

**Aerobic and anaerobic enzyme activity during visceral degeneration and  
regeneration in *Parastichopus californicus***

**By**

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A Thesis Proposal

Submitted to

Walla Walla University

In partial fulfillment of

Requirements for the degree of

Master of Science

**April 2014**

## Table of Contents

### Introduction

|  |   |
|--|---|
| Seasonal organ atrophy and dormancy..... | 3 |
| Respiration in holothurians.....         | 4 |
| Hypoxia responses.....                   | 5 |
| Purpose.....                             | 6 |
| Figure 1.....                            | 7 |
| Research Questions.....                  | 8 |

### Methods

|                                |    |
|--------------------------------|----|
| Timeline.....                  | 9  |
| Location & Animal Care.....    | 9  |
| Tissue Sample Preparation..... | 9  |
| Figure 2.....                  | 10 |
| Enzyme Analyses.....           | 11 |
| Metabolite Analysis.....       | 16 |
| Hemoglobin Analysis.....       | 17 |
| Data Analysis.....             | 17 |
| Figure 3.....                  | 19 |

### Results

|                          |    |
|--------------------------|----|
| Enzyme Analyses.....     | 20 |
| Metabolite Analysis..... | 21 |
| Hemoglobin Analysis..... | 21 |
| Figure 4.....            | 22 |
| Figure 5.....            | 23 |
| Figure 6.....            | 24 |

### Discussion

|                          |    |
|--------------------------|----|
| Enzyme Analyses.....     | 25 |
| Metabolite Analysis..... | 25 |
| Hemoglobin Analysis..... | 26 |
| Figure 7.....            | 28 |
| Figure 8.....            | 29 |
| Further Questions.....   | 30 |

|                       |    |
|-----------------------|----|
| Literature Cited..... | 31 |
|-----------------------|----|

|                 |    |
|-----------------|----|
| Appendixes..... | 36 |
|-----------------|----|

**Introduction:****Seasonal organ atrophy and dormancy**

Many echinoderms have remarkable regenerative capacity. Asteroids, for example, can use regeneration for repair purposes as well as for asexual reproduction. Regeneration in holothurians serves as part of a sacrificial defense mechanism, in which they eviscerate in response to threats by predators and then regenerate their internal organs (Patruno, 2001).

*Parastichopus californicus*, the California sea cucumber, is native to the west coast of North America, being found in low intertidal and subtidal waters from Baja California to the Gulf of Alaska (Paltzat et al., 2007). Swan (1961) reported the phenomena of seasonal evisceration in *P. californicus* in the Friday Harbor area of Washington, where he found an absence of respiratory trees and intestinal organs in late fall. Because holothurians are known to eviscerate under predatory attack or other stressors, it was assumed that this absence represented evisceration, only on a seasonal basis.

Fankboner and Cameron (1985) reported that the missing organs in *P. californicus* were not a result of evisceration, but seasonal atrophy. This organ atrophy is now known to be an indicator of seasonal dormancy in *P. californicus*. Dormancy is a period of reduced metabolic activity that has been described in several holothurians, including *Parastichopus* (Klanian, 2013; Yang, 2006; Bertolini 1930, 1932; Swan 1961; Mosher 1965; Jespersen and Liitzen 1971; Dimock 1977; Muscat 1982; Fankboner and Cameron 1985).

In *P. californicus*, the degeneration process begins in the fall, when animals decrease food intake and display soporific behavior. When atrophy begins, all feeding and movements cease. Approximately 2-4 weeks after the loss of their organs, regeneration

begins, starting with the gut. Once the regeneration of the gut has been completed, feeding begins, and regeneration of the rest of the internal organs, including the respiratory trees, proceeds (Fankboner & Cameron, 1985).

### Respiration in holothurians

Many sea cucumbers possess a dual gas-exchange system, respiring across their body surface and also by using their specialized respiratory organs, the respiratory trees (Astall and Jones, 1991). The respiratory trees spread out within the coelom and reduce diffusion distances to internal tissues. Cloacal pumping facilitates tree-filling in holothurians. Water is drawn into the cloaca by a negative pressure created by the dilation of the relaxed cloacal wall through contraction of the suspensor muscles. After this, the anal sphincter contracts and the muscles relax, causing the water to be forced into the respiratory trees. Exhalation is accomplished by relaxing sphincters in the trees and allowing the coelomic pressure to collapse the respiratory trees, forcing the water out. (Shick, 1983).

Some echinoderm species have hemoglobin-containing coelomic cells called hemocytes in their perivisceral fluid. These oxygen-carrying cells have been found in *Cucumaria frondosa* and *Molpadia arenicola*, but are absent in *Sclerodactyla briareus*, *Holothuria forskali*, and *Pteraster tesselatus* (a sea star) (Shick, 1983). *P. californicus* also does not contain hemocytes (Boolootian & Giese, 1958). Interestingly, while *S. briareus* does not have perivisceral hemocytes, they have high affinity intracellular hemoglobin confined to their water vascular system, found in their tube feet (Roberts et al., 1984; Brown and Shick, 1979). *S. briareus* is a respiratory tree-containing burrowing sea cucumber that will extend its body into the water column under declining P<sub>O<sub>2</sub></sub> conditions.

This allows the water vascular system to pump water in and out of the tube feet, facilitating gas exchange (Shick, 1983; Brown and Shick, 1979). Amongst the echinoderms, hemoglobins have been found primarily in some burrowing sea cucumbers and a few brittle stars (Kitto et al., 1998). The possibility of *P. californicus* also having hemoglobin-containing cells in their water vascular system has not yet been explored.

### Hypoxia responses

During dormancy, *P. californicus* is devoid of its internal organs and does not eat. As it lacks respiratory trees, it is unable to obtain oxygen through that means. It does not have oxygen stores, so how do they obtain enough oxygen for their survival, even if oxygen needs are low? Studies on respiration through cloacal occlusion have shown that more than 50% of gaseous exchange may be accomplished via the respiratory trees in *Holothuria forskali*, *Sclerodactyla briareus*, *Psolus fabrkii*, and *Stichopus mollis* (Astall & Jones, 1991). Therefore it is reasonable to assume that their respiration rate would decrease after evisceration or seasonal atrophy. Many oxygen dependent tissues may switch over to anaerobic metabolism, potentially prompting an increase in the activity of enzymes involved in anaerobic metabolic pathways (Mangum and Van Winkle, 1973).

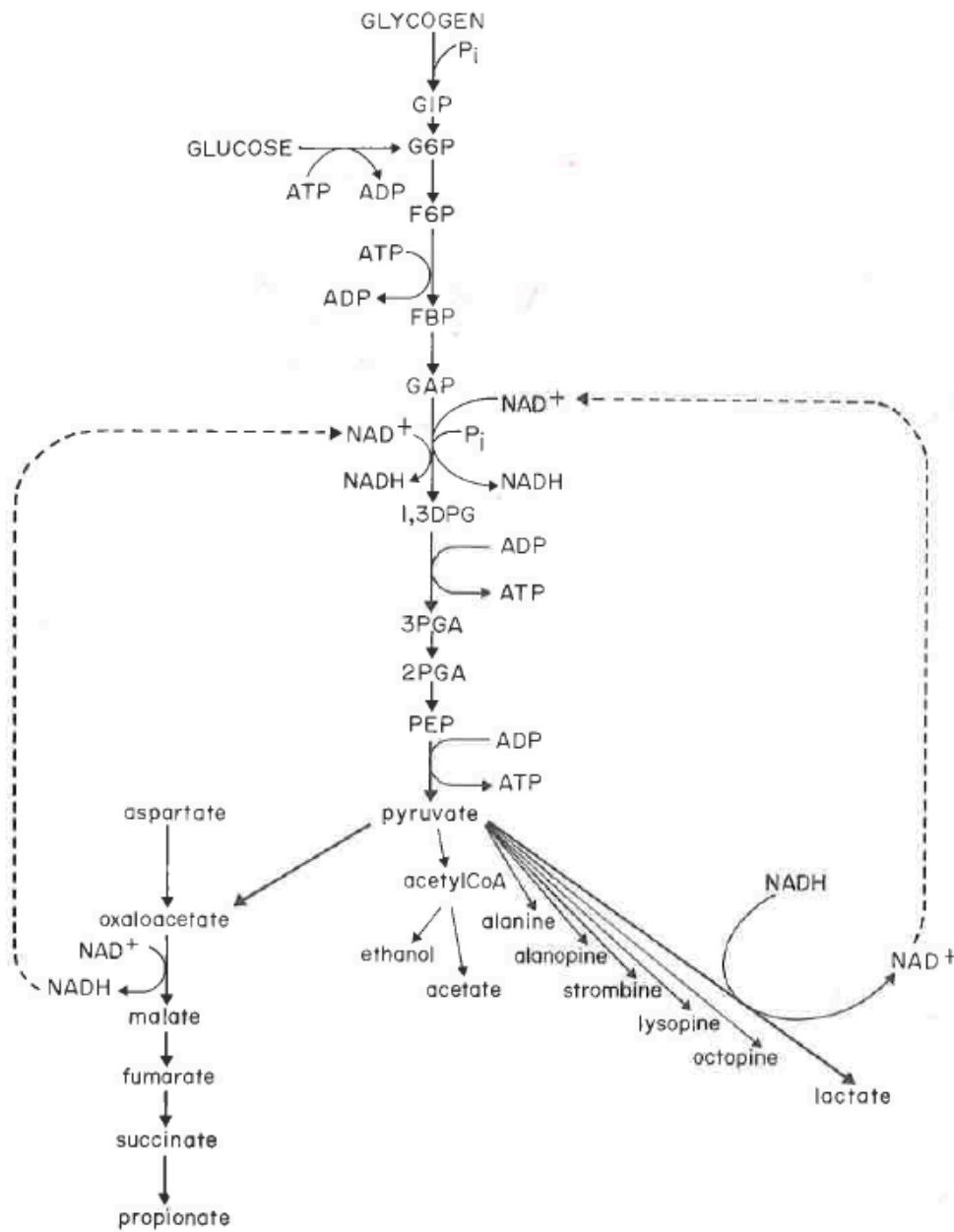
Chronically hypoxic echinoderm tissues utilize the lactate pathway over other anaerobic pathways, such as the succinate or opine pathways, which are more energetically efficient and more efficient in maintaining the NAD<sup>+</sup>/NADH ratio, respectively. While other pathways may be more efficient, one main advantage of lactate formation is the easy diffusion of lactate from tissues into the body fluids, which facilitates the continuous reduction of pyruvate as the reaction product is eliminated (Harcet et al., 2013). However,

the presence of anaerobic pathways other than lactate production in echinoderms has not been definitively ruled out (Shick, 1983).

Opines are end products of anaerobic glycolysis in mollusks and other invertebrates, biosynthesized by opine dehydrogenases that catalyze the reductive condensation of pyruvate and amino acids (figure 1). Opine dehydrogenases have a physiological role analogous to lactate dehydrogenase of vertebrates in the maintenance of redox balance during anaerobic glycolysis. Examples of opine pathways include the octopine pathway, alanopine pathway, strombine pathway, tauropine pathway, and B-alanopine pathway. Studies have shown that, in general, opine dehydrogenases seem to be in the lower marine invertebrate phyla and essentially absent in the higher phyla (Sato et al., 1993). However, the presence of tauropine dehydrogenase was discovered in the starfish *Asterina pectinifera* (Kan-no et al., 1998).

### Purpose

The purpose of this study is to determine if there is a difference in activity between aerobic and anaerobic metabolic pathways during the yearly cycle of visceral degeneration and regeneration in *Parastichopus californicus*. I will establish which pathways are available to be used by the sea cucumber during its seasonal cycle and the degree that they are used relative to each other. The results should provide a more conclusive answer to the question of how an animal without functional respiratory organs is able to survive extensive periods of apparent hypoxia.



**Figure 1.** Major fermentation pathways in animal tissues (Hochachka and Somero, 1984). The most important of these fermentation and anaerobic pathways are:

1. Glucose → lactate, energy yield 2 mol ATP/mol glucose
  2. Glucose → octopine, energy yield 2 mol ATP/mol glucose
  3. Glucose → succinate, energy yield 4 mol ATP/mol glucose
  4. Glucose → propionate, energy yield 6 mol ATP/mol glucose
  5. Glucose → acetate, energy yield 4 mol ATP/mol glucose
  6. Aspartate → succinate, energy yield 1 mol ATP/mol glucose
  7. Aspartate → propionate, energy yield 2 mol ATP/mol aspartate
- (McFadden, 2004)

Research Questions:

- What anaerobic pathways are used by *Parastichopus californicus*?
- When are aerobic and anaerobic metabolic pathways most active and inactive during *P. californicus*' cycle of organ atrophy and regeneration?
- Is the lactate pathway the main metabolic pathway functional in *P. californicus* during its seasonal organ atrophy?
- Do tissue-specific differences occur in aerobic and anaerobic potential?
- Do *P. californicus* have hemoglobin-containing cells in the water vascular system of their tube feet?

## **Methods**

### **Timeline:**

This experiment will be conducted during three seasons over the course of eight months:

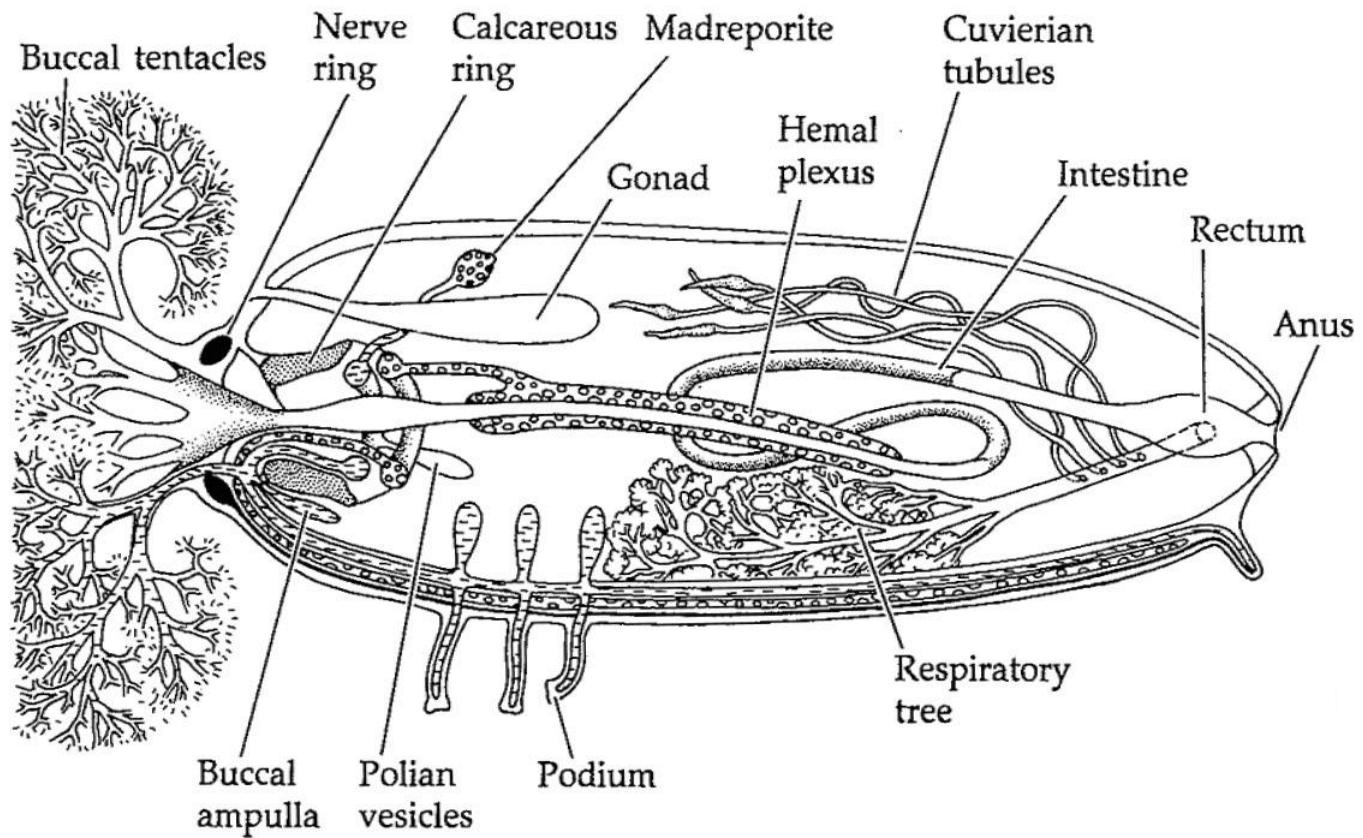
1. July-August 2014 (Summer) – *P. californicus* contains its visceral organs
2. October-November 2014 (Fall/Winter) – *P. californicus* will be undergoing visceral organ degeneration, or will have already done so and be empty of internal organs.
3. January-February 2015 (Spring) – Internal organs should be undergoing the regenerative process.

### **Location & Animal Care:**

*Parastichopus californicus* will be collected using SCUBA off the coast of the Rosario Beach Marine Laboratory in Anacortes, WA, USA (48°25'22"N 122°40'21"W). Upon arrival back at the marine lab, animals will be immediately dissected and samples of tissue will be frozen and stored in liquid nitrogen. Proposed tissues to be sampled include those that are present at all times (longitudinal muscle, dorsal and ventral sides of the body wall) and organs that typically atrophy and regenerate (respiratory trees, digestive tract, and gonads) (figure 2). A sample of coelomic fluid will also be taken for metabolite analysis, as well as a sample of fluid from the water vascular system for hemoglobin analysis. These samples will also be frozen and stored in liquid nitrogen.

### **Tissue Sample Preparation**

Tissue samples for enzymatic analyses will be prepared by first grinding approximately 0.3 g of frozen tissue to a fine powder in liquid nitrogen using a mortar and pestle. Ground tissue will be incubated in a centrifuge tube with a lysis buffer consisting of



**Figure 2.** A diagram of the internal organs of a holothurian (Brusca and Brusca, 2009).

50 mM Tris at pH 7.5, 1 mM Dithiothreitol, 0.15 NaCl, 1 mM EDTA, 0.1% Tween-20, 10% glycerol, and 1.3% protease inhibitor cocktail at a 1 g tissue to 5 mL of fluid dilution. The tubes will incubate on ice for 10 min and will be vortexed for ten 10 sec every minute. After 10 min, the tubes will be centrifuged at 10,000 g for 15 min at 4°C. The supernatant containing the enzymes will be pipetted off the pellet, and stored in liquid nitrogen (Parker, 2014).

Tissues to be used for lactate assays will be deproteinized and homogenized in 1 N perchloric acid at an approximate 1 g tissue to 30 mL of fluid ratio. The homogenate will be centrifuged at 3000 g for 10 min at 0°C to separate the metabolite (supernatant) from the rest of the cellular structures (pellet). The supernatant will be removed and neutralized to pH 3.5 with 5 M K<sub>2</sub>CO<sub>3</sub>. The supernatant will be frozen and stored in liquid nitrogen for further analysis (McFadden, 2004).

### Enzyme Analyses

#### **Citrate synthase**

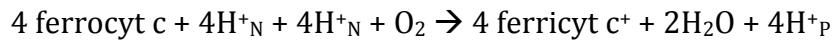
Citrate synthase is found at the beginning of the citric acid cycle and is used as an indicator of the aerobic metabolic ability of a tissue. Citrate synthase catalyzes the formation of citrate from acetyl-coA and oxaloacetate, with coenzyme A as a by-product. The reduction of 5,5-dithiobis (1-nitrobenzoic acid) (DTNB) by free coenzyme A will be monitored by the measuring the increase in light absorption by TNB at 412 nm to determine the activity of citrate synthase. The effectively irreversible chemical interaction between coenzyme A and DTNB is shown below (McFadden, 2004).



The reaction mixture will consist of 0.5 mM oxaloacetate, 0.25 mM DTNB, 0.4 mM acetyl-CoA, and 75 mM Tris-HCl at pH 7.8. Reactions will be initiated by adding oxaloacetate. A background rate of deacylase activity will be measured and subtracted from the citrate synthase activity (protocol from Tullis et al., 1991).

### **Cytochrome c oxidase**

Cytochrome c oxidase is one of the enzymes involved in the electron transport system, providing energy for the cell by coupling electron transport through the cytochrome chain with the process of oxidative phosphorylation. It catalyzes the following reaction:



The enzymatic activity of cytochrome oxidase will be used as an indicator of the activity of the electron transport system in the tissues, and will be measured using the Cytochrome C Oxidase Assay (COX) from ScienCell.

### **Hexokinase**

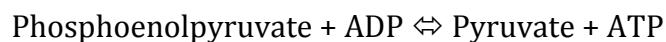
Hexokinase is the enzyme functional in the first step of glycolysis, and is therefore essential in the regulation of carbon flow in glycolysis (Saito & Watts, 1989). It catalyzes the phosphorylation of glucose to glucose 6-phosphate in the following reaction:



Activity of hexokinase will be assayed in a medium containing 7.5 mM MgCl<sub>2</sub>, 0.8 mM EDTA, 1.5 mM KCl, 0.4 mM NADP<sup>+</sup>, 2.5 mM ATP, 1.0 mM D-glucose, 10.0 mM creatine phosphate, 0.9 units/mL creatine phosphokinase, 0.7 units/mL glucose-6-phosphate dehydrogenase, and 75 mM Tris-HCl at pH 7.2. The activity of hexokinase will be determined by measuring the rate of NADP<sup>+</sup> reduction to NADPH by exogenous glucose-6-phosphate dehydrogenase at 340 nm. Reactions will be initiated by the addition of glucose (protocol from Tullis et al., 1991).

### **Pyruvate kinase**

Pyruvate kinase is another rate limiting enzyme in glycolysis. It catalyzes the reversible conversion of phosphoenolpyruvate to pyruvate in the reaction seen below:

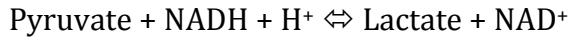


The activity of pyruvate kinase will be monitored by measuring the rate of NADH oxidation to NAD<sup>+</sup> during the reduction of pyruvate to lactate by lactate dehydrogenase at 340 nm. The reaction mixture will consist of 150 mM KCl, 1 mM KCN, 10 mM MgSO<sub>4</sub>, 0.15 mM NADH, 5 mM ADP, 0.02 mM fructose-1,6-diphosphate, 2.5 mM phosphoenolpyruvate, 10 units/mL lactate dehydrogenase, 50 mM imidazole at pH 6.7. Reactions will be initiated by the addition of phosphoenolpyruvate (protocol from Tullis et al., 1991).

### **Lactate dehydrogenase**

The lactate pathway is the best known and most researched of the anaerobic pathways and is present in most, if not all, phyla (Livingstone, 1983). Lactate dehydrogenase catalyzes the production of lactate from pyruvate in one of the main

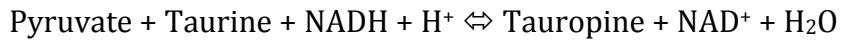
anaerobic metabolic pathways in echinoderms (Ellington, 1982). The full reaction catalyzed by lactate dehydrogenase is as follows:



The reaction rate between lactate dehydrogenase and pyruvate will be measured by monitoring the oxidation of NADH at 340 nm during the reduction of pyruvate to lactate. The reaction mixture will consist of 0.15 mM NADH, 1 mM KCN, 50 mM imidazole, and 2.5 mM sodium pyruvate at pH 7.2. Reaction will be initiated by the addition of pyruvate (protocol adapted from Tullis et al., 1991). The decrease in absorbance due to the oxidation of NADH will be recorded (McFadden, 2004).

### **Tauropine dehydrogenase**

Tauropine dehydrogenase is the terminal enzyme of anaerobic glycolysis in an opine pathway, the tauropine pathway. The reaction catalyzed by TDH is as follows:



Echinoderm tissues generally have relatively large pools of free amino acids (Ellington, 1982). Detectable levels of taurine have been found in the holothurians *Thyone* sp. and *Stichopus japonicas* (Simpson et al., 1959; Severin et al., 1972). Generally, the amino acid present in the highest tissue concentration forms the amino acid moiety of the opine that is predominately accumulated by the organism (Harcet et al., 2013). While thought not to occur in higher marine phyla, tauropine dehydrogenase activity has been found in the sea

star *Asterina pectinifera* (Kan-no et al., 1998). However a study done by Sato et al. (1993) did not find any activity of tauropine dehydrogenase activity in *Stichopus japonicus*. While *S. japonicus* is a closely related species to *P. californicus*, the presence or absence of this pathway has not been studied in *P. californicus*.

The enzymatic activity of tauropine dehydrogenase will be measured by monitoring the rate of NADH oxidation at 340 nm. The assay mixture will contain 100 mM Tris-HCl buffer at pH 7.2, 6 mM sodium pyruvate, 100 mM taurine, and 0.3 mM NADH. Reactions will be initiated by addition of taurine (protocol from Sato et al., 1991 and Kan-no et al., 1998). The background activity of lactate dehydrogenase will be measured before the addition of taurine.

### **Malate dehydrogenase**

Malate dehydrogenase catalyzes the conversion of oxaloacetate to malate in the presence of NADH in the glucose-succinate pathway, as well as in the citric acid cycle. The reaction is as follows:



In this pathway, glucose is catabolized through glycolysis, but flux is diverted towards the end of the glycolytic pathway by carboxylation of phosphoenolpyruvate, producing oxaloacetate. Oxaloacetate is converted to malate by malate dehydrogenase, and malate is further metabolized to succinate and then on to propionate and other volatile fatty acids (Livingstone, 1983). While energy production is faster in pathways using pyruvate reductases (lactate dehydrogenase and opine dehydrogenases), the glucose-

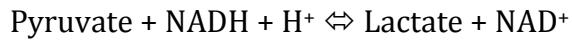
succinate pathway is more efficient in producing energy (Harcet et al., 2013; Livingstone 1983). Incubation of the longitudinal muscle of the sea cucumber *Thyonella gemmata* under hypoxic conditions produced lactate, but also produced succinate after prolonged incubation (Ellington, 1982). Other studies have indicated production of propionate in the urchin *Strongylocentrotus droebachiensis'* ovary tissue when exposed to anoxia for more than eight hours (Bookbinder and Shick, 1986). These results suggest that there may be a possibility of anaerobic succinate pathways in some echinoderm species.

The oxidation of NADH will be measured at 340 nm in order to determine the activity of malate dehydrogenase. The reaction mixture will contain 4 mM NADH, 2 mM oxaloacetate, and 87 mM Tris-HCl at pH 7.4. Oxaloacetate will be added to the mixture to initiate the reaction (protocol from Ellington and Lawrence, 1972).

### Metabolite Analysis

#### **Lactate**

The amount of lactate in the surveyed tissues will be determined by oxidation with lactate dehydrogenase in the presence of NAD<sup>+</sup> as shown in the following equation:



The amount of lactate present in the tissues is proportional to the amount of NADH produced in the reaction (McFadden, 2004).

The reaction mixture will contain 2.5 mL hydrazine/glycine buffer (0.5 M glycine, 0.4 M hydrazine, pH 9.0) and 0.20 mL 40 mM B-NAD<sup>+</sup>. 200 ul of sample homogenate will be added to the mixture, and twelve units of LDH added to initiate the reaction. Total NADH

production will be read at 340 nm after two hours of incubation at 37°C. Lactate concentrations in tissues will be determined by the generation of a standard curve using known lactate concentrations (protocol from McFadden, 2004).

### Hemoglobin Analysis

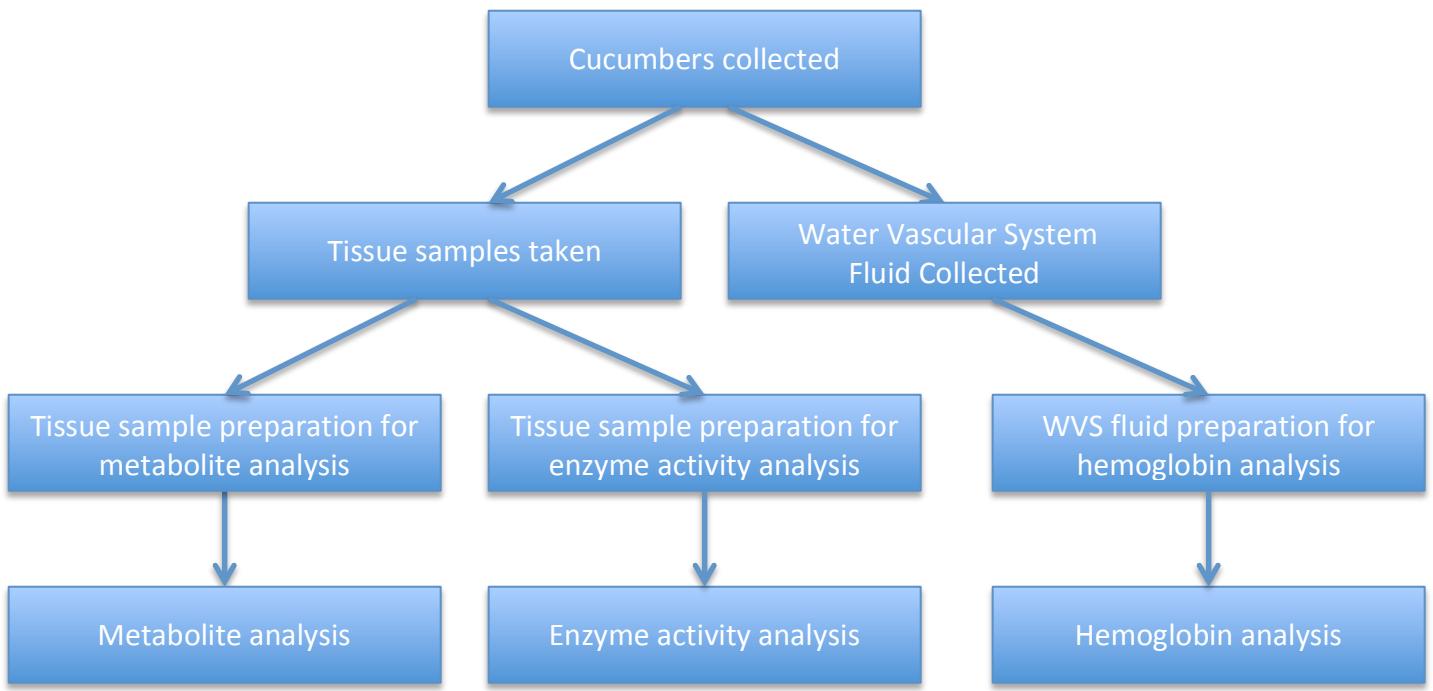
Fluid from the water vascular system will be obtained by puncturing the podial ampullae and draining the contents into collection containers chilled on ice. Fluid containing potential hemoglobin-containing cells will be centrifuged in a refrigerated centrifuge at 130 x g for 10 minutes. Pelleted hemoglobin-containing cells will be washed with filtered seawater and then lysed with ice-cold distilled water for 15 min. The lysate will be centrifuged at 13,000 x g for 10 minutes and the supernatant collected. If the supernatant is reddish in colour, hemoglobin-containing cells should be present (protocol from Roberts et al., 1984). The supernatant will be analyzed for [hemoglobin].

If hemoglobin is present, for determination of total hemoglobin, the absorption spectrum of the solution will be recorded. The hemoglobin concentration, as heme, will be calculated using the Beer-Lambert law and the extinction coefficient at 512 nm. The concentration will be multiplied by the total sample volume to give total hemoglobin (as heme) in millimoles, and then divided by animal wet weight to obtain total hemoglobin per gram of wet weight (protocol from Christensen et al., 2003).

### Data Analysis

A two-way ANOVA will be used to analyze enzyme activity during the summer, fall, and winter in each tissue. The amount of metabolites in each tissue during each sampling period will also be analyzed using a two-way ANOVA. Two-way ANOVAs will be used in order to determine if there are significant differences between time periods and between

tissues. The concentration of hemoglobin containing cells in the water vascular system during the three time periods will be analyzed using a one-way ANOVA.



**Figure 3.** Summary of the experimental procedure that will be followed during each sampling season: summer, late fall, and winter.

**Results:****Enzyme Analyses:**

I expect to find that the activities of anaerobic enzymes (lactate dehydrogenase, malate dehydrogenase, tauropine dehydrogenase) will be higher during the degeneration and regeneration phases than in the summer, when the sea cucumber has fully active respiratory trees. In the summer, I expect to find higher activities of aerobic enzymes (citrate synthase and cytochrome C oxidase) due to the normal functioning of the respiratory trees. As to the anaerobic pathway used by *Parastichopus californicus*, I expect that the lactate pathway will be the most active compared to other proposed anaerobic pathways based on other reports of anaerobic metabolism in holothurians.

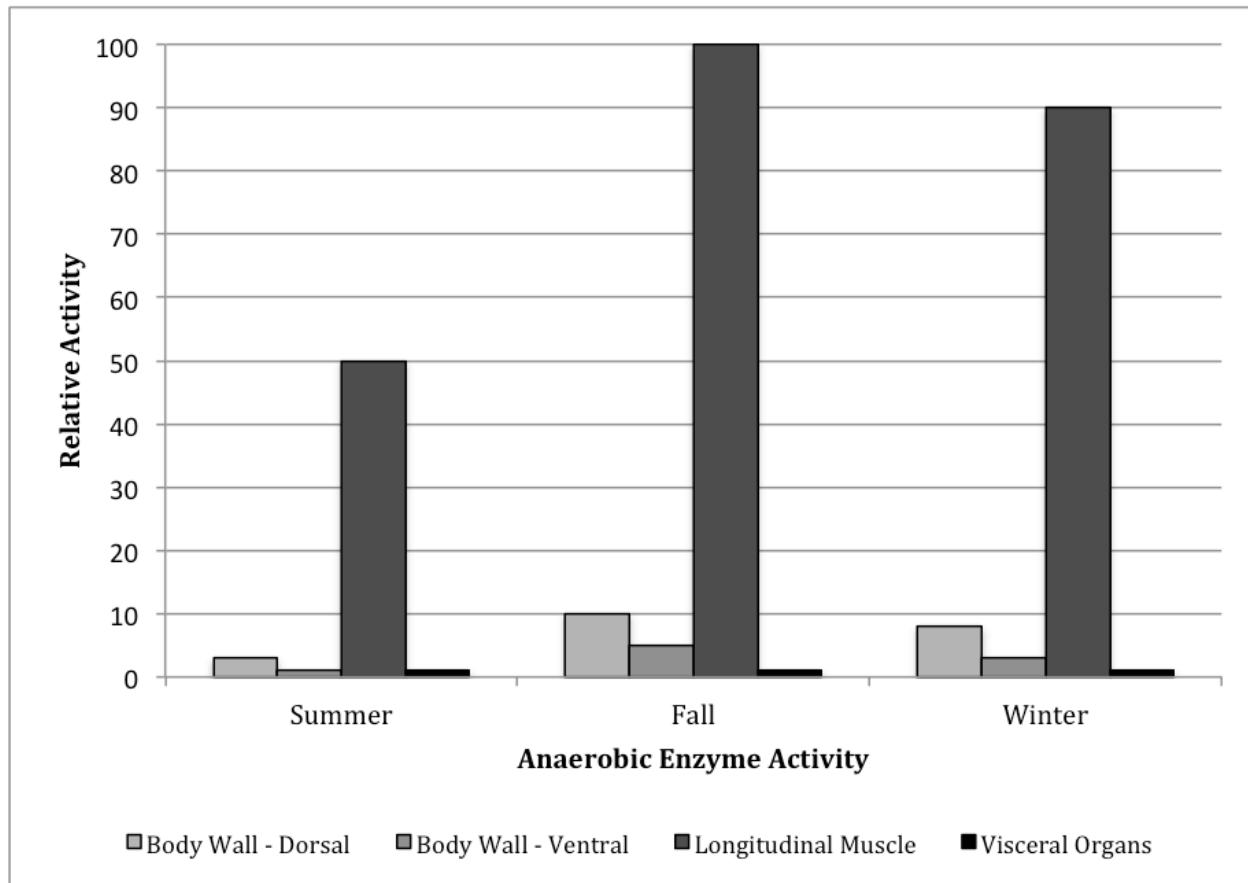
I expect to find higher activities of anaerobic enzymes in the longitudinal muscle than in the body wall tissues and internal organs (figure 4). With respect to dorsal and ventral body wall tissues, I expect to find lower levels of anaerobic enzyme activity, if any, in the ventral tissue sample due to the proposed functioning of the tube feet in gas exchange. Therefore, I expect to find higher activities of aerobic enzymes in the body wall tissues (figure 5). During the summer when the visceral organs are fully functioning and being supplied oxygen via the respiratory trees, I expect to mainly find aerobic enzyme activity. During the winter, there will be very little internal organs to sample from, and those that are there would be in the atrophy stage. While enzyme activity in these organs might not be a fair representation of normal functioning, any present will be sampled. I expect to find low levels of anaerobic enzyme activity in the internal organs.

Metabolite Analysis:

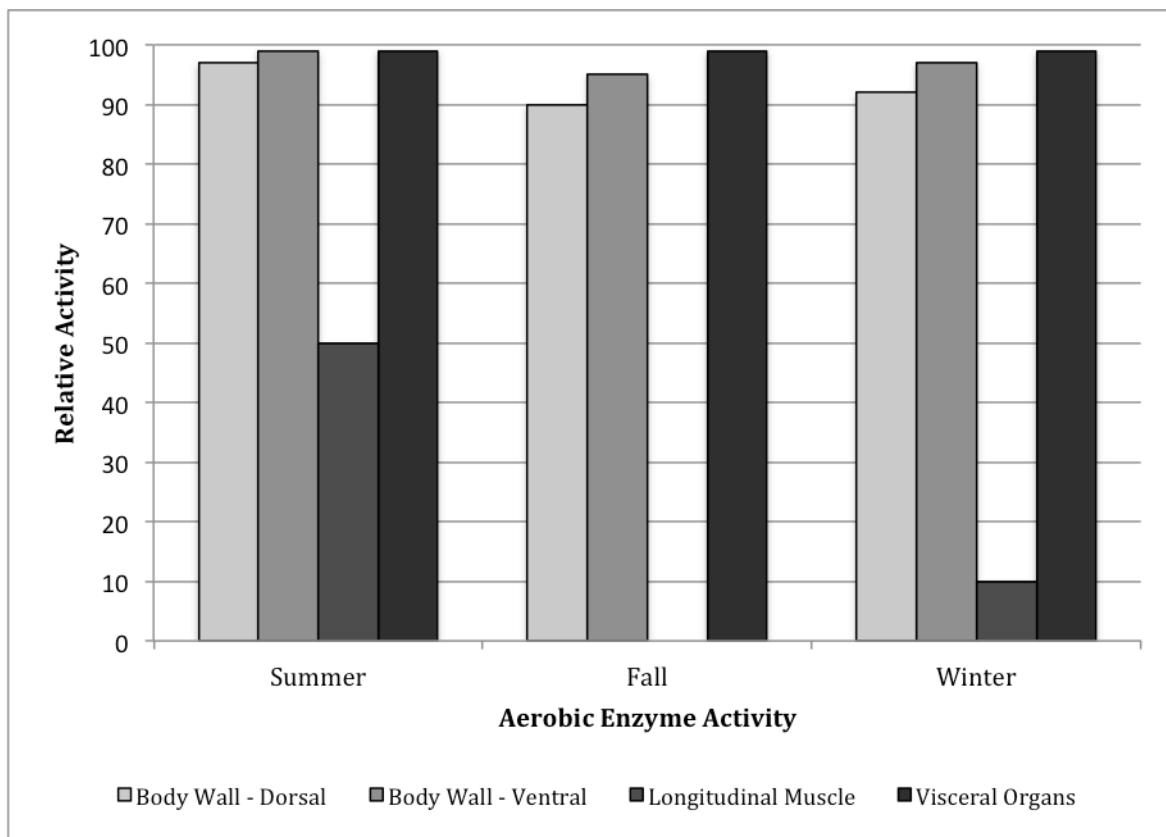
I expect that levels of lactate (or any other anaerobic metabolite) in body tissues will be higher in the fall and winter compared to the summer due to proposed increased activity of the lactate pathway during dormancy (figure 6). I also expect to find higher concentrations of lactate in muscle tissues compared with the body wall or any other tissue. I also expect to find higher levels of lactate in coelomic fluid during the fall and winter compared to the summer.

Hemoglobin Analysis:

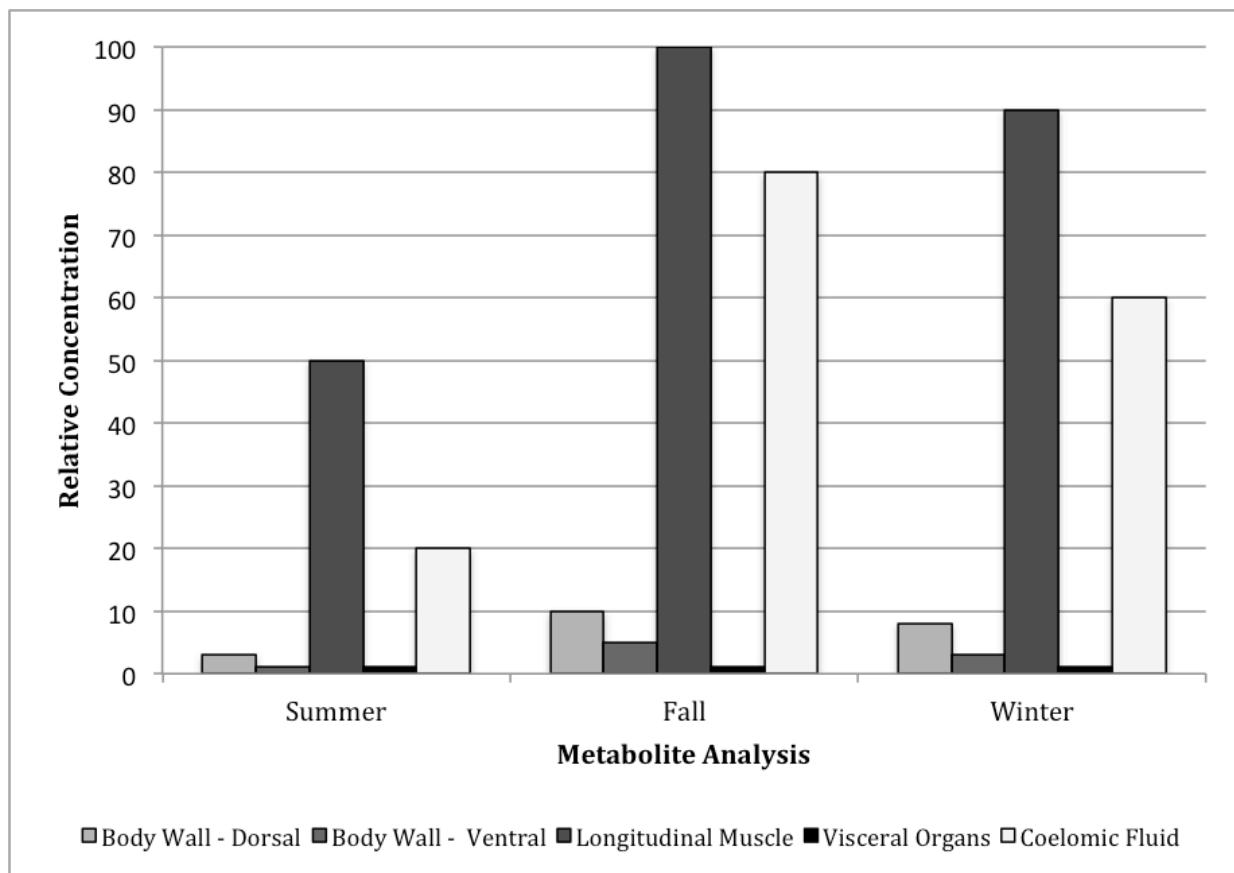
I do not expect to find detectable levels of hemoglobin containing cells in *P. californicus'* water vascular system. However, as far as I know, no one has measured the presence or absence of them in *P. californicus* or in any other *Parastichopus* species, therefore any data regarding this at all is helpful.



**Figure 4.** Proposed results of anaerobic enzyme activities during three seasons in *Parastichopus californicus*' cycle.



**Figure 5.** Proposed results of aerobic enzyme activities during three seasons in *Parastichopus californicus*' cycle



**Figure 6.** Proposed results from metabolite assays during three seasons in *Parastichopus californicus*' cycle.

**Discussion:****Enzyme Analyses**

The longitudinal muscle of holothurians has been characterized by a reduced capacity for aerobic energy metabolism due to an absence of cytochrome activity found in *Parastichopus tremulus* and low mitochondrial density in *Isostichopus badionotus*. These results imply that the aerobic metabolic capacity of the longitudinal muscle is impaired and that it may chronically rely on anaerobic energy production (Ellington, 1982). Thusly, I expect to find activity of anaerobic enzymes in all sampling periods. However, because of the significant reduction of oxygen available to internal tissues during the late fall and winter due to a lack of respiratory trees, an increase in activity is expected.

The body wall of echinoderms is considered to be the primary consumer of oxygen due to its large contribution to the total body weight of the organism (Lawrence & Lane, 1982). The body wall has been shown to be a major source of glycolytic activity in the starfish *Asterias vulgaris*, and as it has been postulated that holothurians also respire across their body surface, investigation into enzymes that are active during their seasonal cycle should be informative as to the source of its energy (Saito & Watts, 1989; Astal & Jones, 1991). If there is a difference in aerobic and anaerobic enzyme activity between sampling periods, I would expect it to be small, as gas exchange across the body surface will continue, and perhaps be elevated while *P. californicus* is without its respiratory trees.

**Metabolite Analysis**

As previously noted, one main advantage of lactate formation is the easy diffusion of lactate from tissues into the body fluids, which facilitates the continuous reduction of pyruvate as the reaction product is eliminated (Harcet et al., 2013). I would expect to find

higher levels of lactate in tissues during periods when respiratory trees are absent, as well as higher levels of lactate in the coelomic fluid due to transportation of lactate out of cells.

### Hemoglobin Analysis

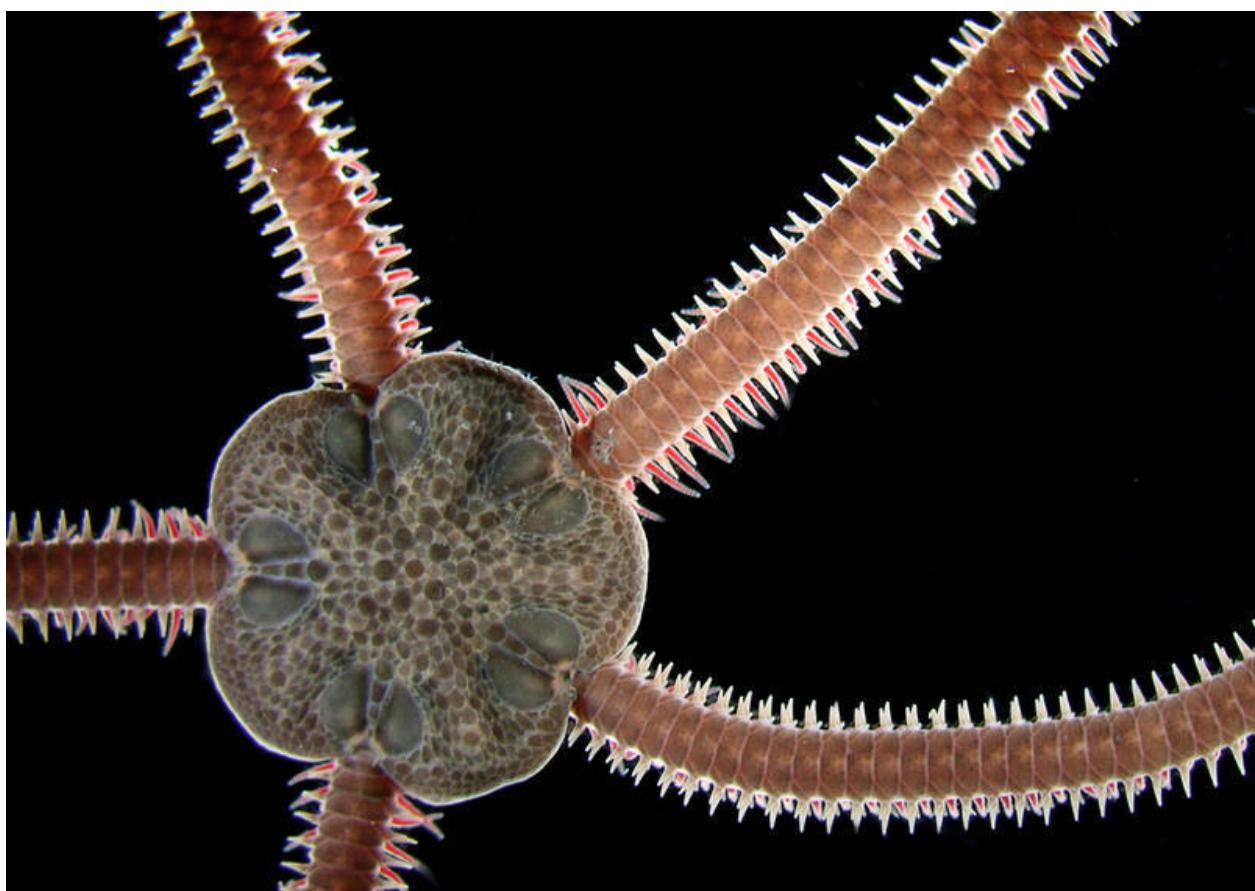
Tube feet are located on the ventral side of the sea cucumber. The tube feet of echinoderms have been implicated in a gas exchange type role as components of the water vascular system (Mangum, 1994). In echinoids, one type of these specialized feet are partitioned isopores. These feet have large pores, thin walls, and a septum that extends approximately three quarters of the length of the lumen of the tube foot. These factors allow for efficient gas exchange due to a large one-way flow, a minimized diffusion distance, and separation between incurrent and excurrent streams (Shick, 1983).

*Sclerodactyla briareus* uses this gas exchange option in low oxygen conditions as the water vascular system in their tube feet contains a high affinity intracellular hemoglobin (Brown and Shick, 1979).

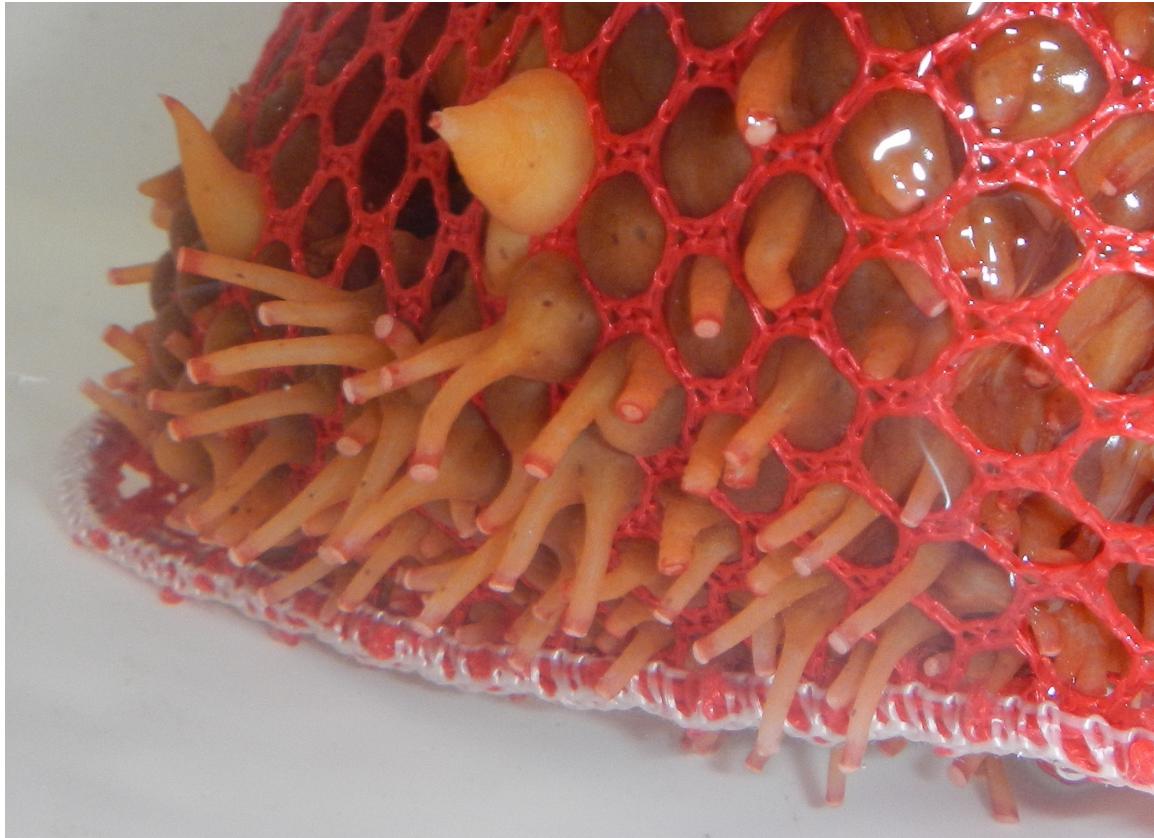
While *P. californicus*, as *S. briareus*, does not have hemocytes in its coelomic fluid, the possibility of gas exchange occurring in the water vascular system of specialized tube feet containing intracellular hemoglobin might contribute to lower levels of facultative anaerobiosis in dermal podia during seasonal organ atrophy. Another echinoderm, the brittle star *Hemopholis elongate*, has hemoglobin in anucleate ceolomocytes present in its water vascular system as does *S. briareus*. The large number of these red blood cells in the water vascular system gives the tube feet a bright red color (figure 7) (Christensen et al., 2003). Upon examination of the tube feet of *P. californicus*, a red ring surrounding the end of each foot can be seen (figure 8). While this red ring could be due to pigmentation, it could also be due to the presence of oxygenated hemoglobin-containing cells.

Vertebrate and invertebrate hemoglobins have the ability to bind with sulfide to form sulfhemoglobin. Many invertebrates show reversible binding with sulfide and, in many cases, the binding of sulfide is vital to the organism because it harbors endosymbionts that require sulfide as an energy source. The use of hemoglobin in sulfide uptake would challenge the proposition of it being used in oxygen exchange. Endosymbionts such as these have not been found in *H. elongata* and during oxygen equilibrium experiments, no abnormal spectral changes were observed when the hemoglobin was exposed to sulfide, indicating that either the hemoglobin is insensitive to sulfide or that there are detoxifying enzymes present. The presence of detoxifying enzymes was unlikely, as any enzymes present would have been greatly diluted in the hemolysate solution (Christensen et al., 2003).

Due to the fact that *P. californicus* is not a burrowing sea cucumber and that it has other methods of gas exchange, I do not expect to find hemoglobin in its water vascular system.



**Figure 7.** *Hemipholis elongata*, the burrowing brittle star. The bright red tube feet can be clearly seen, coloured by oxygenated hemoglobin in anucleate coelomocytes transported in the water vascular system. Image credits to Migotto 2002.



**Figure 8.** The tube feet of *Parastichopus californicus*, the California sea cucumber. A red ring can be seen surrounding the end of each foot. Could it be due to hemoglobin-containing cells?

### Further Questions

- Is respiration across *P. californicus'* body surface elevated during periods without respiratory trees?
- Are hemoglobin-containing cells in water vascular systems only found in burrowing echinoderms?

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**Appendix A – Proposed Budget:**

| <b>Proposed Budget</b>  |                |   |
|---|----------------|---|
| <b>Product</b>  | <b>Amount</b>  | <b>Justification</b>  |
| Liquid nitrogen   | \$180.00       | Preserve and grind specimens  |
| Citrate synthase assay reagents   | \$6.00         | Indicator of the aerobic metabolic ability of a tissue                                      |
| Cytochrome C Oxidase Assay, 100 tests, cat# 8278                                | \$280.00       | Indication of the functioning of the electron transport system                              |
| Hexokinase assay reagents   | \$130.00       | Key regulatory enzyme in glycolysis   |
| Pyruvate kinase reagents  | \$780.00       | Key regulatory enzyme in glycolysis   |
| Lactate dehydrogenase assay reagents  | \$2.00         | Probable anaerobic pathway used by <i>Parastichopus californicus</i>                        |
| Tauropine dehydrogenase assay reagents  | \$15.00        | Possible anaerobic pathway used by <i>P. californicus</i>                                   |
| Malate dehydrogenase assay reagents   | \$5.00         | Main enzyme in succinate pathway, possible anaerobic pathway used by <i>P. californicus</i> |
| Lactate analysis reagents   | \$1000.00      | Product of lactate dehydrogenase reaction   |
| Hemoglobin analysis reagents  | Negligible     | Exploratory analysis of water vascular system   |
| Transportation to Rosario Beach Marine Lab (Anacortes, WA) from Walla Walla, WA | \$602.70       | Two round trips (1,470 total miles) at \$0.41/mile  |
| <b>Total Expenses:</b>  | <b>\$3,000</b> |   |