**Microbiome diversity in the sea cucumber *Parastichopus californicus*during its yearly degeneration/regeneration cycle**

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**Abstract**

**Introduction**

Echinoderm Tissue Regrowth

All multicellular species can repair damaged tissue. However, the degree and capacity to which organisms can regenerate missing tissue is considerably different across many groups of vertebrates and invertebrates. Organisms may regrow tissue to recover from disease, predation, or the loss of body parts. For example, lizards can regrow tails while arthropods can regenerate appendages following autotomy. Other organisms undergo annual cycles of atrophy and tissue regrowth, such as antlers in deer or reproductive organs in birds.

Echinoderms have remarkable regenerative capabilities, as they can regenerate all body parts (Tsonis 2000). Autotomy, the spontaneous loss of body parts, is well documented and widespread in echinoderms. In Asteroidea, lost rays, or arms, can be cast off and regenerated. Some Asteroids can generate a new individual from the basal end of a single ray, without the central disk, while other Asteroids need the central disk to reproduce asexually by fission (Edmondson 1935).

In some holothurians, internal organs are eviscerated as part of a sacrificial defense mechanism in response towards predators (Patruno et al. 2001). Holothurians are known to have 76 predators, with fish (26 species), sea stars (19 species), and crustaceans (17 species) being the most common (Francour 1997). While most predators avoid holothurians because of their toxicity, other highly specialized organisms, such as gastropod *Tonna perdix,* can secrete sulphuric acid to paralyze its prey. *T. perdix* as well as some sea star species seem to have immunity against holothurin, a group of toxins belonging to the class of chemical compounds known as saponins (Caulier et al. 2011).

Other holothurians undergo annual cycles of atrophy of visceral organs and tissue regrowth. *Apostichopus japonicas* is an epibenthic holothurian species found in temperate coastal waters of southeast Asia. *A. japonicas* is the most commercially important and one of the best studied holothurians, as it has long been exploited as an important fishery resource in Russia, China, Japan, and North and South Korea (Sloan 1984). Farming of sea cucumbers is a key part of the aquaculture sector in northern China and the total production reached over 5800 tons in 2002 (Chen 2004). As a result, many biological aspects of *A. japonicas* have been studied including feeding and digestion, reproduction, larvae development, juvenile growth and nutrients, and metabolism (summarized in Yang et al. 2005).

*A. japonicas* is a sediment feeder, ingesting organic matter which contains bacteria, protozoa, diatoms, and detritus from plants and animals (Zhang et al. 1995). *A. japonicas* also reutilizes residual food and feces (H.-S. Yang et al. 2000). In the summer, *A. japonicas* undergoes a period of estivation when the water temperature heats up excessively (Choo 2008). *A. japonicas* becomes inactive at water temperatures above 18°C and undergoes estivation around 20-24.5°C (Sui and Liao 1988; Liu et al. 1996). In the laboratory, medium animals (72.3–139.3 g) were found to estivate at a lower threshold temperature between 24.5 and 25.5 °C, while the estivation temperature of small animals ranged from 25.5 and 30.5 °C (H. Yang et al. 2005). Yang et al. (2005) also observed higher threshold temperatures for *A. japonicas* in more southern areas.

In contrast, *Parastichopus californicus,* found in the low intertidal to subtitle waters from the Gulf of Alaska to Baja California, undergoes seasonal organ atrophy in the fall when water temperature may be decreasing.

Seasonal Organ Atrophy, Dormancy, and Tissue Regrowth in *P. californicus*

*P. californicus* undergoes a seasonal cycle of atrophy, dormancy, and tissue regrowth. Atrophy occurs in the fall, typically in late September. *P. californicus* appetite and movement decrease while displaying soporific behavior (Fankboner and Cameron 1985). Winter decreases in feeding and metabolic rate have also been observed in Antarctic holothurian *Heterocucumis steineni* (Fraser et al. 2004). However, McCloskey (2006) found that metabolic rate increased during winter in *P. californicus.* These results likely indicate *P. californicus* collected byMcCloskey were not undergoing atrophy, but instead, were regenerating visceral organs.

Approximately 2-4 weeks after atrophy, internal organs, including the digestive tract, respiratory trees, and gonads, are regenerated (Fankboner and Cameron 1985). In *Apostichopus* (closely related genus to *Parastichopus*), free edges of the mesenteries are left behind after atrophy. Gut regeneration begins with the thickening of the free edges of the mesenteries along the length of the body, eventually forming the intestinal lumen (García-Arrarás and Greenberg 2001; Murray and García-Arrarás 2004).

While the functions of this seasonal cycle are largely unknown, two clear beneficiaries have been observed. Symbionts live in the coelom of *P. californicus* where they lay eggs (Frankboner 2002). During atrophy, parasitic worms, parasitic gastropods, and parasitic protozoans are expelled through transrectal coelomoducts (Frankboner 2002; Shinn 1985; Shinn et al. 1990). When evicted, new generations can colonize regrown tissue. Parasitic protozoans *Ozametra* sp. and *Anoplodium hymanae* live in the coelom of *P. californicus,* consuming the intestines and coelomocytes (Shinn 1985). Visceral atrophy allows *P. californicus* to regrow tissue damaged from parasites.

Additional hypotheses for seasonal visceral atrophy include osmoregulation to combat salinity extremes from seasonal increases in precipitation (Frankboner 2002), seasonal availability of food, or a possible immune response stemmed from “cross-talk” with microbes.

Background: Importance of Microbiomes

The vertebrate gut microbiome is a complex microhabitat comprised of bacteria, fungi, and archaea. While some bacteria in the gut are pathogenic, the majority of gut flora are normal and essential for human health, helping produce metabolites which contribute to biosynthetic pathways and immunity (Heijtz et al. 2011; Li et al. 2009). Recent studies have shown that microbes contribute to anabolic processes, such as the production of serotonin, which is an important regulatory factor in the gastrointestinal tract and inhibitory neurotransmitter (Yano et al. 2015).

Studies have shown that gut bacteria contribute to immune responses (Heijtz et al. 2011). As a result, probiotics, which positively influence immune responses, are commonly used to prevent and treat active illness (Isolauri et al. 2001). The symbiotic relationship between a vertebrate host and the microbiota has a significant impact on shaping the host’s immune system (Round et al. 2010). In many animals, the immune system and microbiota can exchange chemical signals in “cross-talk.” This allows the immune system to detect and combat harmful bacteria, while helping normal bacteria carry out their functions (Cahenzli et al. 2013).

Additional studies have shown a relationship the microbiome has between mental health and cognition. Probiotic treatment with *Lactobacillus* and *Bifidobacterium* significantly reduced psychological distress, leading to preliminary conclusions that mood and anxiety can be controlled by regulating the microbiome (Messaoudi et al. 2011).

Echinoderm Microbiome Background: What is known

Starcevich (2014) used culture-depended methods to isolate bacterial strains from *P. californicus.* Samples were taken during the spring, summer, and winter to observe seasonal fluctuations in the microbiota.

Results indicated there was higher CFUs (colony forming units) in the late summer and autumn (prior to atrophy) followed by a reduction of CFUs in the winter (during regeneration). A total of 174 unknown isolates were characterized for a total of 27 genera. The most abundant phylum was Proteobacteria, accounting for 93 of the 174 isolates. High numbers of Proteobacteria were contributed to *Vibrio* and *Pseudoalteromonas*. Firmicutes were the second-most represent phylum with 73 isolates, 55 of which were *Bacillus,* the most abundant genus encountered. Most isolates were obtained during autumn and winter (61 and 62 isolates respectively), followed by late summer (35 isolates) and early summer (15 isolates). *Vibrio* accounted for 30-50% of bacteria in early and late summer, however, fell below 17% in the Autumn and Winter. In contrast, *Bacillus* was higher in the Autumn and Winter (21 and 32 isolates respectively), however, was only found twice in early and late summer.

In 2010, investigators looked at the gut microbiome of the sea cumber *Apostichopus japonicues.* PCR-DGGE was used with 16S rDNA V3 fragments to find bacterial diversity (Gao, F et al. 2010). However, bacterial populations discovered using DGGE, and using other culture-dependent methods, may represent less than 1 percent of the total bacterial community (Muyzer et al. 1993).

More recently, the microbiome of *A. japonicues* has been investigated using 454-pyrosequencing and the 16S rRNA gene (Gao et al. 2014). Pyrosequencing technology has higher capacity than DGGE and culture-dependent methods at exploring microbiome diversity (Vaz-Moreira et al. 2011). This study found ambient sediment contained more OTUs (operational taxonomic units) and harbored different bacterial communities than the foregut or hindgut. Investigators attributed lower diversity in the gut to selective feeding. In both ambient sediment and gut contents, Proteobacteria was the most predominant phylum. Potential probiotics found in the gut included Pseudomonas, Bacillus related sequences, and lactic acid bacteria (Lactobacillus, Lactococcus, and Streptococcus).

Studies on the microbiome of other echinoderms demonstrate the importance microbes have on digestive health and innate immunity. Recently, a study showed warming ocean temperatures alter microbiome functionality in sea urchin *Lytechinus variegatus* (Brothers et al. 2016). Investigators harvested sea urchins grown at 26°C and 30°C (to emulate warming ocean waters) and compared the differences between microbiomes. While elevated seawater temperatures did not produce statistically significant changes in microbiome diversity, many modest shifts were observed. For example, OTUs assigned to Vibrionaceae, *Vibrio* sp., and *Alteromonas* sp. increased between ten and twenty-fold. These taxa include pathogenic bacteria which have been linked to disease and stress in corals (Meron et al. 2011). Furthermore, the proportion of OTU’s assigned to class Actinobacteria decreased six-fold. Many bacteria in this class are known to produce antibiotics while suppressing pathogenic function (Meron et al. 2011). These combined shifts in gut microbiota could potentially increase the risk of disease and decrease digestive health in sea urchins.

Microbial diversity was four-fold lower in the intestines of *L. variegatus* than in the food or surrounding sea water (Brothers et al. 2016). Unlike *A. japonicas* in the Gao et al. 2014 study, *L. variegatus* was not allowed to eat selectively. Thus, the lack of intestinal microbial diversity is likely from selective pressures in the intestines. A recent study found 95 percent of the gut microbiota of *L. variegatus* was comprised of order Campylobacterales (Hakim et al. 2015). Because most species in Campylobacterales are microaerophilic, *L. variegatus* likely selects for microbes which can live in low oxygen and/or higher levels of carbon dioxide (Garrity 2000).

Recent studies have shown important roles microbes play in digestion. For example, oceanic sea star class, Asteroidea, depend on photosynthetic bacteria to provide nutrients (Galac et al. 2016). At both developmental stages, bipinnaria and brachiolaria, Family Oreasteridae had a photosynthetic Cyanobacteria *Synechococcus* sp. as the most common bacteria.

Research Purpose/Importance

Understanding the gut microbiome of *P. californicus* is especially important due to their yearly cycle of visceral organ atrophy during the fall, and regeneration of organs in the spring. It would seem that *P.californicus* would lose important microbial diversity as organs, especially the digestive tract, atrophied.

Changes in the host or microbiota genome, such as under environmental stress, can cause rapid changes in the microbial community (Zilber-Rosenberg and Rosenberg 2008). Since *P.californicus* likely goes through stages of varying microbial diversity, *P.californicus* would likely be more sensitive to environmental stress, especially on microbial populations, during the regeneration period. This proposed study seeks to understand the fluctuations of colonizing microbial species in the visceral organs of *P.californicus* throughout the yearly degeneration/regeneration cycle. Understanding the fluctuations in in the microbiome will elucidate the microbiome’s role in the overall health of *P.californicus.*

Research Objectives/Questions

* How much does microbial diversity change in *P. californicus* during the atrophy/regeneration cycle?
* Which microbal species are more common during different phases of the cycle?
* Does *P. californicus*harbor any microbial species known to supplement nutrition or provide antibiotics? If so, in what stages of organ development do these species appear?
* Which microbes are resident, normal, or transient?

**Methods**

Collection of Samples

*P. californicus* will be collected by SCUBA near Rosario Beach Marine Laboratory, Anacortes, WA. Five animals will be collected in the summer, fall (during degeneration), and winter (during regeneration). Samples will be collected from at least six locations for each animal: (1) Seawater where animal was collected, (2) ambient sediment where animal was collected (Gao et al. 2014), (3) the mouth (foregut), (4) the anus (hindgut), (5) coelomic fluid, and (6) feces. Seawater and ambient sediment samples will provide an inventory of microbes *P. californicus* may ingest through feeding. Foregut, hindgut, and coelomic samples will be compared with seawater and ambient samples to determine which microbial species were obtained from selective feeding. Feces will be collected to determine transient bacteria.

To collect foregut and hindgut samples, the entire intestinal tract will be removed from the animal and washed with sterile seawater. All digested food will be rinsed from the intestine, presumably leaving behind microbial communities which are resident (Brothers et al. 2016). After rinsing the intestines, they will be freeze-dried with liquid nitrogen (N2) and ground with mortar and pestle. Coelomic fluid will be collected in 2 ml aliquots by puncturing the perisomal membrane with a syringe. All samples will be stored in centrifuge tubes. If time constrains do not allow for DNA extraction at Rosario, the samples will be transported to the main campus of Walla Walla University (WWU) on dry ice.

DNA Extraction Method

Microbial DNA will be extracted using a fecal DNA kit from Zymo Research (catalog #D6010). DNA will be extracted after tissues have been dried and ground using liquid nitrogen. Extracted DNA will be assessed using a 1% agarose gel. Sufficient DNA will be obtained to preform PCR. Purified DNA will be transported to WWU on dry ice if there are time constraints or Rosario is not set up for PCR.

PCR for Amplification of 16S rRNA

PCR will be used to amplify the V4 region of the 16S rRNA gene (Kumar et al. 2014). Unique bar coded primers DG74 (5'-AGGAGGTGATCCAACCGCA-3') and RW01 (5'-AACTGGAGGAAGGTGGGGAT-3') will be used for amplification. DNA will be amplified using a 100 μl reaction consisting of 1 μl template DNA, 1 μl of a 10 μM solution of both primers, 47 μl of sterile water, and 50 μl of 2X DreamTaq Green PCR Master Mix (Thermo Scientific). GenElute™ PCR Clean-Up Kit from Sigma-Aldrich will be used to purify PCR products. Sufficient PCR product will be generated to use for Illumina Sequencing.

DNA Sequencing Method (Deep Sequencing)

The metacommunity 16S rRNA genes will be sequenced using the Illumina MiSeqTM platform (also called NextGen Illumina MiSeq platform). Samples will be sent to the University of Oregon and sequenced using the HiSeq 4000. One lane of the HiSeq 4000 will be used to run single read 100bp.

Each HiSeq 4000 has 2 flow cells and 8 lanes per flow cell. 5,000,000,000 reads per run pass through filter/16 lanes = 313,000,000 reads per lane. 312,000,000 reads per lane/3,400,000 reads per sample = 91 total samples. If 5 animals are sampled, 3 times a year, at 6 sample sites, 90 total samples will be generated. If an additional sample site was added (5\*3\*7), 105 samples would be generated. This would lower the reads per sample to 2,971,429. Thus, if additional samples are desired, this would lower the reads per sample. As demonstrated, the desired coverage (in reads per sample) will indicate how many samples can be read.

Statistical Analysis of Sequencing Results

Rarefaction curves will assess species richness in the samples. If the total diversity of the bacterial community is represented in the samples, the rarefaction curves will reach an asymptote. Next-generation reads will be clustered to operational taxonomic units (OTUs) at a 97% identity threshold. UCLUST, an algorithm for OUT clustering, will align sequences into clusters by exploiting the use of USEARCH (Edgar 2010). Greengenes v13.8 database will be used to assign taxonomy to the OTUs by comparing the sequences to a reference database with known sequences (Kumar et al. 2014).

*Alpha diversity vs Beta diversity*

Alpha diversity will be calculated using Shannon’s diversity index compared between the three different times of year within the different sample types (e.g. hindgut, foregut) using a Student’s t-test. Beta diversity will be calculated using Bray-Curtis values which measure the dissimilarity in composition between different sites.

**Results/Discussion**

Microbiota composition is expected to be distinct according to sample location. I expect microbial diversity to be higher in ambient sediment and surrounding sea water, containing more OTUs than the *P. californicus* foregut, hindgut, or coelomic fluid. Selective feeding (Gao et al. 2014) as well as selective pressures in the intestines (Brothers et al. 2016) have been proposed to decrease microbial diversity in other echinoderms. For example, Brothers et al. proposed *L. variegatus* selects for microaerophilic Campylobacterales which can live in low oxygen and/or high carbon dioxide levels present in the gut.

Seasonal shifts in microbiota composition are also expected. Starcevich (2014) found a greater number of CFUs in in late summer and autumn, followed by a reduction in winter, during regeneration. Additionally, more isolates were obtained in autumn and winter than in summer. However, *P. californicus* collected during early and late summer were held in salt water tanks for 24-48 hours under starvation conditions. During this processing time *P. californicus* presumably passed the majority of their feces. Starcevich proposed that the remaining bacteria in the gut were more likely to be part of the resident microbiota. In the autumn and winter, *P. californicus* were only held in salt water tanks for about 2 hours, which may not have been long enough to pass the majority of transient bacteria in the fecal matter. As a result, the difference in bacterial diversity between early & late summer and autumn & winter may be contributed to transient bacteria in the fecal matter. The proposed study will aim to clarify results from the previous experiment. Intestines will be washed with seawater, presumably leaving behind microbial communities which are resident. Fecal samples will be obtained, which are likely comprised of transient bacteria.

Microbial species have been observed to fluctuate in abundance throughout the year within the intestines of *P. californicus*. For example, Starcevich (2014) found almost all *Bacillus* isolates during the autumn and winter. Starcevich consequently hypothesized that *Bacillus* may not be a member of the resident microbiota in the summer. The proposed experiment will attempt to explain why *Bacillus* is commonly found in the intestines of *P. californicus* in the autumn and winter, but not in early and late summer. Collecting seawater and ambient sediment samples will determine the abundance of *Bacillus* in the environment throughout the year. If *Bacillus* levels remain consistent, it can likely be concluded that selective feeding or selective pressures in the intestines of *P. californicus* change throughout the year.

The second most identified genus, *Vibrio*, also underwent seasonal changes in abundance (Starcevich 2014). *Vibrio* became more abundant from early summer (5 isolates) to late summer (19 isolates), but decreased in autumn (10 isolates) and again in winter (1 isolate). Starcevich proposed that *Vibrio* may be involved in the atrophy/regeneration cycle. *Vibrio* is widespread in the marine environment and is known to include pathogenic species which have been associated with stress and disease in other aquatic invertebrates (Meron et al. 2011; summarized in Brothers et al. 2016). The buildup of *Vibrio* over summer may be one of the triggers leading to atrophy. *P. californicus* only has an innate immune system and much of the immune system involves phagocytic coelomocytes which destroy foreign materials in the phagosome (Gliński and Jarosz 2000; summarized in Starcevich 2014). The innate immune system of *P. californicus* may not have the ability to clear *Vibrio* over the course of summer. Thus, the atrophy/regeneration cycle may be necessary to regenerate new, uncolonized intestines. Starcevich additionally proposed that a stable gut population of *Bacillus* in autumn and winter may be able to exert a probiotic effect, improving immunity and disease resistance to *Vibrio*, as demonstrated in *A. japonicas* (Zhao et al. 2016).

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**Proposed Budget**

|  |  |  |  |
| --- | --- | --- | --- |
| **Item** | **Purpose** | **Amount** | **Cost** |
| Bleach | Cleaning mortar and pestle |  | $5 |
| Liquid Nitrogen | Grounding/drying tissue samples | 1 tank is $40? | $100 |
| Dry Ice | Transportation of Purified DNA and/or PCR products |  | $40 |
| Quick-DNA™ Fecal/Soil Microbe Miniprep Kit from Zymo Research (catalog #D6010) | DNA extraction | Each kit has 50 preps ($216 each) | $432 |
| Bar coded primers: primer pair DG74 (5'-AGGAGGTGATCCAACCGCA-3') and 65ab (5'-AACTGGAGGAAGGTGGGGAY-3'). Use RW01 instead? (5'-AACTGGAGGAAGGTGGGGAT-3'). Order custom from IDT. | Amplify the V4 region of the 16S rRNA | Won't cost more than $10-15 each | $30 |
| GenElute™ PCR Clean-Up Kit NA1020-1KT (Sigma-Aldrich) | Purify PCR products | 70 reactions is $121 | $121 |
| HiSeq 4000 (Illumina MiSeqTM platform) | DNA sequencing of 16S rRNA genes | 1 lane: single read 100 bp | $1,440 |
|  |  |  |  |
|  |  |  | **TOTAL** |
|  |  |  | $2,168 |