. (2001). Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *280*(1), R100-R107.

Martin, J. L. (2016). Collecting and processing crustaceans: an introduction. J. Crustacean Biology 36(3), 393-395.

McLarty, S. (2015). Gut content and pigment analysis in the marine isopod *Pentidotea resecata.* M.S. thesis. Walla Walla University, College Place, WA.

Morris, R. H., Abbott, D. P., & Haderlie, E. C. (1980). Intertidal invertebrates of California. Stanford University Press, Stanford, CA. 690 pp.

Nilsson, G. E., & Östlund-Nilsson, S. (2004). Hypoxia in paradise: widespread hypoxia tolerance in coral reef fishes. *Proceedings of the Royal Society of London B: Biological Sciences*, *271*(Suppl 3), S30-S33.

Ng, P. K. L. (2017). Collecting and processing freshwater shrimps and crabs. J. Crustacean Biology 37(1), 119-121

NOAA National Estuarine Research Reserve System (NERRS). System-wide Monitoring Program. Data accessed from the NOAA NERRS Centralized Data Management Office website: http://cdmo.baruch.sc.edu/.

Parrilla-Taylor, D. P., & Zenteno-Savín, T. (2011). Antioxidant enzyme activities in Pacific white shrimp (*Litopenaeus vannamei*) in response to environmental hypoxia and reoxygenation. *Aquaculture*, *318*(3), 379-383.

Penhale, P. A. (1977). Macrophyte-epiphyte biomass and productivity in an eelgrass (*Zostera marina* L.) community. *Journal of Experimental Marine Biology and Ecology*, *26*(2), 211-224.

Potters, G., Pasternak, T. P., Guisez, Y., Palme, K. J., & Jansen, M. A. (2007). Stress-induced morphogenic responses: growing out of trouble? *Trends in plant science*, *12*(3), 98-105.

Sand-Jensen, K., Pedersen, O., Binzer, T., & Borum, J. (2005). Contrasting oxygen dynamics in the freshwater isoetid *Lobelia dortmanna* and the marine seagrass *Zostera marina*. *Annals of Botany*, *96*(4), 613-623.

Sharma, P., Jha, A. B., Dubey, R. S., & Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany*, *2012*.

Short, F., Carruthers, T., Dennison, W., & Waycott, M. (2007). Global seagrass distribution and diversity: a bioregional model. *Journal of Experimental Marine Biology and Ecology*, *350*(1), 3-20.

Storey, K. B. (1996). Oxidative stress: animal adaptations in nature. *Brazilian Journal of Medical and Biological Research*, *29*, 1715-1733.

Suzuki, N., & Mittler, R. (2006). Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. *Physiologia plantarum*, *126*(1), 45-51.

Welker, A. F., Moreira, D. C., Campos, É. G., & Hermes-Lima, M. (2013). Role of redox metabolism for adaptation of aquatic animals to drastic changes in oxygen availability. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *165*(4), 384-404.

Wu, R. S. (2002). Hypoxia: from molecular responses to ecosystem responses. *Marine Pollution Bulletin*, *45*(1), 35-45.

APPENDIX I

<u>Observations from field research</u>

From observations in the field, *P. resecata* tend to have many diatoms growing on them. Subjectively, we mostly observed *P. resecata* clasped onto the underside of the blade of the eelgrass when they were undisturbed. Perhaps these isopods live on the underside of the eelgrass blade to limit exposure to UV or to keep diatoms from growing on them.

P. resecata were rarely seen swimming, except when they were dislodged from the eelgrass by our nets, suggesting that they may have low activity, which may result in low metabolism. In controlled experiments done by colleagues, *P. resecata* mostly remained still and moved slowly in a tank during the day and at night, suggesting low metabolic rates (Anderson et al. 2017).

Although hemolymph color was unusually bright green for a crustacean, tissue color was clear and didn't seem to have any pigments. The cuticle color also seemed to be nearly clear, therefore the color of the animal comes primarily from its brightly colored hemolymph as a result of its consumption of *Z. marina*, unlike the description noted previously in the literature (Lee and Gilchrist 1972).

APPENDIX II

The date, time of day, tidal height, and weather conditions on the days that oxygen and temperature profiles were collected at four locations in Padilla Bay, corresponding to the profiles in Figures 6 and 7.

	Date	Time	Tide (ft)/(m)	Weather
A	Jul 29	3:30 PM	+7.0/+2.1	Sunny
B	Aug 9	6:15 AM	+3.0/+0.9	Cloudy
C	Aug 4	1:00 PM	-0.8/-0.2	Sunny
D	Jul 31	8:45 AM	-1.5/-0.5	Cloudy