# ANTIOXIDANT CAPACITY IN THE HEMOLYMPH OF THE MARINE ISOPOD *PENTIDOTEA RESECATA*

By

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**ABSTRACT**

The isopod *Pentidotea resecata* inhabits *Zostera marina* eelgrass beds. Examination of oxygen levels in a *Z. marina* bed indicated that *P. resecata* frequently experience hyperoxia and potential hypoxia reperfusion events in these beds, which may lead to enhanced reactive oxygen species (ROS) production and increased oxidative damage if the antioxidant defenses cannot sufficiently suppress these toxic oxygen intermediates. The total antioxidant capacity of *P. resecata* hemolymph was compared to that of *Ligia pallasii*, a semi-terrestrial isopod living in normoxic conditions, and to that of *Pandalus danae*, a shrimp that lives below the photic zone. The hypothesis was that *P. resecata* hemolymph would have stronger antioxidant defenses than the other crustaceans because this isopod faces a more hostile oxygen environment. LCMS analysis of *P. resecata* hemolymph confirmed the presence of antioxidants including pheophorbide a, lutein, and β-carotene, while *L. pallasii* hemolymph contained pheophorbide a and lutein but no β-carotene. *Pandalus danae* hemolymph had no carotenoids or pheophorbide. Although *L. pallasii* hemolymph was missing β-carotene, it had a significantly higher total antioxidant capacity than that of *P. resecata*. Hemolymph from *P. danae* had an intermediate antioxidant capacity even though it contained none of the antioxidants detected in the other species. The unexpected antioxidant activities among the species could be explained by differences in metabolic functions or environmental factors that were not examined in this study; or perhaps *P. resecata* isopods use alternate mechanisms to mitigate oxidative damage.

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## INTRODUCTION

*Reactive Oxygen Species*

Oxygen, although essential for aerobic organisms, often converts to toxic reactive forms called reactive oxygen species (ROS), which can severely damage an organism. ROS are created when oxygen is partially reduced, and include free radicals, which are molecules that contain an unpaired electron, as well as other reduced oxygen intermediates. Some common reactive oxygen species and molecules with similar properties include superoxide radicals ($O_2$-), singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$), hydroxyl radicals (HO•), and nitric oxide (•NO) (Lesser, 2006).

Although ROS participate in normal cellular functions, in excess amounts or in inappropriate locations they considerably damage cells and tissues. About 0.2 percent of oxygen acquired by cells during respiration is converted to ROS that are required for cell functions such as cell signaling, immunity, and redox reactions. However, surplus ROS can have deleterious effects when they react with cellular components, often causing apoptosis and organ malfunction (Li et al., 2016). Lipid peroxidation, which adversely affects membrane fluidity and can also negatively affect enzyme function and ATP production in the mitochondria, is often associated with oxidative stress. The cytotoxic effects of lipid peroxidation commonly trigger apoptosis. Oxidative damage can also alter amino acid structure and change electrical charges within a cell, promoting inappropriate peptide associations and causing a higher chance of protein degradation and enzyme dysregulation. Reactive oxygen species may also cause genetic changes in DNA by creating lesions and mutations (Lesser, 2006).

Some ROS are produced endogenously within an organism through aerobic respiration processes, particularly during normal function of the electron transport chain in the mitochondria as well as during detoxification processes in the endoplasmic reticulum (Li et al., 2016). However, production of ROS is enhanced under several physiological and environmental conditions. One such condition is temporary oxygen limitation to the tissues (hypoxia) followed by reperfusion of oxygenated blood. This reperfusion can lead to ROS generation as the mitochondrial electron transport chain restarts (Clanton, 2007). During hypoxia, electrons accumulate and can react with any available oxygen. During reoxygenation, an increased influx of oxygen will react with any accumulated electrons, leading to an increase in ROS. For example, the shrimp *Litopenaeus vannamei* experiences increased oxidative stress during reperfusion events, particularly DNA damage in the hemolymph, gills, and hepatopancreas (Li et al., 2016).

Another condition that enhances ROS production is exposure to unusually high concentrations of oxygen (hyperoxia). Hyperoxia can be problematic because as oxygen levels increase, so does the concentration of ROS (Lesser, 2006). Studies of some arthropods have shown that hyperoxia can cause reduced tolerance to environmental stressors, most likely due to the increased formation of ROS, which leads to oxidative stress (Verberk et al., 2016). Hyperoxia can lead to increased oxidative damage to the tissues. In goldfish, for example, exposure to hyperoxia and normoxic recovery led to an increase in protein carbonyl formation during both the hyperoxic and the recovery periods, indicating elevated oxidative damage during hyperoxia. Peroxides and thiobarbituric acid (byproducts of lipid peroxidation and indicators of oxidative stress)

also accumulated during initial exposure to hyperoxia (Lushchak et al. 2005). Other fish such as salmon also accumulated thiobarbituric substances following long term moderate exposure to hyperoxia (considered to be 140 – 150% saturation), indicating hyperoxia-induced oxidative stress. An increase in oxygen saturation to 280% for a week resulted in severe mortality (Lygren et al., 2000).

Studies have shown that both hypoxia and hyperoxia lead to a change in antioxidant levels in fish species, which indicates that antioxidant capacity can be regulated under different environmental conditions (Lushchak & Bagnyukova, 2006). Environmental factors other than hypoxia and hyperoxia that affect ROS formation in organisms inhabiting marine environments include stressful conditions such as unfavorable or fluctuating water pH, temperature, ultra violet radiation (UVR), and salinity (Lesser, 2006).

Examples of pro-oxidative habitats include polar surface waters (Camus & Gullisken, 2005), hydrothermal vents (Lesser, 2006), and highly photosynthetic marine environments such as plankton blooms (Kim et al., 1999), algae beds, and seagrass beds (Lesser, 2006). Hyperoxia due to primary production can cause an increased photochemical generation of ROS in the sea water, especially when UVR reacts with dissolved organic matter to form ROS. ROS in the water column can diffuse into marine organisms, especially during respiration, and disturb the balance between ROS and antioxidants (Lesser, 2006). For this reason, highly productive marine habitats such as *Zostera marina* eelgrass beds may contain harmful concentrations of ROS.

*Antioxidants*

Antioxidants are an important defense against ROS. They prevent ROS formation by soaking up excess electrons, quench ROS by donating electrons to molecules with unpaired electrons, and break down ROS into less harmful forms. These processes prevent ROS from reacting with cell components and causing oxidative damage. Non-enzymatic antioxidants include plant pigments such as carotenoids, which are produced by plants but must be acquired by animals through diet. They protect both plants and animals against oxidative damage. Carotenoids effectively quench ROS, largely because of their molecular structure, which consists of highly conjugated double bond systems. These bonds allow carotenoids to donate electrons to reduce ROS and to soak up extra electrons to prevent ROS formation without becoming reactive themselves (Lesser, 2006). Carotenoid pigments include carotenes and xanthophylls. Xanthophylls play a substantial role in photoprotection via the interconversion between the following three xanthophylls: violaxanthin, antheraxanthin, and zeaxanthin (Young, 1991). Chlorophyll and chlorophyll derivatives such as pheophytin and pheophorbide a also have some antioxidant activity (Lanfer-Marquez et al., 2005; Hsu et al., 2013; Cho et al., 2011).

Ascorbic acid, another type of non-enzymatic antioxidant, can be synthesized by all plants and by most animals (Lesser, 2006). Many crustacean species have at least a limited capability to synthesize their own ascorbic acid, however in some species, dietary supplementation is required in order for them to have sufficient ascorbic acid levels (Dawood & Koshio, 2016). Ascorbic acid directly scavenges reactive oxygen species without enzyme catalysts by donating an electron to a ROS, and can indirectly scavenge

by converting alpha tocopherol to its reduced form so that it can then oxidize ROS and neutralize them. Tocopherol, which is also an antioxidant, is made by plants but must be obtained by animals through their diet. Glutathione, a tripeptide found in both animals and plants, also has antioxidant activity. There are also small molecule antioxidants such as uric acid, mannitol, dimethylsulfide, and mycosporine-like amino acids. Some mycosporine-like amino acids (MAAs) not only scavenge free radicals, they also absorb UVR, preventing the formation of new ROS. Most are synthesized in plants and are obtained through diet in animals. An interesting study showed that the concentration of MAAs in reef building corals increases with decreasing depth, which correlates in proportion with an increase in photo-oxidative potential due to increased UVR and to hyperoxia exposure from photosynthesis. This correlation between antioxidant concentration and photo-oxidative potential suggests that some animals can change their antioxidant capacity based on environmental factors (Lesser, 2006).

Antioxidants produced by algae and plants, including carotenoids, flavonoids, ascorbate, and MAAs, play an important role in blocking UVR and quenching ROS. In response to high levels of UVR, which increases ROS production and causes DNA damage, these antioxidants accumulate in plants and algae, providing increased levels of photoprotection. Studies indicate that similar photoprotective effects occur in animals that eat the antioxidant-producing plants and algae. For example, *Tigriopus brevicornis*, a marine copepod that often experiences intense UVR in the upper intertidal zone, has an increased tolerance to UVR (determined by an increased survival time during UVR exposure) when fed UVR-acclimated algae compared to those eating yeast or non UVR-

acclimated algae. This differential tolerance indicates that dietary metabolites, possibly including carotenoids and chlorophyll derivatives, are important for photoprotection in some crustaceans (Corcora et al., 2016). These plant pigments may also have a role in protecting isopods such as *Pentidotea resecata*, the species of interest in this study, from oxidative damage.

In addition to non-enzymatic antioxidants, most organisms also have enzymatic antioxidants. Some examples of enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), and peroxidases such as glutathione peroxidase (GPx). SOD neutralizes superoxide anion radicals, converting them into oxygen and hydrogen peroxide. CAT and peroxidases catalyze the conversion of hydrogen peroxide to water and oxygen, creating less harmful forms of oxygen. GPx catalyzes the oxidation of the tripeptide thiol glutathione with hydrogen peroxide, reducing hydrogen peroxide to water (Lesser, 2006).

*Antioxidant Capacity*

An organism's total antioxidant capacity (TAC) represents the sum of all antioxidant activity in the organism, including the activity of both enzymatic and non-enzymatic antioxidants. TAC can be regarded as the ability of an organism to suppress ROS. There are many examples of animals adjusting their antioxidant capacity in response to environmental conditions. Some sponges and lugworms increase total oxidative scavenging capacity and activity of antioxidant enzymes such as catalase seasonally during the summer months, when UVR and ROS levels are high. This increase in ROS is largely due to a temperature-correlated increase in mitochondrial substrate

oxidation and electron transport chain proton leakage in the lugworms, and to elevated tissue $pO_2$ in the sponges from increased photosynthesis by their symbiotic cyanobacteria (Lesser, 2006).

Antioxidant capacity can change when an organism is exposed to variable environmental oxygen concentrations. For example, goldfish that experienced hyperoxia showed an initial increase in lipid peroxidation byproducts such as lipid peroxidases and thiobarbituric acid, followed by a reduction of these byproducts, indicating that prolonged exposure to hyperoxia led to increased defenses against lipid peroxidation or to increased catabolism of these toxic byproducts. Examination of enzymatic antioxidants showed increased activity of both catalase and GPx during normoxic recovery from hyperoxia, which suggest that these fish have the ability to adjust the activity of enzymatic antioxidants during and after hyperoxic events (Lushchak et al., 2005). *Litopenaeus vannamei* shrimp significantly upregulate SOD and GPx activity in the gill tissue and hemolymph during hypoxia and during the early phases of reoxygenation in order to suppress the accompanying increase in ROS generation (Li et al., 2016). Some other animals that frequently experience hypoxia have continuously high antioxidant capacities to cope with reoxygenation. In order to survive, it is imperative for an organism to have enough antioxidants to balance out ROS concentrations to avoid severe tissue damage (Geihs et al., 2016). Because crustaceans live in a wide range of habitats and are fairly sensitive to oxygen concentrations, as they seem to have lower hypoxia tolerance than many other aquatic groups (Vaquer-Sunyer & Duarte, 2008), there is most

likely an extensive range of adaptations utilized by different species in response to varying oxygen concentrations (Geihs et al., 2016).

*Immunity*

The crustacean immune system utilizes the generation and quenching of ROS to destroy pathogens without harming self tissues. Isopod immune responses are not well studied. Most crustaceans use phenoloxidase, a hemocyanin derivative, to destroy pathogens. Phenoloxidase catalyzes the destruction of pathogens by initializing a series of chemical reactions that ultimately lead to the formation of melanin. During this process, pathogens are bombarded with high concentrations of ROS and other cytotoxic molecules, which often directly kill the pathogens. Pathogens can also be encapsulated and destroyed by the melanin produced during the phenoloxidase cascade (Babin et al., 2010). Crustaceans also have phagocytic hemocytes that can engulf pathogens (Roth et al., 2010). Not only is the phenoloxidase system important during immune responses, it also has a major role in sclerotizing the crustacean exoskeleton (King et al., 2010).

The phenoloxidase immune cascade is activated in response to foreign antigens such as microbial or viral pathogens. This cascade leads to a rapid generation of melanin, quinone metabolites, and ROS at concentrations that are often lethal to small pathogens, making phenoloxidase an effective immune defense (Babin et al., 2010). However, phenoloxidase activity can also lead to auto-reactivity, since high concentration of ROS can cause excessive oxidative damage to self tissues. Organisms such as scallops and crabs that frequently experience increased ROS and oxidative stress have depressed

phenoloxidase activity and overall immune impairment, most likely because of the extreme oxidative damage that can result from the phenoloxidase immune cascade (Hannam et al., 2010; Tanner et al., 2006). In order to prevent auto-reactivity, antioxidants such as CAT and SOD are often upregulated during the immune response (Gopalakrishnana et al., 2011). *Gammarus pulex* amphipods have enhanced phenoloxidase activity when their diet is supplemented with carotenoid antioxidants compared to those that do not receive carotenoid supplementation. This elevated phenoloxidase activity was accompanied by an increase in hemolymph carotenoids. The upregulation of phenoloxidase is attributed to the antioxidant properties of the dietary carotenoids, and this upregulation allows increased melanization of pathogens. Carotenoids are also thought to be the reason that the enhanced phenoloxidase activity was not accompanied by a decrease in survival during infection (Babin et al., 2010). It is possible that isopods that experience increased oxidative stress, such as those living in *Zostera marina* eelgrass beds, may downregulate phenoloxidase in order to reduce additional oxidative stress that would otherwise be generated by the phenoloxidase cascade, leading to lower immunity and a weaker anti-pathogen response. However, the presence of antioxidants such as carotenoids may help alleviate ROS damage produced by the phenoloxidase cascade, allowing phenoloxidase activity to be maintained during an immune response, and potentially having an immunostimulatory effect.

*Zostera marina as a Producer of ROS*

*Zostera marina*, a species of eelgrass, is a submerged monocot that lives in shallow coastal marine and estuarine environments (Fonseca & Uhrin, 2005). It is found in the northern temperate zone, and extends into the Arctic Circle (Bellingham et al., 2003). *Z. marina* is prevalent in the temperate Atlantic, the Mediterranean, and the temperate North Pacific from Alaska to Baja California (Short et al., 2007). This vascular plant has an underground root and rhizome system, and lives in sandy or muddy sediments. *Z. marina* needs light for photosynthesis, and therefore can only grow in depths that allow it to photosynthesize. It is also susceptible to desiccation, and thus needs to live deep enough where it has at least some water coverage most of the time (Fonseca & Uhrin, 2005). Through the processes of photosynthesis and respiration, *Z. marina* influences oxygen levels in the surrounding environment, releasing oxygen during the day and consuming it at night. Oxygen concentrations in the water column surrounding *Z. marina* can vary depending on the rate of photosynthesis, which is largely dependent on light levels (Sand-Jensen et al., 2005). Oxygen levels can also change with water currents (Hume et al., 2011). In one study, oxygen concentrations in an eelgrass bed varied from 15 to 36 kPa (~ 80-125% of oxygen saturation in air) throughout the day (Sand-Jensen et al., 2005).

*Z. marina* beds are productive marine habitats that provide an important environment for a variety of species to live, feed, and breed (Fonseca & Uhrin, 2005), including the isopod species of primary interest in this thesis, *Pentidotea resecata*. These

animals are potentially exposed to high and fluctuating oxygen levels present in these photosynthetic eelgrass beds.

*Pentidotea resecata*

Isopods, an order of crustaceans, have diverse lifestyles and physical characteristics. They live in a variety of habitats in marine, freshwater, and terrestrial environments. Different isopod species have a wide array of feeding preferences, including herbivorous and omnivorous scavengers, parasites, plant feeders, and detritivores. They can range in size from 0.5 to 500 mm long (Brusca & Brusca, 2003). *Pentidotea resecata* (Figure 1), previously classified as *Idotea resecata*, is a coastal marine isopod species that typically resides in rocky littoral habitats with prominent algae cover (Brusca & Wallerstein, 1977) or in seagrass beds, especially in bays and estuaries (Lee & Gilchrist, 1972). Its geographic range spans most of the North American Pacific Coast, from southeast Alaska to Baja California (Brusca & Wallerstein, 1977). This isopod is fairly large and can grow over 6.0 cm long (McLarty, 2015; Cowles, 2015). Common substrates that it lives on include *Macrocystis pyifera*, a brown-colored kelp, and *Z. marina*, a green eelgrass. The overall color of the individuals of this species closely matches the color of the substrate on which they live and feed. Some studies have found that the brown color morph exclusively lives on *Macrocystis* and the green color morph is found only on eelgrass. This exclusivity suggests that they may retain pigments from the eelgrass or algae on which they live. The animals used in this study were green (Figure 1) and lived on *Z. marina.* Other factors that influence the coloration of


**Figure 1. The isopod *Pentidotea resecata.*** (A) The dorsal side of the isopod. (B) The ventral side of the animal. Note the branchial chamber at the posterior end of the isopod.

*P. resecata* include chromatophore expansion, as well as cuticle and body color (Lee & Gilchrist, 1972). The hemolymph of the animals used in this study which, unlike most crustaceans is green in color, may also influence isopod coloration (McLarty, 2015).

According to one study, *P. resecata* whole animal extracts contain numerous plant pigments, many of which have antioxidant properties. Pigments found in whole body extracts of this isopod included ten carotenoids (alpha and beta carotene, echinenone, canthaxanthin, mono-hydroxyl-beta carotene, 4-hydroxy-4'-keto-beta carotene, lutein, zeaxanthin, violaxanthin, flavoxanthin). Xanthophylls seemed to be the most abundant type of carotenoid, especially lutein (Lee & Gilchrist, 1972). These isopods may also retain chlorophyll and chlorophyll breakdown products (McLarty, 2015). Many of these pigment molecules scavenge ROS (Young, 1991; Lanfer-Marquez et al., 2005), thus serving as antioxidants. Retention of plant pigment antioxidants may be important in mitigating the damaging effects of ROS in *P. resecata*, as these isopods potentially live in super-oxygenated environments and have thin bodies that light can penetrate, as well as oxygen diffusion through the gills and cuticle, making them susceptible to oxygen toxicity.

Gas exchange in these isopods occurs mainly across the rami of the ten biramous pleopods, which are located below the abdomen in the branchial chamber (Figure 1B). Gas exchange usually occurs across the flat endopods of the pleopods, which have a thin cuticle to facilitate gas diffusion. The uropods of *P. resecata* and other valviferans meet at the ventral midline to form an operculum, which covers the branchial chamber to protect the gills (Ruppert et al., 2004).

Oxygen transport from the gills occurs via hemocyanin, an oxygen transporting molecule dissolved in the hemolymph of *P. resecata.* Hemolymph bathes the isopod tissues in an open circulatory system. It contains many proteins, hemocytes, and other components necessary for circulatory and immune functions. Hemolymph is typically clear or light blue in color due to oxygenated hemocyanin (Bolton et al., 2009). The hemolymph of *P. resecata* in eelgrass beds, however, seems to have an unusual green color, as well as some fluorescence such as would be seen in chlorophyll (McLarty, 2015). This color is possibly due to retention of eelgrass pigments in the hemolymph. These pigments may serve as non-enzymatic antioxidants. The hemolymph would be an ideal location to store antioxidants, as it is exposed to high levels of oxygen that could potentially become toxic, and it circulates throughout the body and bathes all the organs and tissues.

*Ligia pallasii*

*Ligia pallasii* is a semi-terrestrial isopod species ranging from the Aleutian Islands to northern California (Figure 2). Individuals can grow up to 6.0 cm long. This species, although semi-terrestrial, must live in moist environments, as gas exchange occurs across the pleopods which must be damp in order to work properly. They typically live in supralittoral environments, but rarely range more than a few meters above the high tide line because they are prone to evaporative water loss that can lead to desiccation and death. These animals will feed in the upper intertidal region when the tide is low, but will not completely submerge themselves because they must breathe air. They typically eat

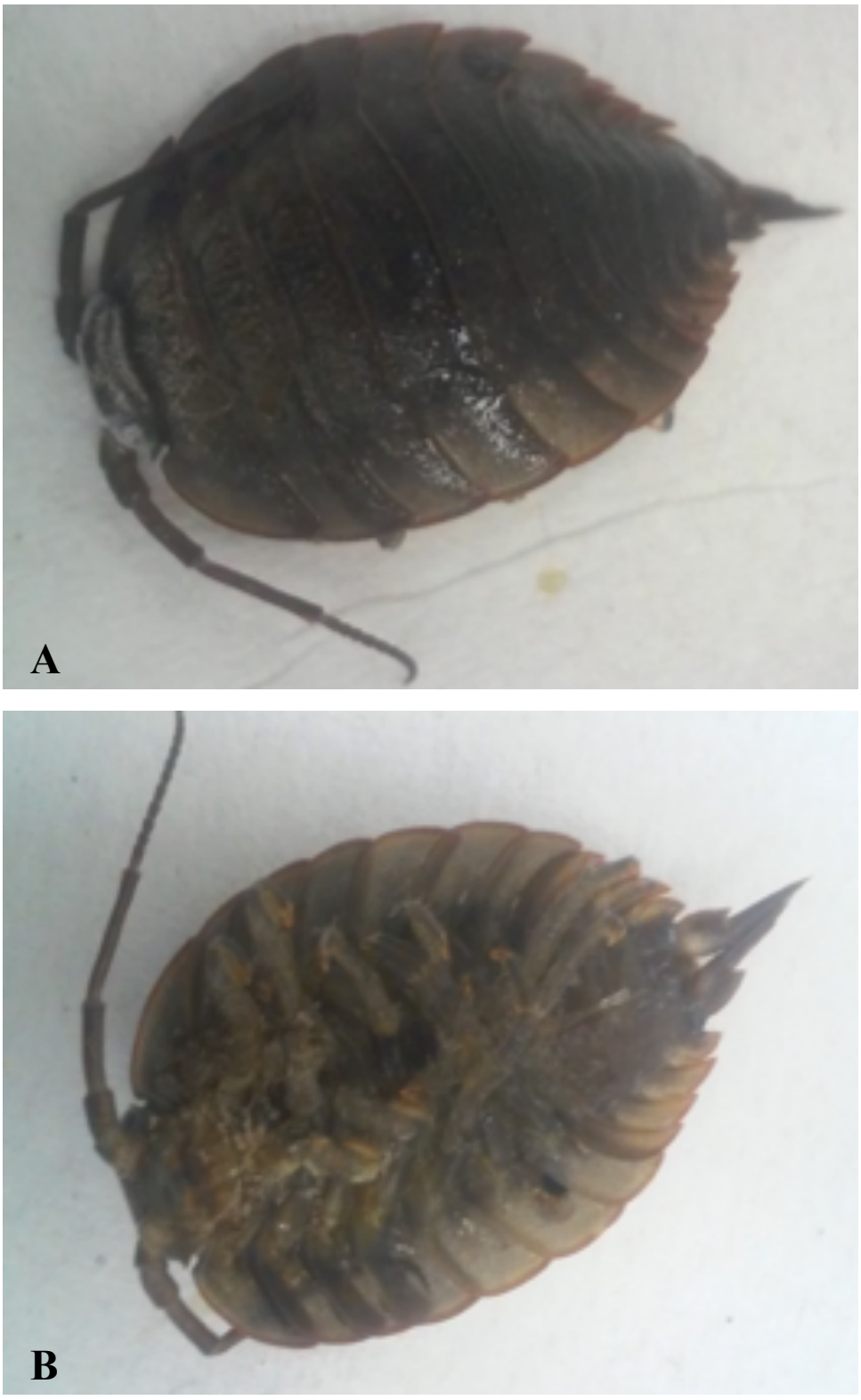


**Figure 2. *Ligia pallasii* isopod.** (A) Dorsal side. (B) Ventral side.

seaweeds and encrusting diatoms, and usually feed at night, spending most of the daylight in cracks in vertical cliffs or under rocks (Carefoot et al., 2000). Therefore, this isopod species, though continually exposed to oxygen at normal atmospheric pressure, is unlikely to have any substantial exposure to the hyperoxic or hypoxic conditions that *P. resecata* may experience.

*Pandalus danae*

*Pandalus danae,* commonly known as the dock shrimp or coonstripe shrimp, can also be found from Alaska to southern California (Figure 3). These crustaceans typically live subtidally on sandy or gravel ocean floors at depths from 10-180 meters. They can also be found on dock and jetty pilings (Bauer, 1975). *Pandalus danae* used in this study were collected from about 100 m depth, which is below the photic zone. No photosynthesis occurs at the depth from which these shrimp were collected, making it unlikely that these animals experience high levels of oxygen or UVR. They therefore probably experience low levels of ROS compared to *P. resecata.*

**Figure 3. *Pandalus danae* shrimp.**

*Objectives & Hypotheses*

The objectives of this study were to 1) determine whether *P. resecata* experience oxygen conditions that are conducive to the formation of high levels of endogenous ROS, 2) compare the total antioxidant capacity and phenoloxidase activity of *P. resecata* hemolymph to that of *L. pallasii* and *P. danae,* and 3) determine whether the hemolymph of these crustaceans contains chlorophyll and carotenoid antioxidants. I hypothesized that *P. resecata* would experience stressful environmental conditions, and would therefore have much higher hemolymph antioxidant activity than the other species. The antioxidant defenses of *P. resecata* could be aided by a variety of plant pigment antioxidants in the hemolymph, which the other crustaceans may not have. Higher antioxidant activity may also allow for a stronger phenoloxidase response because it reduces the risk of autoimmunity. I expected that *P. resecata* would have the highest antioxidant capacity and that the species with the highest TAC would also have the strongest phenoloxidase activity.

## METHODS

*Study Site*

Padilla Bay, the study site from which *P. resecata* were collected and oxygen profiles were measured, is a part of the Salish Sea located north of Bay View, Washington (Figure 4). It is a large, shallow protected bay that contains one of the most extensive eelgrass beds on the west coast of North America—about 3,200 hectares of eelgrass, although the distribution varies seasonally. The predominant eelgrass species in Padilla Bay is *Z. marina*, which supports a wide diversity of fauna. The eelgrass, which is found intertidally from -3.0 m to +0.8 m, remains fully submerged in deeper areas of the bay, but in shallower areas it is often exposed to air, especially during low tides (Bulthuis & Shull, 2006).

*Oxygen Profiles*

To ascertain the oxygen environment routinely experienced by *P. resecata,* vertical depth profiles of oxygen saturation in a *Z. marina* bed located in Padilla Bay, WA and inhabited by the isopods were determined at various tides and light levels using a YSI 550A oxygen field electrode. Oxygen and temperature readings were taken at low, high, and medium tides (ranging from -0.52 m to +2.44 m); at dawn, mid-morning, and afternoon (ranging from 6:20am to 4:00pm); and on cloudy and sunny days. Data for each of the various tide and light conditions were collected. Measurements were taken by wading into Padilla Bay when the tide was low enough or by using a boat when the tides were higher. Oxygen saturation was read at depth increments of 10 or 20 cm until the

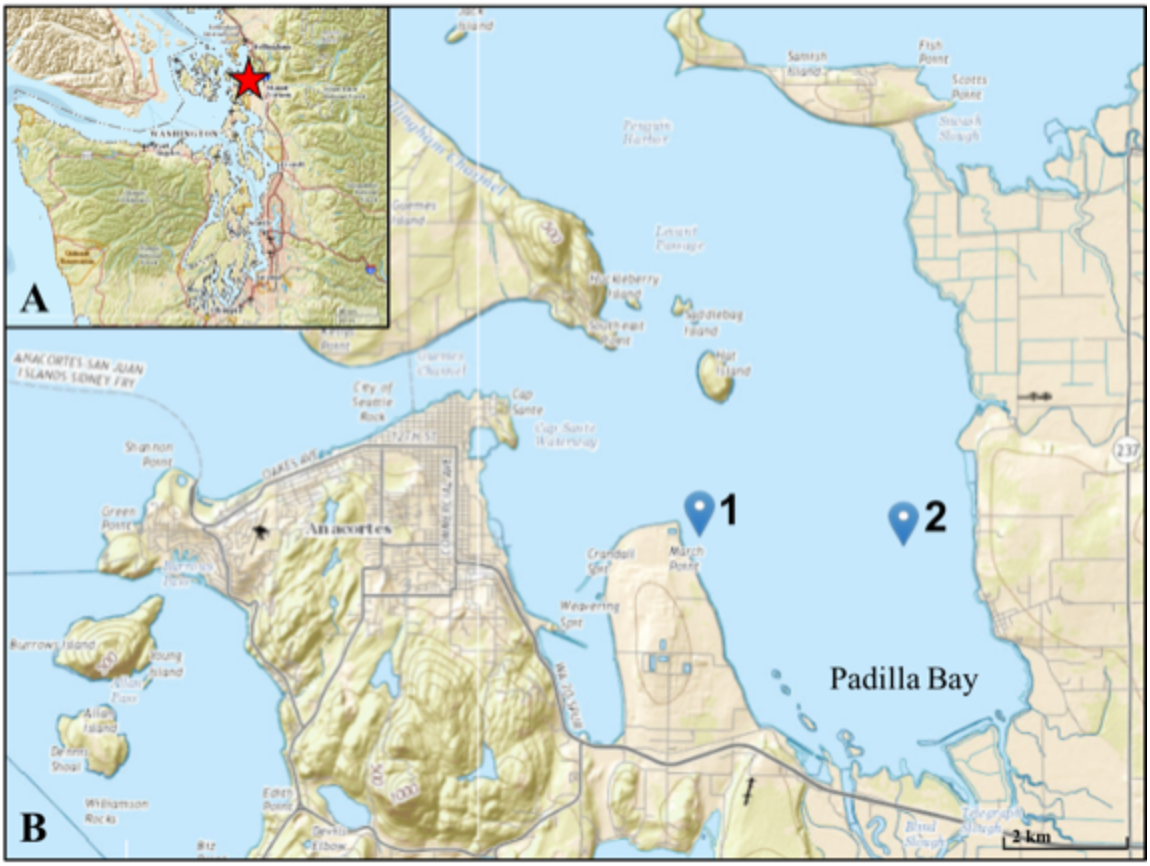


**Figure 4. Map of Padilla Bay.** The inset (A) shows the location of Padilla Bay in Washington State. The main map (B) shows a zoomed in image of Padilla Bay. Location 1 shows the study site at which oxygen profile measurements were taken and from which *P. resecata* were collected (Latitude/Longitude: 48.49765 N 122.55216 W). Location 2 shows the location of the Padilla Bay National Estuarine Research Reserve sonde, where annual oxygen data were collected (Latitude/Longitude: 48.4963 N 122.5005 W). Maps were taken from the US Geological Survey topographic maps.

probe reached the bottom sediment. Light levels were measured in photosynthetic photon flux density units (μmol photons $m^{-2}$ $sec^{-1}$) using a $360^{o}$ light sensor (Li-cor spherical sensor) connected to a Li-cor LI-1400® data logger. All measurements were taken during July and August of 2016. Year-long records of oxygen concentrations in Bayview Channel, a narrow channel near eelgrass beds and located about 3.8 km from the sampling site, were also downloaded from a YSI 6600 sonde located -1.1 m below the zero tide line and operated by the Padilla Bay National Estuarine Research Reserve (Figure 4) (Porter et al., 2016).

*Animal & Hemolymph Collection*

All animals were collected during the months of June, July, and August 2016. *P. resecata* isopods were taken from the eelgrass bed at Padilla Bay (Latitude/Longitude: 48.49765 N 122.55216 W) by dragging a hand net through the eelgrass during low tides and by trawling a plankton net to catch them from a boat at higher tides. Only animals greater than 3.0 cm in length were used because smaller animals had inadequate hemolymph volume. Animals big enough to use were predominantly males. Isopods were immediately transported to the Rosario Beach Marine Laboratory in an aerated container and placed in ice-cold seawater to make them lethargic prior to dissection. *P. resecata* were killed by using a razor blade to decapitate them approximately at the eyes, ahead of the gut to allow hemolymph to be drained without any gut fluid leaking out. Hemolymph was dripped into a microcentrifuge tube, which was then placed on dry ice until all

dissections were complete, at which point samples were stored in a -80 $^{o}$C freezer (about 20 - 250 µL of hemolymph was extracted per individual).

*L. pallasii* isopods were collected from cracks in rock faces on Northwest Island, WA (Latitude/Longitude: 48.4234 N 122.6539 W) and taken to the lab, where they were killed in a dry ice chamber because it was too difficult to decapitate them without the gut fluid mixing with hemolymph. Hemolymph was extracted with a syringe in order to extract only hemolymph and not gut fluid, placed in microcentrifuge tubes on dry ice, and stored at -80 $^{o}$C (about 20 - 250 µL of hemolymph was collected per individual).

*P. danae* shrimp were collected subtidally by otter trawl at a depth of about 100 m in San Juan Channel (Latitude/Longitude: 48.6479 N 122.1349 W). They were transported to the Rosario Beach Marine Laboratory in an aerated cooler and killed in a sealed container filled with seawater and dry ice. The tip of the tail was removed, as it was the easiest location from which to collect exclusively hemolymph, and hemolymph was dripped into a microcentrifuge tube on dry ice and stored in a -80 $^{o}$C freezer (about 100 - 400 µL of hemolymph was collected per individual). Due to the small hemolymph sample size, different individuals were typically used for each assay and pigment analysis.

*Total Antioxidant Capacity Assay*

TAC assays quantitatively measure the ability of a sample to quench reactive oxygen species, allowing the overall sum of all antioxidants (both enzymatic and non-enzymatic) in a sample to be determined. A common method used to quantify TAC

entails attempting to create a reactive oxygen species and measuring the ability of the sample to suppress or inhibit formation of this ROS. Cayman's Antioxidant Assay (used in this study) measures the capability of antioxidants in a sample to prevent the oxidation of 2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate], or ABTS , to $ABTS^{*+}$, a radical cation, by metmyoglobin and hydrogen peroxide. The less $ABTS^{*+}$ produced, the higher the antioxidant capacity. The concentration of $ABTS^{*+}$ can be measured at an absorbance of 750 nm, and this concentration is proportional to the concentration of antioxidants. Values are compared to a standard curve of known concentrations of Trolox (a tocopherol/vitamin E antioxidant analogue) and are representative of the total antioxidant capacity of the sample.

The Cayman Chemical Antioxidant Assay Kit® was used to measure the TAC of the hemolymph, providing a quantitative measure of the hemolymph's ability to suppress ROS formation. Reagents were prepared immediately prior to conducting the assay according to the directions provided in the manufacturer's assay protocol. Hemolymph was thawed on ice and centrifuged at 4 $^{o}$C for 15 minutes at 2300 g. Samples were placed on ice and diluted 1:20 in antioxidant assay buffer. 10 µL of Trolox standards or diluted hemolymph supernatant was added to each well of a 96-well plate in duplicate or triplicate, depending on sample volume. 10 µL of metmyoglobin and 150 µL of chromogen were added using a multichannel pipette. 40 µL of hydrogen peroxide, the activator, was then added with a multichannel pipette. The plate was read at 25$^{o}$C on a SpecraMax 190 Microplate Reader every minute for 10 minutes at 750 nm with shaking between reads.

Statistical analyses were performed in R version 3.2.2. TAC data were analyzed using Shapiro-Wilk tests to determine normality and a Levene's test (lawstat package) to determine homoscedasticity, followed by an ANOVA with a Tukey *post hoc* test to determine significant differences among the three species.

*Pigment Analysis*

Hemolymph absorbance scans were conducted on a 96-well Bio-Tek uQuant Universal Microplate Spectrophotometer at wavelengths from 200 to 850 nm and compared to absorbance scans of common plant pigments to provide a preliminary indicator of whether chlorophyll and/or carotenoids were present in the hemolymph. 90 μL of whole hemolymph was added to each well and scanned at room temperature. Hemolymph samples from at least three different individuals of each species were scanned along with a PBS control.

Liquid-chromatography-mass spectrometry (LCMS) analysis was used to determine the presence of chlorophyll and carotenoids in the hemolymph. Samples were mailed overnight on dry ice to Washington State University. Dr. Anna Berim from Washington State University's Gang Laboratory prepared the samples and conducted the LCMS analysis. Samples from the same species were pooled if necessary to bring the hemolymph volume of each sample up to 200 μL (only one sample was used if possible, at most 3 samples were pooled). 500 μL of chloroform was used to initially extract the metabolites from these samples. The aqueous phase was then extracted again, but this time with 500 μL ethyl acetate. The aqueous phase of this extraction was then re-

extracted with 1 mL of isooctane:methanol, 1:1 concentration by volume. Samples were vortexed for 20 seconds, left to settle for 15 minutes, after which a 10-minute phase separation was conducted at 8500 g as described by Westerlund & Hoffmann (2004). Organic extracts were saved from all of these extraction phases, combined, and vacuum dried. They were reconstituted in 120 μL of acetonitrile-methanol solution (70:30 by volume). Reconstituted samples were centrifuged at 21,000 g at 4 $^{o}$C to remove particles. 2 μL of each of these extracts were used for LCMS analysis.

A Waters Synapt® G2-S quadrupole time-of-flight mass spectrometer coupled to a Waters Acquity® ultra performance liquid chromatography system was used to analyze each sample. Target metabolites were ß-carotene, lutein, and the chlorophyll breakdown product pheophorbide a. 2 μL of each sample, as well as lutein and pheophorbide a standards from Cayman Chemical were run on the column at 40 $^{o}$C using a mobile phase system of water with 0.1% formic acid and acetonitrile with 0.1% formic acid. Solvents were run on the column at a flow rate of 400 μL per minute and added at different time points to form a concentration gradient (Table A2-1). The mobile phase solvents used to isolate ß-carotene were acetonitrile methanol, 70:30 by volume, and water. The gradient can be seen in Table A2-2. Initial flow rate was 400 μL per minute, and was increased to 600 μL per minute from minute 8 to minute 11, at which point it was returned to 400 μL per minute until 14 minutes had passed (Berim, personal communication, 2017).

Chromatographic behavior and mass spectra of all four metabolites were compared to those of the standards and of the isotopic models, which compare the combination of pigment isotopes that most precisely matches the variety of elements of

different masses present in the hemolymph and in the standard samples, to determine which pigments were present (Berim, personal communication, 2017). Figure A2-1 shows the LCMS analysis of pheophorbide a in a *Ligia pallasii* hemolymph sample. An example of the occurrence of β-carotene and lutein in *P. resecata* hemolymph can be seen in Figure A2-2 and Figure A2-3 in Appendix II, respectively. Relative amounts were determined using area under the curve in the mass chromatogram. Only samples in which pigments could be confidently identified as present were used in the analysis. Units are arbitrary. Randomization t-tests for each pigment were conducted in R (version 3.2.2). Relative amounts were only compared among species for a single pigment. Amounts could not be compared among the different pigments due to different sensitivity of LCMS to the different pigments.

*Phenoloxidase Assay*

Activity of the phenoloxidase immunoenzyme was determined by measuring maximum change in absorbance over time as an indicator of relative enzyme activity. Hemolymph samples were centrifuged at 4$^{o}$C. The phenoloxidase assay was conducted by adding 135 μL of deionized water and 20 μL PBS (pH 7.4) to each well of a 96-well plate kept on ice. 20 μL of hemolymph supernatant was added to each well except for the control wells, which contained water instead. 5 μL of chymotrypsin (5mg/mL) was added to each well. Chymotrypsin was used to activate the inactive form of the phenoloxidase enzyme so that the total potential immune response could be measured. The plate was incubated on a shaker for 6 minutes at room temperature, and then 20 μL of L-DOPA

(3,4-dihydroxy-L-phenylalanine, 4mg/mL) was added to all wells. The absorbance was measured every 6 minutes by a SpectraMax 190 Microplate Reader set to 25 $^{o}$C at 490 nm for at least 90 minutes with shaking between reads. A phenoloxidase assay that measured phenoloxidase activity already present in a given sample without an artificial activator was also conducted to determine whether phenoloxidase was active prior to the addition of chymotrypsin. This assay followed the same methods as the phenoloxidase assay that measured all potential phenoloxidase activity in response to a foreign antigen, except that 140 μL of deionized water was used per well, no chymotrypsin was added, and there was no 6 minute incubation. However, no notable phenoloxidase activity was seen for any of the three species, so only the data from the activated phenoloxidase assay were used in the final analysis (Cornet et al., 2009; Rigaud & Moret, 2003).

Phenoloxidase activity was calculated as slope during the linear phase of the reaction expressed in units of absorbance change per minute. Figure A3-1 in Appendix III shows an example of the typical phenoloxidase activity curves. Statistical analyses were conducted using R (version 3.2.2). Shapiro-Wilk tests were used to determine normality and a Levene's test (lawstat package) was used to determine homoscedasticity. Not all of these assumptions were met, so a Kruskal Wallis test was used to determine statistical differences, followed by a Dunn post hoc test with a Bonferroni correction.

## RESULTS

*Light & Oxygen Profiles*

Photosynthetic photon flux density in Padilla Bay decreased as depth from surface increased (Figure 5). Examination of oxygen levels at selected locations and times in the Padilla Bay *Z. marina* bed during the experimental period indicated fluctuating concentrations from 74.6% to 156.0% saturation. The water column tended to have higher oxygen saturation on sunny days than it did on cloudy days, suggesting a correlation between light/photosynthesis and oxygen levels (Figure 6). Annual oxygen levels in 2016 ranged from 15.1% to 229.3% (Figure 7). Oxygen saturation was much more extreme and variable from May to August compared to the rest of the year. Dissolved oxygen in the water column varied greatly even during 24 hour periods in the summer. Overall, oxygen tended to be highest in the mid afternoon, and lowest in the evenings and very early mornings.

*Total Antioxidant Capacity Assay*

*Ligia pallasii* hemolymph had a significantly higher total antioxidant capacity (TAC) than that of *P. resecata. Pandalus danae* hemolymph TAC was not significantly different from either isopod. The mean TAC for *P. resecata, L. pallasii,* and *P. danae* hemolymph, was 0.77 ± 1.69 mMol, 2.41 ± 1.64 mMol, and 1.25 ± 1.39 mMol, respectively (Figure 8).

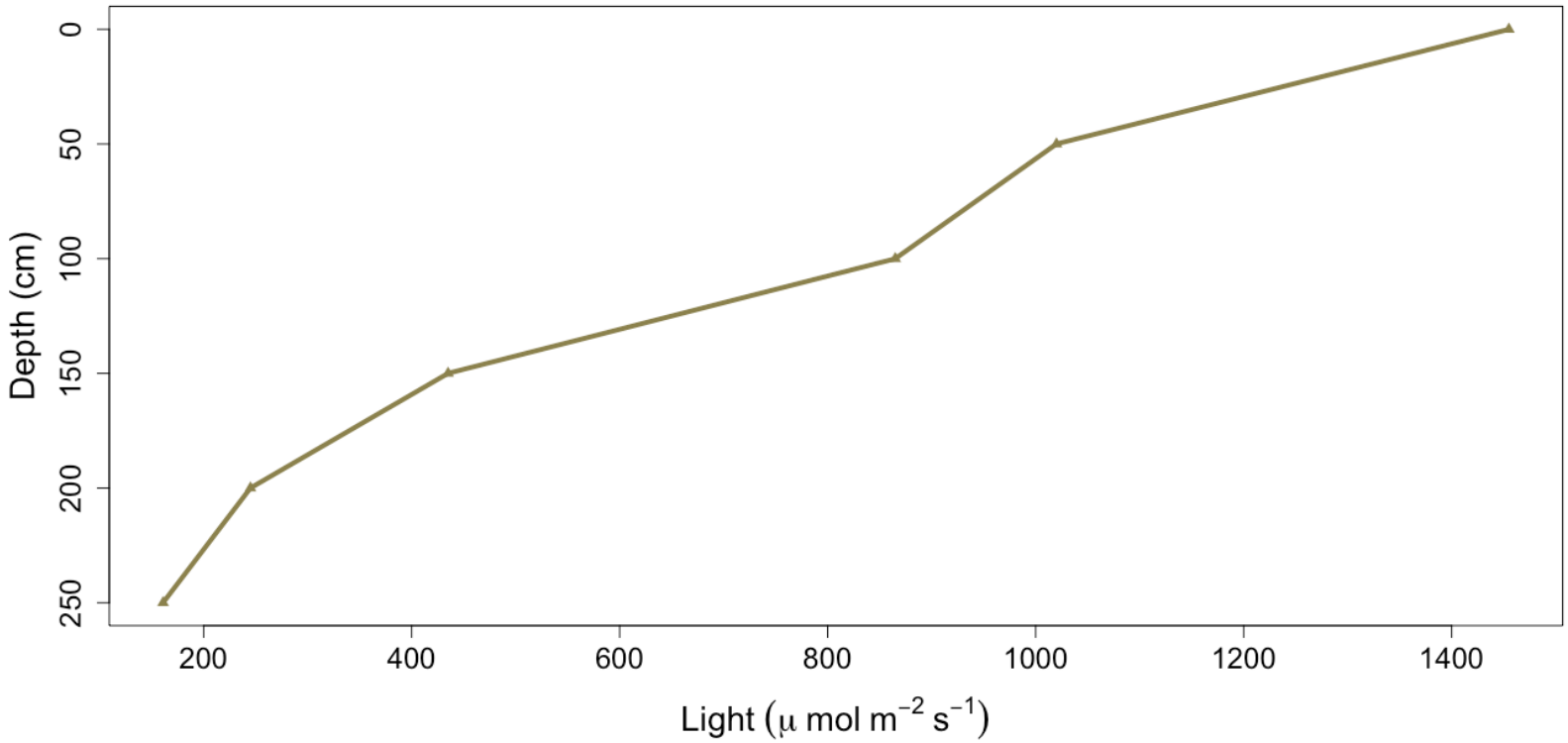


**Figure 5. A daytime depth profile of photosynthetic photon flux density in Padilla Bay.** Measurements were taken in the Padilla Bay eelgrass bed at high tide on a sunny afternoon (3:15pm) during the experimental period (July 29, 2016). Light levels decreased with depth.

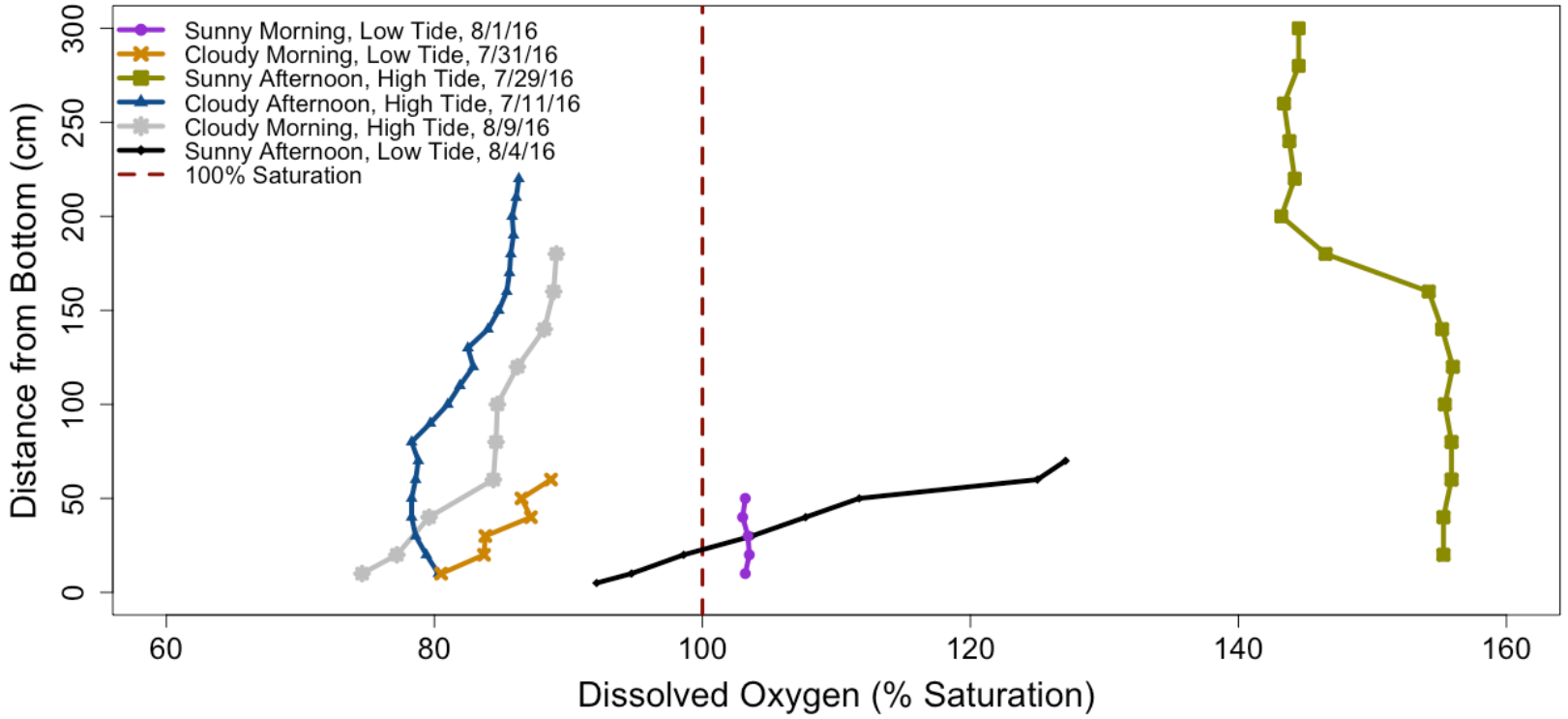


**Figure 6. Oxygen depth profiles at various tides, times of day, and cloud coverage.** All measurements were taken in Padilla Bay. The Y axis shows water height, with 0 cm representing the floor of the bay. The highest distance from the bottom measurement indicates the surface of the water, or the depth of the water at that time. The X axis shows the percent saturation of dissolved oxygen in the water column. The vertical dotted line represents 100% oxygen saturation, or oxygen saturation equal to that of the air. All other lines show oxygen profiles.

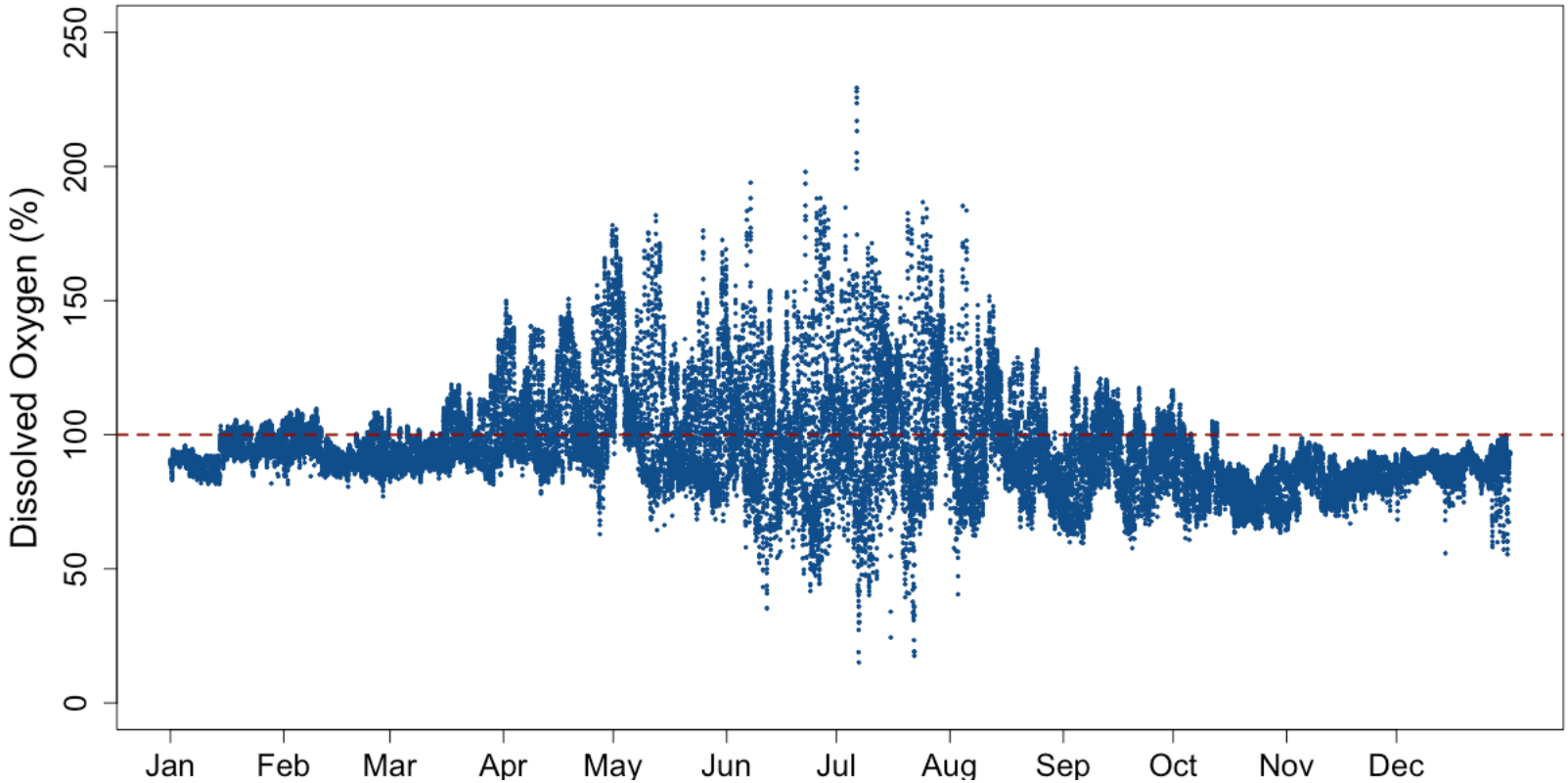


**Figure 7. Annual oxygen fluctuations in Padilla Bay during 2016.** The dots indicate the percent saturation of dissolved oxygen in the water column, and the dashed line indicates 100% saturation. The maximum oxygen saturation was 229.3%, the minimum was 15.1%. Data taken from the Padilla Bay National Estuarine Research Reserve sonde in Bayview Channel.

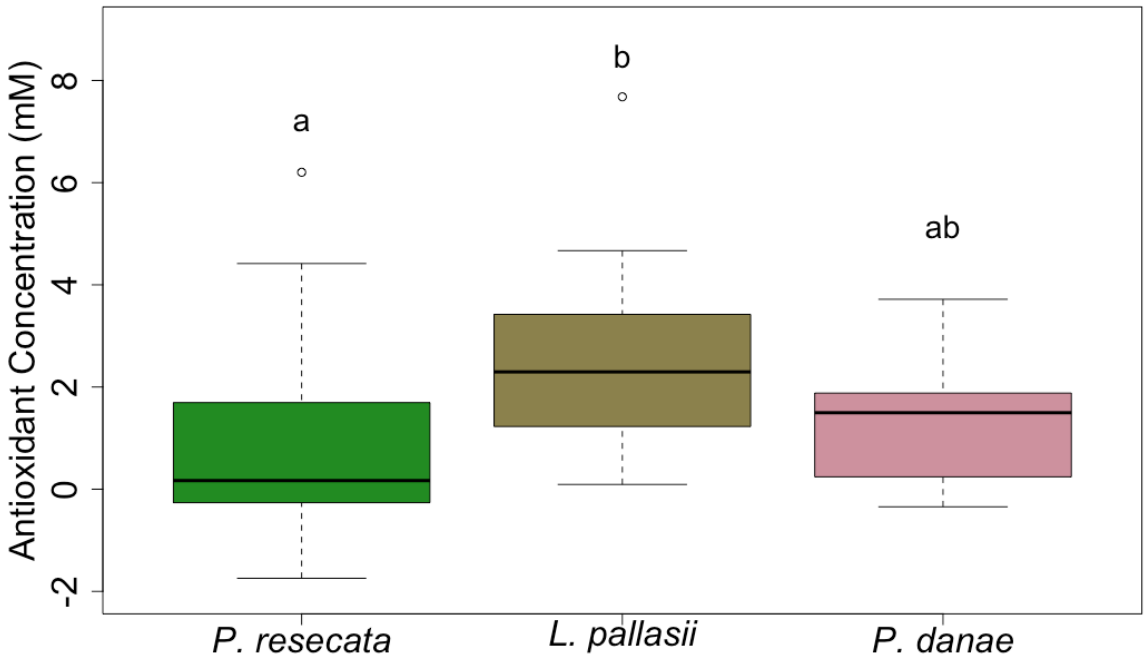


**Figure 8. Box plot of the total antioxidant capacity of *L. pallasii, P. resecata,* and *P. danae* hemolymph.** Letters indicate significant differences among species with different letters. A one-way ANOVA with a Tukey *post hoc* ($p<0.05$, df=2, F value=8.491) was used to determine differences among the species *L. pallasii* (n=31)*, P. resecata* (n=36)*,* and *P. danae* (n=9). *L. pallasii* hemolymph had a significantly higher TAC than that of *P. resecata* ($p=3.2 \times 10^{-4}$), while *P. danae* was intermediate (p=0.16 when comparing to *L. pallasii* and p=0.70 when comparing to *P. resecata*). The dark black middle band represents the median, the area above that line represents the upper quartile and the area below represents the lower quartile, whiskers encompass the nominal range of the data (1.5 times the interquartile range), and circles represent outliers, which are any data points located outside the whiskers.

*Pigment Analysis*

The absorbance pattern of *P. resecata* hemolymph closely matches that of chlorophyll and carotenoids, with a broad peak from 400-500 nm and a sharp peak from 650-700 nm. The absorbance scans of *P. danae* and *L. pallasii* do not have these peaks and are mostly flat in the visible wavelengths (Figure 9). LCMS analysis of *P. resecata* hemolymph confirmed the presence of antioxidants including pheophorbide a, lutein, and β-carotene, while *L. pallasii* hemolymph contained pheophorbide a and lutein but no β-carotene. *P. danae* hemolymph did not contain pheophorbide a, lutein, or β-carotene (Table 1). Relative concentration of hemolymph pigments were determined based on the mass spectrometry signal intensities and can be found in Table 1. *L. pallasii* hemolymph had a significantly higher relative concentration of pheophorbide a than did *P. resecata* hemolymph (Figure 10). The relative concentrations of lutein in *P. resecata* and *L. pallasii* hemolymph were not significantly different (Figure 11). Because of the analysis methods used, relative concentrations for one particular pigment can be compared among the species. However, relative concentrations among different pigments cannot be accurately compared.

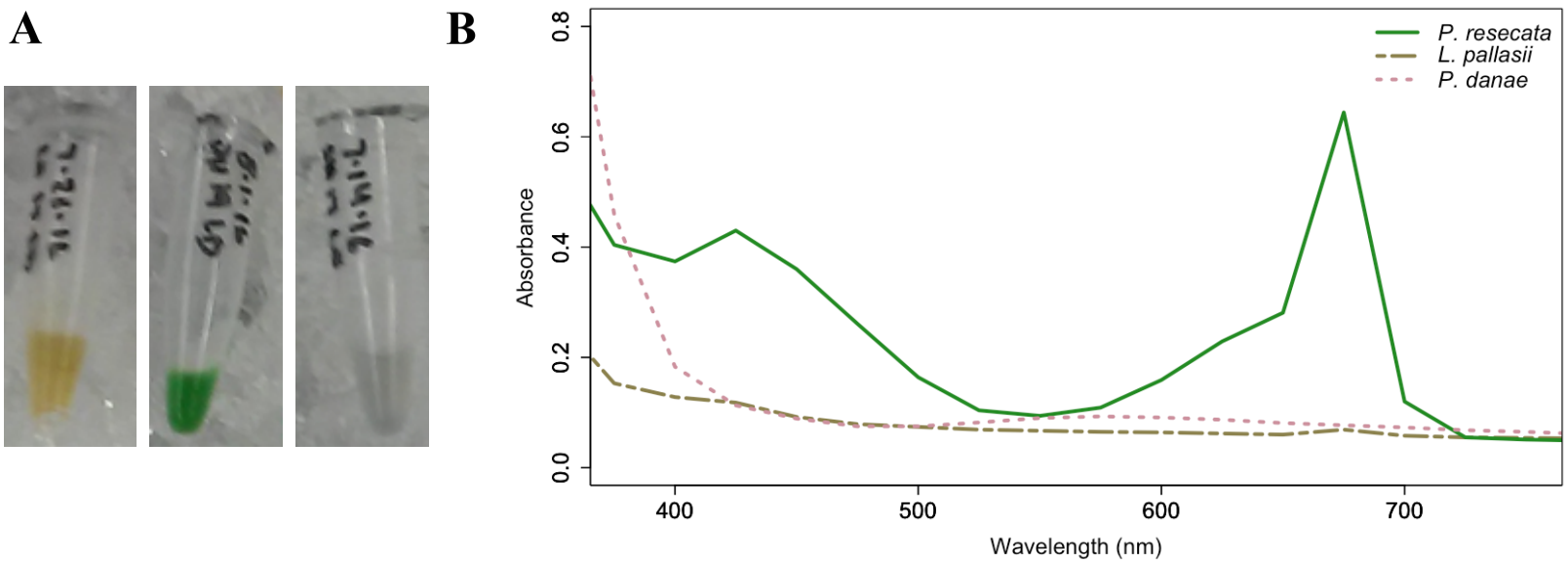


**Figure 9. Hemolymph color and absorbance spectra.** (A) Hemolymph samples collected from *L. pallasii* (yellow), *P. resecata* (green), and *P. danae* (clear). (B) Absorbance spectra (380-750 nm) of *L. pallasii, P. resecata,* and *P. danae* hemolymph. The similarity of the absorbance scan of *P. resecata* to that of carotenoids and chlorophyll indicates the possible presence of these plant pigments.

**Table 1. Liquid chromatography-mass spectrometry results.** Numbers show the relative concentrations of pheophorbide a, β-carotene , and/or lutein in *L. pallasii, P. resecata,* and *P. danae* hemolymph. Concentrations are only comparable among species for a single pigment. Dashes indicate that a particular pigment was not detected in the species.

| | ***P. resecata*** | ***L. pallasii*** | ***P. danae*** |
|---|---|---|---|
| **Pheophorbide a** | 16.9 ± 11.8 | 252.2 ± 265.8 | — |
| **ß-carotene** | 50.0 ± 18.6 | — | — |
| **Lutein** | 3667.1 ± 3400.9 | 1074.9 ± 1251.6 | — |

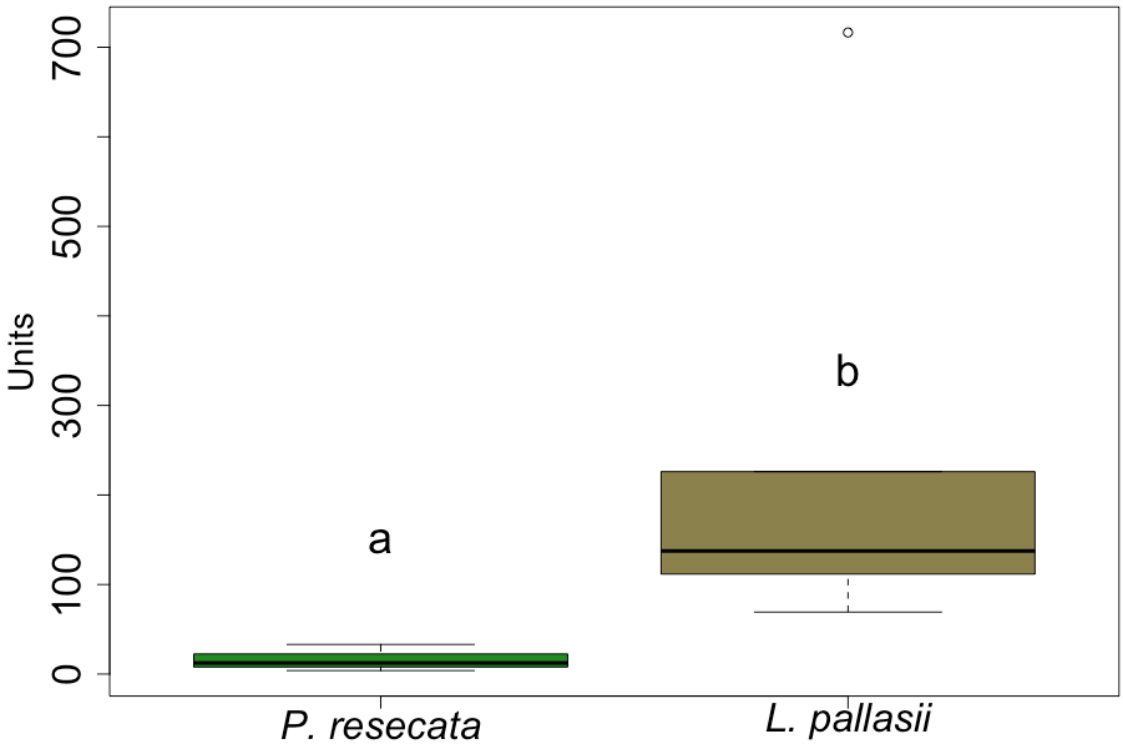


**Figure 10. Relative amounts of pheophorbide a in the hemolymph of *L. pallasii* and *P. resecata* isopods.** *L. pallasii* hemolymph (n=5) had a significantly higher relative concentration of pheophorbide a than did *P. resecata* hemolymph (n=5) (Randomization t-test, $p=7.8 \times 10^{-3}$, 500,000 permutations).

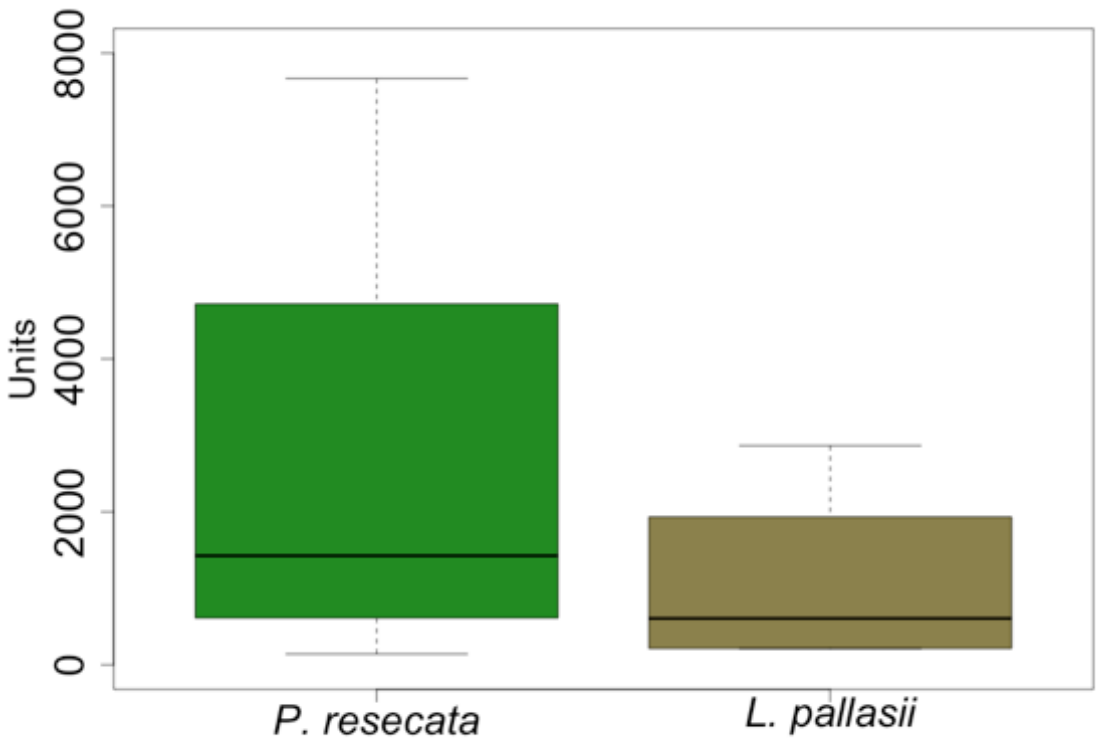


**Figure 11. Relative amounts of lutein in the hemolymph of *L. pallasii* and *P. resecata*.** A randomization t-test was used to determine differences among the species (p=0.54, 500,000 permutations). The relative concentration of lutein was not statistically different between *P. resecata* (n=4) and *L. pallasii* (n=4).

*Phenoloxidase Assay*

*Pandalus danae* hemolymph had significantly higher phenoloxidase activity than did either *L. pallasii* or *P. resecata* hemolymph. The phenoloxidase activities of *L. pallasii* and *P. resecata* were negligible and were not significantly different from each other. Phenoloxidase activity (slope during the linear phase) for *P. resecata* was $1.02 \times 10^{-3} \pm 1.47 \times 10^{-3}$ $\Delta abs_{490}$/min, for *L. pallasii* was $6.29 \times 10^{-4} \pm 6.06 \times 10^{-4}$ $\Delta abs_{490}$/min, and for *P. danae* was $1.89 \times 10^{-2} \pm 1.11 \times 10^{-2}$ $\Delta abs_{490}$/min (Figure 12).

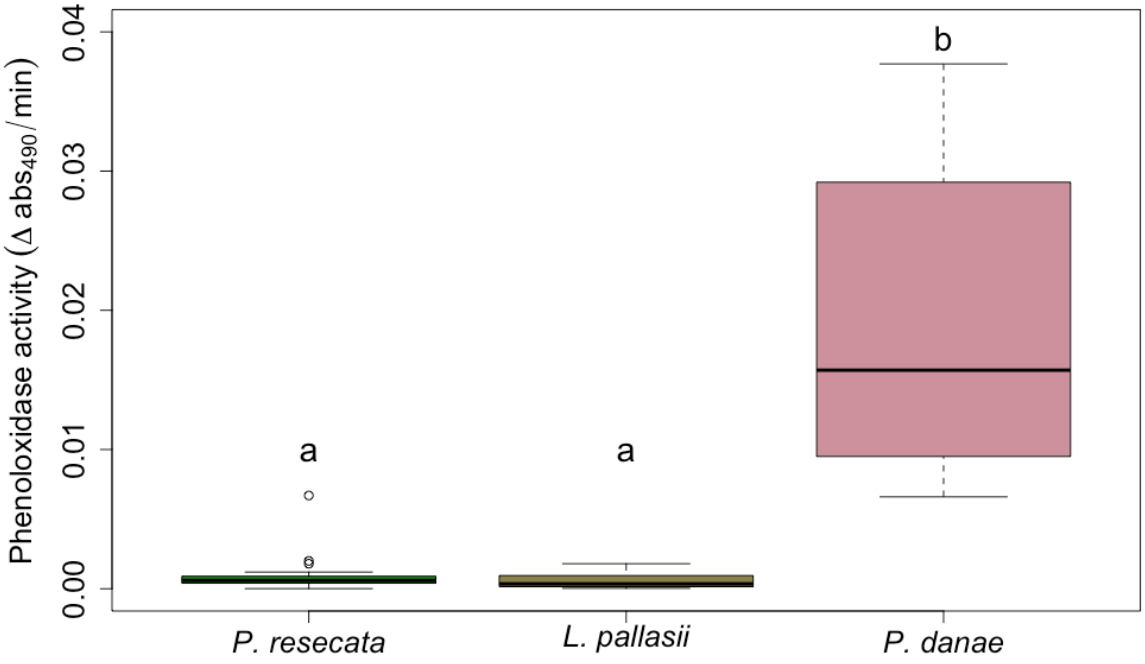


**Figure 12. Box plot of the phenoloxidase activity of *L. pallasii, P. resecata,* and *P. danae* hemolymph.** A Kruskal Wallis test ($p=1.2 \times 10^{-5}$) with a Dunn *post hoc* and Bonferroni correction ($p<0.05$, df = 2, chi-squared = 22.73) was used to determine significant differences between the species *L. pallasii* (n=16)*, P. resecata* (n=19)*,* and *P. danae* (n=9). Results show that *P. danae* hemolymph had a significantly higher phenoloxidase activity compared to that of *P. resecata* ($p=9.5 \times 10^{-5}$) and *L. pallasii* ($p=7.0 \times 10^{-6}$). Phenoloxidase activity of *L. pallasii* and *P. resecata* were not significantly different from each other (p=0.40).

## DISCUSSION

### *Oxygen Profiles*

Oxygen saturation in Padilla Bay tended to be higher when the eelgrass was exposed to more sunlight, both on a daily and seasonal basis (Figure 6 and Figure 7). Oxygen saturation trended higher in the summer compared to the winter as well as on sunny days compared to cloudy days. It also trended lower immediately after sunrise compared to later in the day. The fluctuating oxygen concentrations in Padilla Bay in 2016, both annually and during just the experimental period, show that *P. resecata* experience hostile environmental oxygen conditions including hyperoxia and potential hypoxia reperfusion events. Not only did oxygen saturation in Padilla Bay vary considerably throughout the year, so did pH, temperature, and salinity (Table A1-1 in Appendix I). *Pentidotea resecata* therefore likely experience increased ROS compared to *L. pallasii,* which primarily inhabit environments that are presumably normoxic due to continuous exposure to the atmosphere (Carefoot et al., 2000). Hyperoxia in combination with relatively high UVR in Padilla Bay likely causes *P. resecata* to also experience higher ROS concentrations than do *P. danae*, which were collected below the photic zone.

### *Total Antioxidant Capacity*

It was surprising that *L. pallasii* hemolymph had a significantly higher antioxidant capacity than that of *P. resecata,* and that the hemolymph from *P. danae* was not significantly different from either isopod, because oxygen and light levels in Padilla Bay

indicate that *P. resecata* seems to live in an environment far more conducive to ROS formation than the other two species of crustaceans. Therefore, *P. resecata* would seemingly need stronger antioxidant defenses. The unexpected results showing increased TAC in *L. pallasii* hemolymph are supported by another study that showed that the enzymatic antioxidant activities of SOD, CAT, and GPx in the tissues of *L. pallasii* are all significantly higher than the activities of these enzymes in *P. resecata* tissue (Johnson, 2017).

It is possible that *P. resecata* utilizes alternative preventative mechanisms to protect against oxygen toxicity, such as excreting endogenous ROS and preventing entry of exogenous ROS, or perhaps they have stronger repair mechanisms to mitigate oxidative damage. They may also prevent formation of endogenous ROS (Camus & Gulliksen, 2005). Some species of fish have seemingly low blood CAT and GPx activities proportionate to the oxidative stress they experience, which is thought to be at least in part because they can excrete hydrogen peroxide through their gills via diffusion into the water column (Wilhelm-Filho et al., 1994). Perhaps *P. resecata* has a similar mechanism by which to excrete ROS. It is also possible that this isopod adjusts its respiration and activity level in pro-oxidative environments to prevent ROS entry and formation, respectively. Reduced metabolic rates often result in decreased cellular ROS formation in environments with high $pO_2$. This could lead to more stable internal oxygen levels and ROS concentrations. (Abele & Puntarulo, 2004).

A study examining antioxidant activity in polar amphipods that reside at different depths showed that surface water amphipods, which are exposed to elevated ROS due to

oxygen levels and UVR, had a lower hemolymph total oxyradical scavenging capacity (TOSC) toward hydroxyl compared to the deep sea amphipod, which lives below the photic zone and experiences lower ROS levels (Camus & Gulliksen, 2005). A suggested explanation for why hemolymph TOSC was relatively low in the surface water amphipods even though they inhabit a pro-oxidative environment was that the hydroxyl radical may not be formed much in the hemolymph, possibly because these animals have adapted high SOD activity to prevent hydroxyl radical formation. The surface amphipod also had no significant change in TOSC when exposed to exogenous hydrogen peroxide, whereas the deep water amphipods did. This finding may indicate that the surface water amphipod has developed defenses that either prevent exogenous ROS from diffusing through the gills or mechanisms that allow excretion of hydrogen peroxide and other ROS through the gills, demonstrating that crustaceans may adapt to environments with high oxygen concentrations by developing mechanisms other than increased antioxidant capacity to protect against oxygen toxicity (Camus & Gulliksen, 2005) such as enhancing repair and detoxification processes (Lushchak & Bagnyukova, 2006). Whether any of these mechanisms take place within *P. resecata* merits further investigation.

An alternative explanation is that *L. pallasii* experience higher levels of ROS than *P. resecata* do despite the apparent increased oxygen and UVR in *P. resecata's* habitat. The oxygen and UVR in the habitats of both *L. pallasii* and *P. danae* were not measured during this study, and could potentially be higher than expected, which could lead to elevated ROS in these crustaceans. It is also possible that other environmental factors contribute to these unexpected results. Perhaps the concentration of non-enzymatic

antioxidants in *L. pallasii* is higher than the concentration in *P. resecata* due to differences in diet and food processing, although this is unlikely because the hemolymph absorbance scans seem to indicate lower concentrations of plant pigments in *L. pallasii* (Figure 9). Additionally, *L. pallasii, P. danae,* and *P. resecata* may all have different antioxidant enzyme activities due to physiological and environmental differences that lead to oxidative damage such as thermal stress, pH changes, and pollutants rather than exposure to unfavorable oxygen levels, all of which can affect cellular ROS formation (Lesser, 2006).

Metabolic differences among the three species could also help explain the unexpected TAC. These three crustacean species may utilize oxygen in different ways. Activity levels and other possible behavioral differences may contribute to differences in metabolic functions. Marine crustaceans such as crabs, lobsters, and shrimp have varying antioxidant defenses depending on their level of aerobic metabolism. Some species may have more energy efficient mitochondria, effectively reducing ROS production and therefore reducing the need for antioxidants (Lesser, 2006).

*Pigment Analysis*

Analysis of *P. resecata* hemolymph confirmed the presence of antioxidants including pheophorbide a, lutein, and β-carotene, while *L. pallasii* hemolymph contained pheophorbide a and lutein but no β-carotene. *Pandalus danae* hemolymph had neither carotenoids nor pheophorbide (Table 1). Based on these results, it would be reasonable to suggest that *P. danae* should have the lowest TAC because it has no plant pigment

antioxidants in the hemolymph. However, this was not the case. Instead, *P. danae* had an intermediate TAC. *L. pallasii* hemolymph had a higher TAC than that of *P. resecata* even though it contained only one of the two carotenoids measured whereas *P. resecata* hemolymph contained both (Table 1). It is unlikely that the higher TAC of *L. pallasii* hemolymph compared to the TAC of *P. resecata* hemolymph is due to a higher concentration of plant pigments in *L. pallasii* compared to *P. resecata* because although *L. pallasii* hemolymph had significantly higher concentrations of pheophorbide a (Figure 10), the concentration of lutein was not different between these isopods (Figure 11). Also, *P. resecata* hemolymph contained β-carotene whereas *L. pallasii* hemolymph did not (Table 1). This, together with the peak intensities of the absorbance scans (Figure 9) indicates that *L. pallasii* does not have more plant pigment antioxidants that does *P. resecata.* It is more likely that the differences in TAC are due to differences in the activity of enzymatic antioxidants (Johnson, 2017).

It is important to note that hemolymph color varied substantially among individuals of a species. For example, some *P. resecata* individuals had pale yellow-green hemolymph, whereas others had a bright, rich green hemolymph. *Ligia pallasii* hemolymph ranged from almost clear to yellow in color. *Pandalus danae* hemolymph was consistently clear. The variations in hemolymph color in the isopods may indicate differences in pigment types and concentrations among individuals. There may also be pigments present that were not measured in this study. However, the absorbance scans of different individuals in a particular species had similar patterns to the scans shown in Figure 9, although peak intensity did vary. Diet is most likely the principal reason for the

differences in pigments present. *Pandalus danae's* diet consists mainly of other small animals and detritus rather than plants or algae, so its hemolymph does not contain plant pigments (Rice et al., 1980). *Ligia pallasii* mostly eats macroalgae (Carefoot et al., 2000), which have abundant pigments, whereas *P. resecata* eats primarily eelgrass and the epiphytes present on the blades (Lee & Gilchrist, 1972), another abundant pigment source. The color of *P. resecata* in this study was similar to the green color of the eelgrass. Perhaps differences in pigment concentrations in the algae versus the seagrass affect the types of pigments present in the isopods. *Pentidotea resecata* can also live on kelp, and the populations which do so feed primarily on *Macrocystis* and are brown in color. The composition and relative abundance of carotenoids differs in whole body homogenates of the green color morphs compared to the brown (Lee & Gilchrist, 1972). Perhaps the pigments present in the hemolymph of *P. resecata* populations that reside on algae would be more similar in composition to those in *L. pallasii*.

*Phenoloxidase Assay*

*P. resecata* isopods, which may experience increased oxidative stress, may also exhibit immunosuppression due to the oxidative damage an active phenoloxidase response could add onto an already stressed organism. However, the presence of antioxidants such as carotenoids may help alleviate ROS damage, therefore having an immunostimulatory effect by allowing an enhanced phenoloxidase immune response and increased overall health.

The contrary effects of ROS and antioxidants on the immune response have been demonstrated in many studies. *Idotea balthica* is a marine isopod closely related to *P. resecata,* that also inhabits *Z. marina* beds. *Idotea balthica* eats eelgrass and its accompanying epiphytes, but will eat *Fucus* algae if *Z. marina* is not available. Studies have shown that the immune response of *I. balthica* is sensitive to environmental stressors such as temperature, salinity, and ocean acidification. *I. balthica* isopods collected from a *Z. marina* bed and exposed to a simulated heat wave had significantly fewer phagocytic hemocytes compared to those kept at stable temperatures, indicating lower immunity in the heat wave group (Roth et al. 2010). A study investigating male isopods from three populations of *I. balthica* living in normal salinity and low salinity showed that isopods living in low salinity experienced significantly higher oxidative stress than did the other two populations, likely due to the metabolic cost of coping with low salinity. Oxidative stress was measured by the concentration of protein carbonyls in the tissues, which are byproducts of oxidative damage to proteins that form when ROS react with amino acid side chains and form carbonyl groups such as reactive ketones and aldehydes. The immune response (measured by phagocytic hemocyte activity) in the low salinity population was lower than that of the normal salinity group. The low salinity population had higher mass mortality during infection when placed in high acidity water (an additional stressor that can lead to oxidative damage) compared to the normal salinity population, which indicates that greater oxidative stress to begin with (in this case due to salinity) can impair immunity. The increased mortality of infected isopods that experience increased oxidative stress compared to those that did not also suggests that

ROS can greatly affect crustacean immunity (Wood et al., 2014). *Pentidotea resecata* isopods, which live in environments conducive to increased oxidative stress, may experience reduced immunity if their antioxidant systems cannot compensate for the increased ROS.

Other studies have also shown that oxygen saturation may affect crustacean immunity. Organisms such as scallops and crabs that frequently experience oxidative stress have depressed phenoloxidase activity and overall immune impairment, most likely to prevent the extreme oxidative damage that can result from activating the phenoloxidase immune cascade (Hannam et al., 2010; Tanner et al., 2006).

The presence of antioxidants such as carotenoids may also influence the phenoloxidase immune response. For example, *Gammarus pulex* amphipods that ate carotenoid supplements had decreased auto-reactivity and oxidative damage during the immune response compared to those that did not receive supplements. Carotenoids also led to an improved immune response, including an increase in phenoloxidase activity and in resistance to bacterial pathogens (Babin et al., 2010). *Pentidotea resecata* and *L. pallasii* both have carotenoid antioxidants in their hemolymph, which may enhance phenoloxidase activity, whereas *P. danae* lacks these important non-enzymatic antioxidants.

Antioxidant enzyme activity in many organisms is upregulated during initial stages of infection, which indicates the importance of antioxidants during immune responses. In the *Portunis trituberculatus* crab, superoxide dismutase activity, glutathione peroxide activity, and catalase activity in the cell-free hemolymph and in the

hepatopancreas were significantly higher during initial stages of infection with the white spot syndrome virus as well as during infection with *Vibrio* pathogenic bacterial infections than they were when the crabs were not infected. These antioxidant enzymes have an important role in quenching excessive ROS during the immune response of these crabs to pathogenic infections, and an upregulation of antioxidant enzymes may allow a stronger immune response and increased melanization of pathogens (Ren et al., 2017). The increased TAC of *L. pallasii* hemolymph in this study compared to the hemolymph of the other two crustaceans could potentially allow this isopod species to have stronger phenoloxidase activity.

These studies led me to believe that *P. resecata* would have a lower phenoloxidase activity compared to *L. pallasii* because *P. resecata* has a significantly lower TAC and lives in a pro-oxidative environment. Studies indicate that species with a lower antioxidant capacity tend to have a weaker immune response than those with a high TAC (Ren et al., 2017). The potentially high ROS concentrations experienced by *P. resecata* should have also contributed to a diminished phenoloxidase activity because high ROS levels can lead to immunosuppression through a downregulation of phenoloxidase (Hannam et al., 2010; Tanner et al., 2006). However, the phenoloxidase activity of *L. pallasii* and *P. resecata* were not significantly different from each other (Figure 12). I also thought that *P. danae* would have equal phenoloxidase activity to the isopods due to a similar TAC. However, this was not the case. Instead, *P. danae* had a significantly higher phenoloxidase activity than did *L. pallasii* and *P. resecata*, even though its hemolymph contained no carotenoids (Figure 12).

There are a few factors that could contribute to these unexpected results, the primary reason being differences between shrimp and isopod immune processes. A few isopod species, including *Glyptonotus antarcticus* and *Bathynomus giganteus*, lack phenoloxidase gene transcripts, while most other crustaceans, including shrimp, express these gene transcripts (Jaenicke et al., 2009). Most isopods, however, have some measurable phenoloxidase activity despite not having the phenoloxidase gene. This phenoloxidase activity may stem from hemocyanins rather than from a phenoloxidase protein (Jaenicke et al., 2009). *Ligia pallasii* isopods have some sort of phenoloxidase activity that allows them to digest phenols (King et al., 2010), and *Porcellio scaber* isopod hemocyanins can be converted into a molecule that exhibits phenoloxidase activity when the isopod is immune challenged, allowing them to melanize pathogens (Jaenicke et al., 2009). In *Cirolana harfordi* isopods, phenoloxidase activity is also seen in hemocyanin molecules rather than in phenoloxidase molecules (Terwilliger, 2007). It is possible that the lower phenoloxidase activity seen in *L. pallasii* and *P. resecata* hemolymph compared to that of *P. danae* is not due to ROS exposure or TAC, but to the efficiency of the molecule used in each species to initialize the phenoloxidase cascade.

Another potential contributor to the surprisingly high phenoloxidase activity of *P. danae* compared to the isopods is a difference in phenoloxidase activation. One study suggested that chymotrypsin, which is effective at activating shrimp phenoloxidase, does not properly activate isopod phenoloxidase. Sodium–dodecyl–sulphate (SDS) is a significantly better activator of isopod hemocyanin phenoloxidase activity compared to chymotrypsin because SDS causes a structural change in hemocyanins that allows

phenolic molecules to more readily bind to the otherwise sterically hindered active site to initiate phenoloxidase-like activity. Chymotrypsin was used in the current study, rather than SDS, which may be partially responsible for the difference between phenoloxidase activity in the shrimp compared to the isopods (Jaenicke et al., 2009).

A sex bias may have also affected the results. Roth et al. (2010) determined that male *Idotea balhtica* isopods have significantly lower phenoloxidase activity than females do. Likewise, Sicard et al. (2010) found that male *Armadillidium vulgare* isopods had lower phenoloxidase activity than did females. The *P. resecata* isopods used for the phenoloxidase assays in this study were predominately male, whereas the *L. pallasii* isopods and the shrimp were most likely a mix of males and females.

Phenoloxidase activity can also vary with the molt stages of crustaceans. For example, phenoloxidase activity in *Penaeus stylirostris* shrimp is lower during the premolt stage than it is during the intermolt stage. The decrease in phenoloxidase activity during the premolt cycle correlates with a decline in immunity during premolt, which was measured by a significantly increased mortality percentage in *Vibrio*-infected shrimp during premolt compared to intermolt mortality (Le Moullac et al., 1997). Furthermore, hemocyanins with phenoloxidase activity located in the gastroliths of the crayfish *Cherax quadricarinatus* may be important in sclerotization during intermolt cycles. The expression levels of hemocyanin transcripts during different molt cycles may also vary (Glazer et al., 2013).

*Future Directions*

Additional studies may be useful in comparing the level of oxidative stress these crustaceans experience and how this affects their overall health. Lipid peroxidation and protein carbonyl assays, which measure ROS-induced damage, would be helpful in comparing how much oxidative damage occurs within each species (Lavarías et al., 2011). ROS assays could determine whether there are differences in endogenous ROS concentrations among the three species. An additional experiment that could elucidate the mechanism *P. resecata* utilizes to protect itself from oxidative stress would be to place individuals in environments with controlled oxygen levels to determine if *P. resecata* experience different levels of ROS or oxidative stress and if they are able to adjust their TAC and phenoloxidase activity in these environments. It would be useful to conduct more phenoloxidase assays using different activators (Jaenicke et al., 2009). Identifying additional plant and algae pigments that may be present in the hemolymph could be useful in determining how much these pigments contribute to the overall antioxidant capacity and immunity of these species. Altering the diet of the isopods may change the types of pigments present in the hemolymph. For example, perhaps if the *P. resecata* isopods were fed only macroalgae, they would have more similar pigments to *L. pallasii* (Lee & Gilchrist, 1972). There are many further studies that could be conducted to clarify the interesting results obtained in this research.

## CONCLUSIONS

*Pentidotea resecata* isopods live in habitats that are exposed to UV radiation and that become both hypoxic and hyperoxic. Despite inhabiting an environment conducive to enhanced ROS production, *P. resecata* had a significantly lower hemolymph TAC compared to *L. pallasii*. *Pentidotea resecata* hemolymph also contained pheophorbide a, lutein, and β-carotene, whereas *L. pallasii* hemolymph contained only pheophorbide a and lutein. *P. danae* hemolymph contained none of these pigments, yet its TAC was not significantly different from that of either isopod. Antioxidant capacity did not seem to correlate with the phenoloxidase immune response, as *P. danae* hemolymph had a significantly higher phenoloxidase activity than did either isopod, and the phenoloxidase activity of *L. pallasii* hemolymph was not significantly different from that of *P. resecata*, even though its antioxidant capacity was significantly greater. Altogether, these results suggest that other factors are also likely involved in the antioxidant and immune function of these three crustaceans.

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## APPENDIX I: PHYSICAL CHARACTERISTICS OF PADILLA BAY

**Table A1-1. Oxygen saturation, temperature, pH, and salinity of the water column in Padilla Bay in 2016.**

Data taken from the Padilla Bay National Estuarine Research Reserve buoy in Bayview Channel.

| | Oxygen (%) | Temp. ($^{o}$C) | pH | Salinity (psu) |
|---|---|---|---|---|
| **2016 Average** | 95.1 | 11.5 | 7.9 | 28.9 |
| **2016 Minimum** | 229.3 | 22.9 | 8.7 | 30.8 |
| **2016 Maximum** | 15.1 | 1.3 | 7.2 | 24.2 |

**Table A2-1. Solvent gradient over time used for LCMS analysis of lutein and pheophorbide a.**

| Time (min) | Water + 0.1% Formic Acid (% by Volume) | Acetonitrile + 0.1% Formic Acid (% by Volume) |
|---|---|---|
| **0.00** | 97% | 3% |
| **0.86** | 97% | 3% |
| **9.69** | 1% | 99% |
| **10.52** | 1% | 99% |
| **11.02** | 97% | 3% |
| **14.00** | 97% | 3% |

**Table A2-2. Solvent gradient over time used to isolate ß-carotene for LCMS analysis.**

| Time (min) | 70:30 Acetonitrile:Methanol (% by Volume) | Water (% by Volume) |
|---|---|---|
| **0.00** | 85% | 15% |
| **2.00** | 85% | 15% |
| **3.00** | 99% | 1% |
| **7.00** | 99% | 1% |
| **8.00** | 99% | 1% |
| **11.00** | 85% | 15% |
| **14.00** | 85% | 15% |

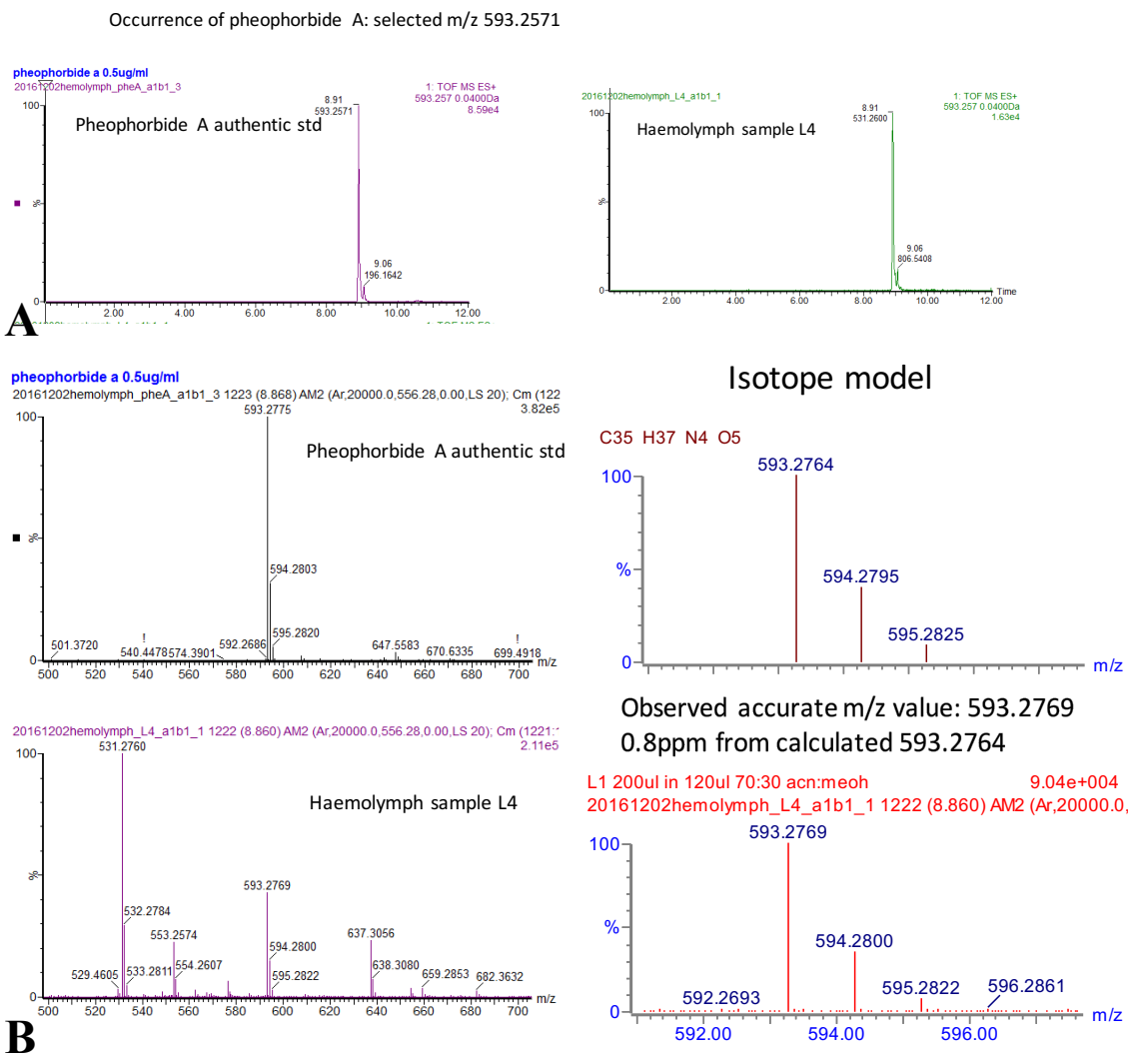


**Figure A2-1. LCMS analysis of pheophorbide a**. (A) Retention time of pheophorbide a in an authentic standard (left) and in *L. pallasii* hemolymph (right). (B) Analysis of mass profiles of an authentic pheophorbide a sample (top left) and a theoretical isotope model of the combination of isotopes one could expect to see for a molecule of the particular mass composition measured experimentally (top right). The mass profile of pheophorbide a in a sample of *L. pallasii* hemolymph can be seen in the lower images. The right image is a more focused version of the left. Figures made by Dr. Berim.

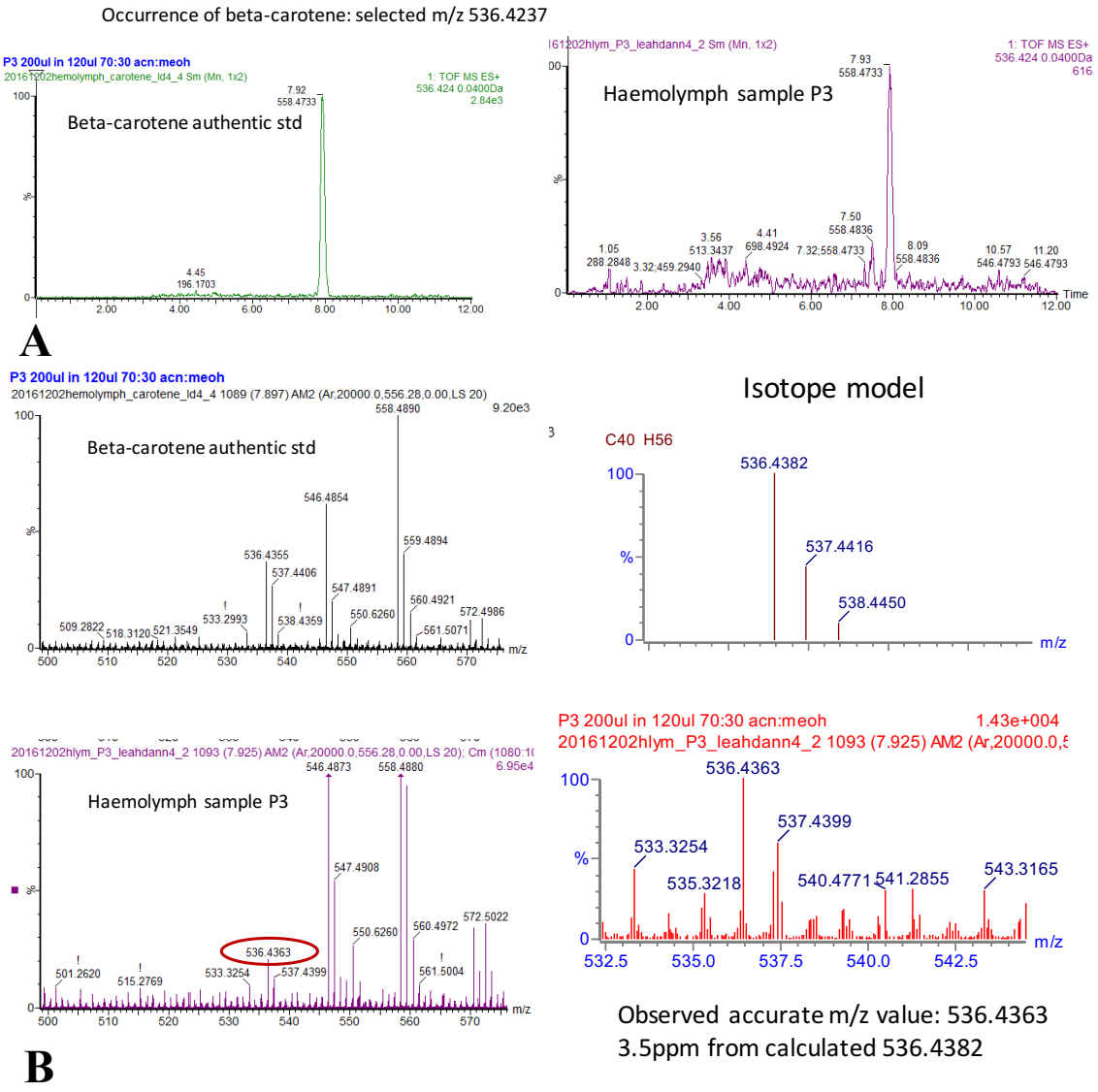


**Figure A2-2. LCMS analysis of β-carotene.** (A) Retention time of β-carotene in an authentic β-carotene standard (left) and in a sample of *P. resecata* hemolymph (right). (B) The mass analysis of the β-carotene standard (top left) and a theoretical model of the masses of isotopes of this molecule (top right). The mass profile of *P. resecata* hemolymph can be seen in the lower images. The right image is a zoomed in version of the left. Figures created by Dr. Berim.

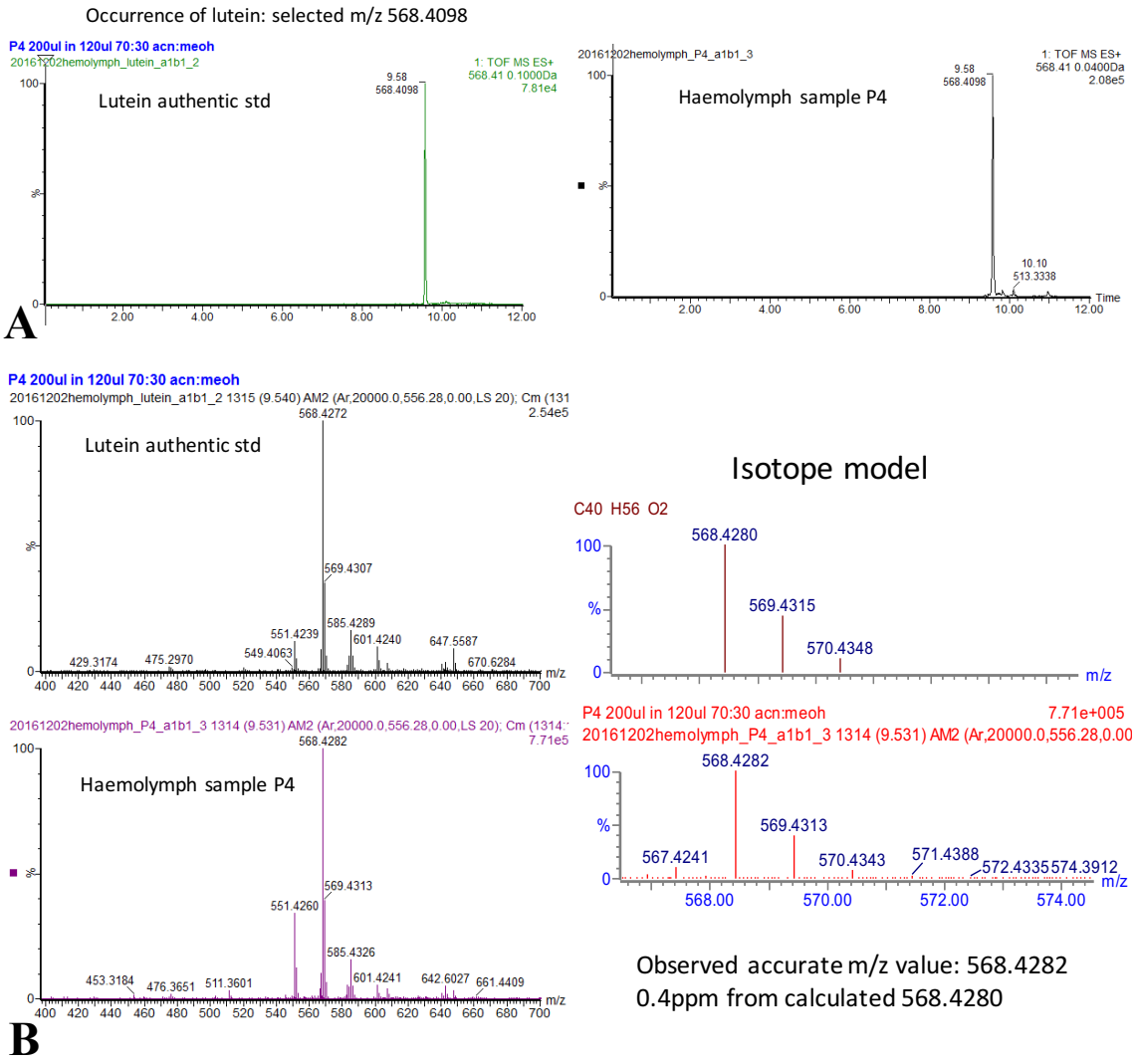


**Figure A2-3. LCMS analysis of lutein.** (A) Retention time of lutein in an authentic standard (left) and in a sample of *P. resecata* hemolymph (right). (B) The mass analysis of the lutein standard (top left) and a theoretical model of lutein mass and its isotopes (top right). The mass profile of lutein in *P. resecata* hemolymph can be seen in the lower images. The right image is a zoomed in version of the left. Figures were made by Dr. Berim.

## APPENDIX III: PHENOLOXIDASE ASSAY

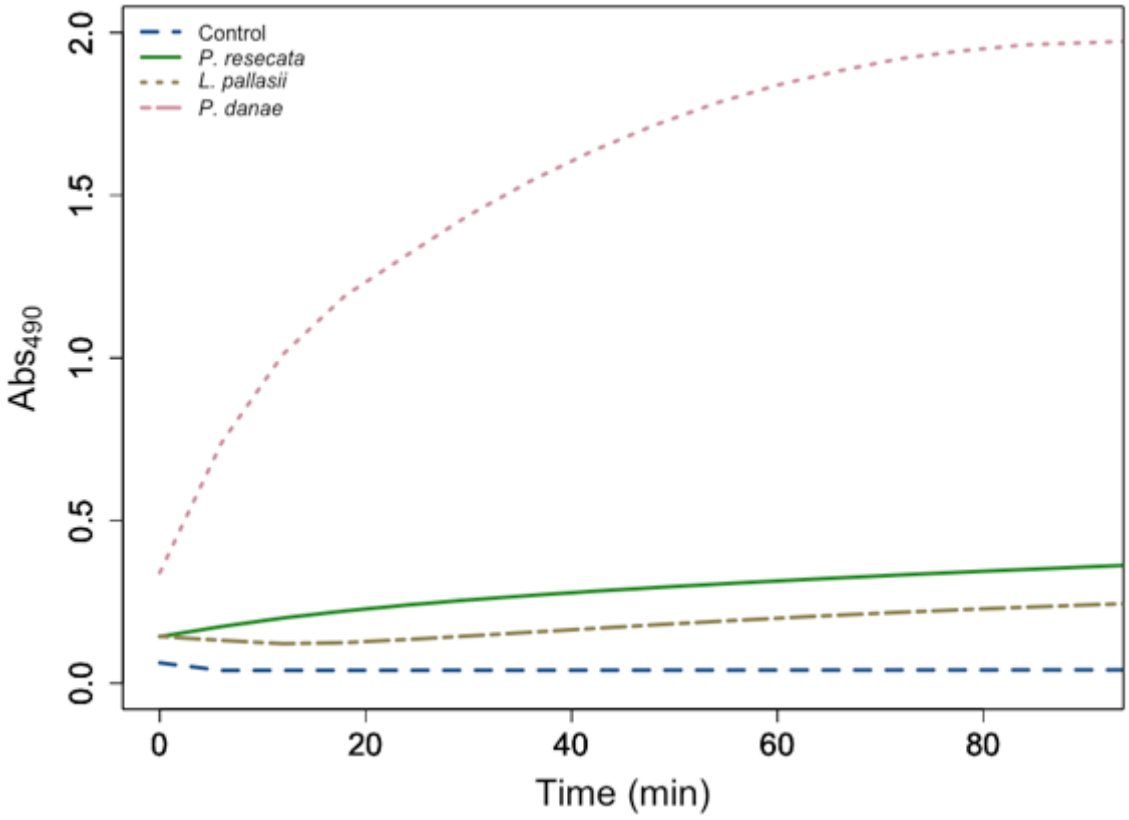


**Figure A3-1. Phenoloxidase activity of *L. pallasii, P. resecata,* and *P. danae* hemolymph.** Absorbance was read at 490 nm. Each line represents the absorbance change over time for a single hemolymph sample. Phenoloxidase activities shown were typical for each of the species.