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MONITORING ORGAN REGENERATION OF SEA CUCUMBER
HOLOTHURIA LEUCOSPILOTA AFTER EVISCERATION

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Abstract. Defense mechanisms have been long recognized as an important factor in establishing the development of many organism life histories. As a result, many processes associated with ensuring survivability have been very well established in organisms that utilize defense mechanisms. How these defense mechanisms have shaped the evisceration and regenerative processes of sea cucumber *Holothuria leucospilota* still remain largely unstudied. In this study it is revealed that *Holothuria leucospilota* remain very consistent in their modes of evisceration, ejecting the same organs in every evisceration event. The consistency is compounded by the finding that approximately 28% of their body mass is eviscerated regardless of organism size. In terms of organ regeneration, the thickening of the mesentery tissues is apparent at Day 4 and regeneration of the digestive tract at Day 16. Sediment feeding also resumes at Day 16 along with the regeneration of the digestive tract. No regeneration of the left respiratory tree or gonads is observed within the 28-day study.

Key words: Echinodermata, sea cucumber, evisceration, regeneration, Holothuria leucospilota, French Polynesia, predation, defense mechanism

INTRODUCTION

Defense mechanisms are widely observed and studied in organisms throughout the tree of life. These mechanisms range from having different hues of coloration for camouflage and warning cues to having protruding spines and parts as weapons against predators to traveling in packs to ward off threats to their young (Caro 1992). However, some most extreme examples in the spectrum of defense mechanisms in animals belong to a category of behavior often expressed in the Echinoderms: autotomy.

Autotomy is the process of self-amputation of body appendages or organs in response to stressful stimuli. Examples of this phenomenon have been studied in starfish (*Asteroidea*), brittle stars (*Ophiuroidea*), crinoids (*Crinoidea*), and sea urchins (*Echinoidea*). Starfish and brittle stars have the ability to detach arms to evade predation (Swan 1966). Arm and stalks have been known to detach in crinoids and spines fallen off of sea urchins in response to stressful predation attempts (Ebert 1967). However, with losses of such crucial body parts such as feeding arms and defensive spines, the capacity for regeneration of those parts has become especially developed in Echinoderms as part of its life history. The source of energy is unique to the organism, with energy for regeneration mostly absorbed from food or from breaking down tissue. Mostly noted in asteroids, the fatty molecules in the pyloric caeca have long been studied as a resource for regenerating arms (Emson & Wilkie 1980). Without quick regeneration of these parts from energy stores, these organisms become more vulnerable to predators as they continue to survive with lesser feeding, locomotion, or defense structures (Drumm 2010).

Though less recognized within echinoderms for autotomy and regeneration, sea cucumbers (*Holothuroidea*) are also extremely well-developed in these processes. One of the most stunning is a process called evisceration, which is a specialized form of autonomy that involves the ejection of the majority of its internal organs through a self-

induced rupture in its body. The phenomenon occurs through a rapid softening of a special type of connective tissue called the catch-connective tissue and violent muscle contractions that rupture the body wall or cloaca and eject the organs out of the body (Dawbin 1949; Bai 1967). A holothurian that has undergone evisceration can eject any or all of its organs based on predetermined breakage zones that are specific to species. These eviscerated organs include the oral structures, digestive tube, the gonadal tubules, hemal system, respiratory trees, and Cuverian tubules (Garcia-Arraras 2001). Even more remarkably, after such a traumatic event of losing most of its organs, the majority of the sea cucumbers survive and will slowly regenerate all of these lost internal organs over a period as short as 7 days to as long as 145 days (Garcia-Arraras & Mashinov 2011) For most species, the regenerated organs return to about the same size and weight as the eviscerated organs and maintain functionality after complete development (Yang 2005).

Evisceration is found only in the holothuroid orders of Aspidochirotida (deposit-feeding sea cucumbers) and Dendrochirotida (filter-feeding sea cucumbers) (Garcia-Arraras 2001). *Holothuria leucospilota (*Brandt 1835) is one of the most common and conspicuous animals found in the shallow, wave-sheltered waters on the island of Moorea, French Polynesia (Tremain 2005). *H. leucospilota* is a black, medium to large (400-500 mm), cylindrical and elongate sea cucumber, its body is usually hidden under rubble and algae and has 20 oral tentacles that actively feed on sediment all day (Endean 1957; Ong Che 1990). As a member of the Aspidochirotida order, *H. leucospilota* is as an attractive model system as it is fairly sensitive to physical touch and can eviscerate under tactile stress without the standard injections of potassium chloride or distilled water as has been recommended in other papers to quicken evisceration time. (Hyman 1955, Dawbin 1949, Byrne 1986) Evisceration in *H. leucospilota* is accomplished through a

rupture in the cloaca and can eject organs from their internal anatomy, which include their digestive tract, gonadal tubules, respiratory trees, oral structures, esophagus, and Cuverian tubules. These organs are collectively called its viscera when they become detached from its body wall. Regeneration of lost viscera occurs in about 20 days (Dolmatov 2012).

However, despite *H.leucospilota* being a good model system for studying autotomy and regeneration, it has only been studied in depth only recently (Dolmatov 2012). Many papers have been published on feeding behaviors, movement, respiration, and modes of evisceration for holothurians, (Uthicke 1999; Ong Che 2000; Mangion 2004; Yu 2011) but very few papers have combined the ideas together in monitoring regeneration and energy resources for *H. leucospilota* after traumatic events such as evisceration.

This study seeks to investigate the regeneration of *H. leucospilota* after evisceration of its digestive tract, reproductive gonads, and respiratory tree. However, to answer this question, many more basic questions needed to be answered about evisceration process and natural habitat of the species on Moorea, French Polynesia:

Where *Holothuria leucospilota* are found around Moorea, French Polynesia? If so, where are the highest densities of *H. leucospilota* from which I can sample from for the study?

A. Do they eviscerate the same organs and dissolve tissue at same location within the body each time? How much of their body weight is lost due to evisceration?

B. How long does it take for the regeneration of the eviscerated organs? In what order are they formed and how quickly are they formed?

C. Given the lack of a digestive system, does the sea cucumber feed after evisceration? If so, how much does it feed and at what rate? If not, how does it get nutrients to sustain energy for regeneration of lost viscera?

It was hypothesized that the sea cucumber would be found best in rocky areas around either of the bays on Moorea. For evisceration, it was predicted that it eviscerates the same organs each time, and loses about half its body mass in the process. It was predicted that the regeneration order will first be mesentery, digestive tract, respiratory tree, and finally gonads. For feeding, it was predicted that they will not feed within the first 2 weeks after evisceration and instead uses its own energy stores to start regeneration by breaking down its body wall tissues. After critical organs have formed (digestive tract, respiratory) after 2 weeks, it will then start consuming sand. It was also hypothesized that the body wall and muscle thickness will decrease over the time of this study.
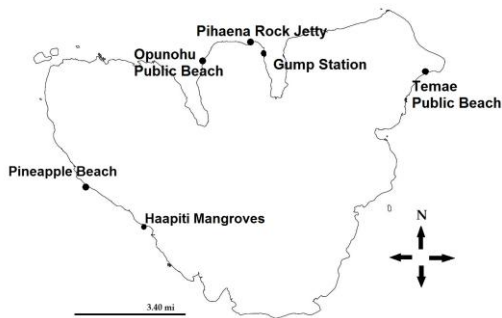
METHODS

Study sites

FIG. 1. Map of sites on Moorea, French Polynesia used for the field survey.

All data was collected between September 25, 2011 and November 15, 2012 at the UC Berkeley Gump Research Station, (17°29'23.10"S, 149°49'31.78"W) in Moorea, French Polynesia.

Several field surveys were performed at various sites around Moorea, French Polynesia to record the presence of *H. leucospilota.* The sites investigated for the field study were located at the Pihaena Rock Jetty (17°48′54.81"S, 149°83′11.38"W), Painapo (Pineapple) Beach near Haapiti (17°33'58.82"S,

149°52'9.19"W), Ta'ahiamanu (Opunohu Public Beach) on the east side of Opunohu Bay (17°29'27.82"S, 149°51'1.10"W), Gump Station (17°29'17.09"S, 149°49'33.88"W), Haapiti Mangroves (17°33'17.92"S, 149°52'44.81"W), and Temae Public Beach (17°29'57.25"S, 149°45'46.26"W) (Fig. 1).

Field survey

To figure out the density of *H. leucospilota* at these sites, 50m-by-2m belt transects were carried out at each study site location with a 1-meter square quadrat at every meter, with the transect tape laid out along the shoreline or jetty (Fig. 2).
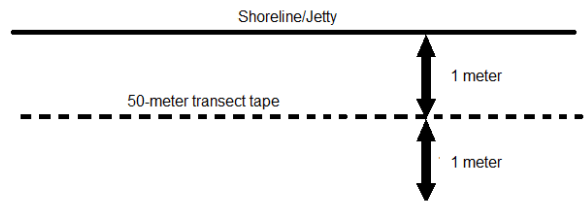

FIG. 2. Diagram of belt transect set-up

From preliminary observation, *H. leucospilota* appeared in the highest densities at the Pihaena Rock Jetty site. The rock jetty site was easily identifiable with a 50-meter stretch of rock rubble stretching outwards through a shallow lagoon area. The substrate at the study site largely consisted of loose algae-encrusted rocks and basalt-dominant sand, as was common in locations near Opunohu and Cook's Bay. All organisms used in the study were collected within the shallow area, up to depths from 0-1 meter and at maximum 3 meters from the jetty.

Holothuria leucospilota were easily found under movable rocks, usually with its posterior end hidden under the rock and the anterior end out with oral tentacles extended (See Appendix A). Fifty sea cucumbers were collected from the study site and carried back via a 10-minute bike ride in gallon-sized, sealable plastic bags back to the UC Berkeley Gump Station to be stored the water tables (2.35x 0.88x0.15 m), which were located under

a roofed water lab that was exposed to open air all day. The average water level was kept at around a depth of 0.1 m during the duration of the experiment and a constant inflow and outflow of seawater kept around 27°C to maintain a fresh flow of water for the organisms at all times. Large cement blocks were placed around inside the table to simulate rocks for the sea cucumbers to burrow themselves under.

Evisceration

Evisceration was induced by prodding the dorsal side of the sea cucumber and performing a peristaltic motion along its body length with gloved hands. If Cuverian tubules were not expelled after a minute, the sea cucumbers would be turned onto its ventral side and the same motions would be performed again until Cuverian tubules were expelled from the individual. This would continue for about 10 minutes, or until a cloaca rupture occurred. Once the digestive tract appeared through the rupture, a light squeeze would be given to the body of the sea cucumber to ensure evisceration and then would be put aside for 10 minutes to allow for complete evisceration.

Experimental Set Up

On Day 1, 32 sea cucumbers were eviscerated on a running seawater table to ensure that toxins emitted by the sea cucumber during evisceration would not accumulate in its immediate surroundings. After evisceration, the sea cucumbers were placed on a separate partitioned water table (Fig. 3) with identical environmental conditions as the original water table water described previously. Sixteen non-eviscerated control sea cucumbers were placed in the same water table to serve as the control for the experiment.

This partitioned table contained a composition of 70% basalt-dominant sand and 30% Alyzerin-Red-dyed sand, used to track
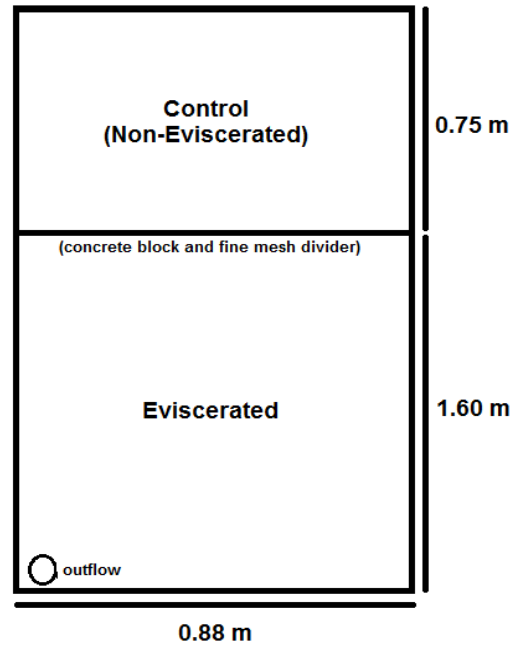

FIG. 3. Diagram of water table layout for eviscerated/control sea cucumbers.

sand consumption within the sea cucumbers after evisceration. The mixed sand was evenly spread on the table until a 1-cm-deep layer of sand coated the bottom of the table. Alyzerin-Red-dyed sand was prepared by collecting coral-based sand and soaking it in a 0.1g/L Alyzerin Red dye solution in seawater solution for 24 hours and washed once with seawater before mixing with the basalt sand (collected from Cook's Bay) and depositing the sand into the table.

Evisceration: Body Mass & Organ Loss

After complete evisceration of each organism on Day 1, the identity of each eviscerated organ was identified and recorded. The wet weights of the organism and its eviscerated organs were also taken by measuring them on a scale. Before weighing, the eviscerated organs and organism were suspended in air for 10 seconds in a separate container so that minimal external fluids would be accounted for in the measurement.

Eviceration: Point of Disconnect

When the sea cucumbers were dissected to track for eviscerated organ regeneration, the length of the esophagus left behind after evisceration and the length of the organism were also measured to determine if the anatomical point of evisceration was consistent to organism length within the species. The coloration was helpful in differentiating between the esophagus and regenerating tract. (Fig. 4)

FIG. 4. Example of a measurement of the location of point of disconnect (taken from Day 28).

Regeneration

The 32 eviscerated sea cucumbers were maintained and tracked regenerating organs and sediment consumption. Every 4 days (Day 1, 4, 8, 12, 16, 20, 24, 28), four eviscerated sea cucumbers and two control sea cucumbers were taken from the water table at the same time of day and were frozen and prepared for dissection.

Regeneration: Tracking Organ Regeneration

During dissection, the presence and absence of the Cuverian tubules, left and right respiratory trees, esophagus, digestive tract, Polian vesicle, mesentery lining, and gonadal tubules were recorded. If present, diameter thickness (if applicable) and lengths of the organs were recorded.

Regeneration: Sediment Consumption

To track if the sea cucumbers consumed any sand during regeneration, any dyed sand that was found anywhere in the esophagus or digestive tract was extracted from and washed with tap water. This was done by putting the sand in a large petri dish and swirling the water around 5 times and dumping out any floating excrement. This was repeated 15 times to ensure that sand was washed thoroughly before leaving the petri dishes to dry for 48 hours. The sand was then weighed and recorded for further analysis.

Regeneration: Body Wall/Longitudinal Muscle Thickness

To determine if energy resources from the body wall or muscle tissues were being used for regeneration, thickness of the body wall and longitudinal muscle tissue were measured. A 1-inch incision was made using scissors at the middle of the sea cucumber perpendicular to the initial longitudinal incision to provide better measuring angle for body wall and longitudinal muscle thickness. These measurements were taken using an analog caliper to the closest tenth of a millimeter.

All statistical analysis and graphs were performed in JMP.10 SAS Institute, Inc. 2012.

RESULTS

Field Survey

The densities of *H. leucospilota* indicate that the Pihaena Jetty location has the highest density found per square meter, followed by the Gump Station and Opunohu Public Beach Sites. Pineapple Beach only had 1 *H. leucospilota* that was abnormally sized. Temae Public Beach and Haapiti Mangroves did not have any *H. leucospilota*.

Due to the high density and number of organisms at the Pihaena Jetty location, it was

chosen as the main field site from which my study organisms would be collected from for experimentation. (Fig. 5)
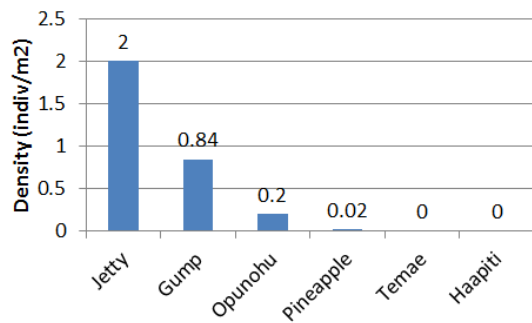

FIG. 5. Densities of *H.leucospilota* individuals per sq. meter at the Pihaena Jetty, Gump Station, Opunohu Public Beach, Pineapple Beach, Temae Public Beach, and Haapiti mangrove field sites.

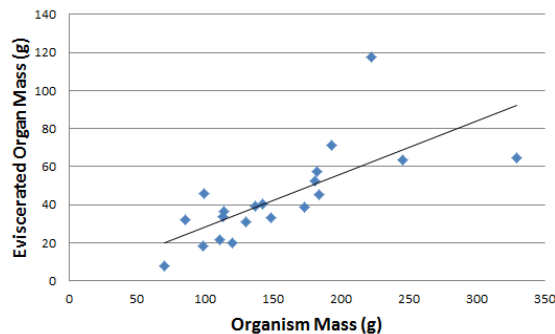*Evisceration: Body Mass & Organs Lost*

FIG. 6. A sea cucumber in this study lost on average about 29% of its body mass through evisceration (Linear regression analysis, F = 20.277, P = 0.0003, R^2 =0.5133)

All 32 individuals ejected the same organs during evisceration. The organs that were eviscerated were consistently the Cuverian tubules, digestive tract, the mesentery, the left respiratory tree, and gonadal tubules.

Regardless of organism mass, the *H. leucospilota* used in this study consistently ejected around 29% of its body mass through the evisceration process. (Linear regression analysis, F = 20.277, P = 0.0003, R^2 =0.5133). The pre-eviscerated organism mass ranged from 70.0 to 329.1 grams, and viscera mass ranged from 8.0 to 117.7 grams. The equation modeling this correlation can be illustrated as the following: (Fig. 6)

Viscera Mass = -2.287251 + 0.2911654*Body Mass

Evisceration: Point of Disconnect

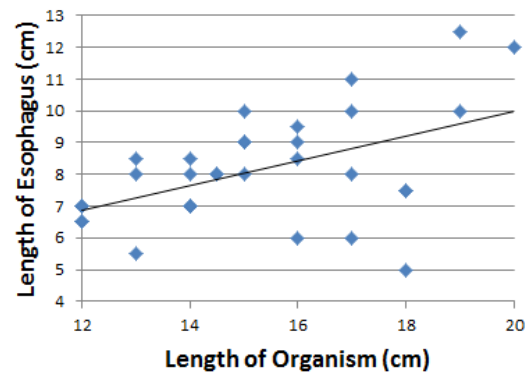

FIG. 7. Regardless of length, the anatomical point of disconnect in *H. leucospilota stays consistent to around 2/5ths the length of the length of the organism* (Linear regression analysis, F= 10.954, P = 0.0024, R^2 = 0.261).

Sea cucumbers eviscerate from around the same approximate anatomical location within their esophagus, which is around 2/5ths down the length of the individual organism. (Linear regression analysis, F= 10.95, P = 0.0024, R^2 = 0.261) (Fig. 7). The lengths of the organism ranged from 12 to 20 centimeters and the esophagus length to the point of disconnect ranged from 5 to 12.5 cm. The equation modeling this correlation can be illustrated as the following:

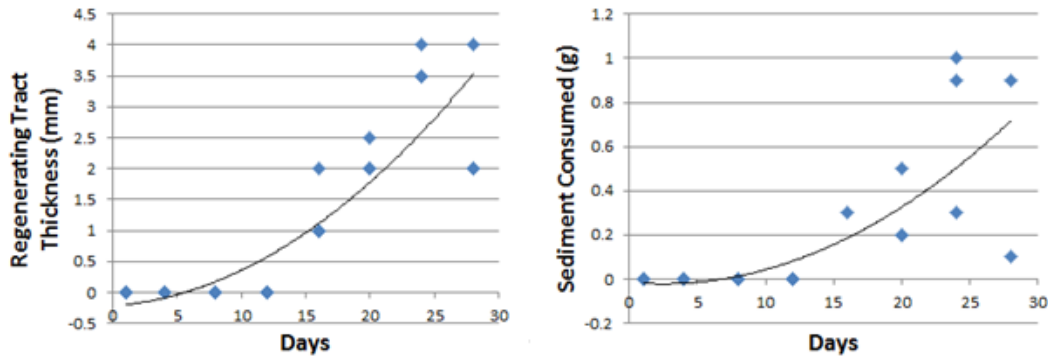Esophagus Length (cm) = 2.161234 + 0.3917914*Organism Length (cm)

FIG. 8. Both the thickening of the digestive tract (Linear regression , F= 16.893, p<0.0001, R^2 = 0.7809) and amount of sediment consumed (Linear Regression, F=48.125, p<0.0001, R^2 = 0.5949) simultaneously started growing exponentially on Day 16.

Regeneration: Organ regeneration

Organ regeneration started on Day 4 with a thickening in the mesentery lining on the inside wall cavity as well as the thickening of the Cuverian tubules within the body cavity. The mesentery tissue thickened anteriorly and posteriorly toward the middle from Day 4 to Day 16, which then formed a unite gut tract by Day 16 and was able to start thickening as a united digestive tract. After Day 16, the digestive tract grew exponentially in thickness over time through Day 28 (Linear regression, F= 16.89, p<0.0001, R^2 = 0.7809) (Fig. 8). By Day 28, the Cuverian tubules had not fully thickened back to original size.

No signs of the regeneration of the left respiratory tree or gonadal tubules were apparent during the 28-day study.

Regeneration: Sand consumption

Sediment consumption did not occur from Days 1 to 12. However by Day 16, dyed sediment appeared within the esophagus and digestive tract and increased exponentially in mass through time through Day 28 (Linear Regression, F=48.12, p<0.0001, R^2 = 0.5949) (Fig. 8). The sediment weights taken from Days 16 to 28 (ranging 0.1-0.9 grams) however did not resemble that of the controls (1.2 to 3.6 grams).

Regeneration: Body Wall/Longitudinal Muscle Thickness

No significant findings were found between the body wall thickness or longitudinal muscle thickness over time after evisceration.

DISCUSSION

The field portion of the study attempted to investigate the densities of *H. leucospilota* around Moorea and found that the highest densities were at the Pihaena Rock Jetty, Opunohu Public Beach, and Gump Station sites and the lowest at Pineapple Beach, Temae Public Beach, and Haapiti Mangroves. This differs from the last published class survey results in 2004 where *H. leucospilota* could only be found at a single location called Point Aroa on the northeastern side of Moorea (Tran 2003).

From preliminary observation, the largest reasoning for this was likely influenced by *H. leucospilota* habitat preference, which includes dense rock and algae cover in low current areas (Ceesay 2012). Based on personal observation and communication with peers who had measured current speeds at these sites, the current speeds at Temae Public Beach and Pineapple Beach were much faster than that of the areas in and around the Gump

Station and Opunohu and Cook's Bays. In the Haapiti Mangroves, though the current was much lower, the habitat was not ideal due to the lack of rock or algae cover at the site. The other sites (Gump Station, Pihaena Rock Jetty, Opunohu Public Beach) all had areas of lower current combined with movable rock substrate that allowed the *H. leucospilota* to populate the area in higher numbers.

The Pihaena Jetty Site the highest number as well as the highest density of *H. leucospilota* of all the sites studied in Moorea. As the regeneration study required high number of organisms to be sacrificed, a site where removing approximately 50 sea cucumbers would not hurt the population significantly was necessary. The Pihaena Jetty was chosen as the most ideal site, as this was the only population where several hundred individuals were present rather than just several dozen. However, since only one population was used for the study, the results produced from this study may only reflect the traits of one specific population of *H. leucospilota* and may differ from that of other populations of *H. leucospilota* around the island.

For evisceration, the individuals consistently ejected the same organs during evisceration, as was expected as it has been suggested that sea cucumbers within the species follow the same evisceration processes and that species within the same order follow similar eviscerated organ trends (Bai 1971; Garcia-Arraras 2001). These trends continue through the other measurable traits of evisceration for *H. leucospilota*, including the averages of 28% of body mass lost and the point of evisceration being 2/5ths down the length of the organism. This consistent location for the point of evisceration suggests that catch connective tissue is crucial in holding most of the viscera that is expelled in evisceration to the body wall regularly. The loss of Cuverian tubules also is important in that its primary defense mechanism of ejecting these toxic tubules are missing and cannot be used immediately while the sea cucumber is in the process of regenerating the Cuverian tubules.

After evisceration, the sea cucumber starts the processes to regenerate its organs again. (Hyman 1955) However, because of a lack of equipment and materials as well as scheduling dissections every 4 days, many cellular developments before the mesentery thickened on Day 4 were missed. In addition, because the mesentery was extremely thin, it was difficult to quantify the thickening of the mesentery before day 16 when the unite gut grew very quickly and became visible enough to measure by microscope or eye alone.

Compared to the only other published study done on regeneration of *H. leucospilota* earlier in 2012 at the South China Sea, this study agrees with the 16-20 day time period of unite gut thickening as published in the data of that study (Dolmatiov 2012). However, this study contradicted with the finding that mesentery growth occurs at 7-8 days instead of at 4 days. This could be an issue of agreeing on the definition of mesentery growth, as different parts of the mesentery (the posterior and anterior ends become thicker as the gut is formed) grow at different rates at different points in the first 16 days.

Sediment consumption started when the unite gut had formed on Day 16, highly suggesting that with a unite gut tract leads to the initiation of sediment consumption. This also confirms that sediment consumption cannot happen with a developing, unconnected digestive tract. However, this also brings into question how the sea cucumber gains the energy for the regeneration of the unite gut.

One other food energy source that was not accounted for in measuring sand was the possibility of scraping off and absorbing microparticles of algae from nearby rocks or even absorbing dissolved organic molecules from their surroundings through their epidermis (J. Nestler, personal communication, November 5, 2012). Another possible energy resource would be from breaking down tissues like the body wall or

muscle tissue, which was tested in this study with no significant results. However, these results could have been negatively affected heavily by how the sea cucumbers were sacrificed. In the freezer environments, the *H. leucospilota* tended to constrict and shrink to a much smaller size, introducing the possibility of thickening both the body wall and muscle that led to variable thickness measurements.

In light of a 2010 study finding positive trend of decreasing body wall weight in response to removal of gonads in *H. leucospilota*, it could be beneficial to reinvestigate body wall changes in response to evisceration through further study (Drumm 2010). This could include sacrificing the sea cucumbers through high doses of anesthetic to relax the muscles and weighing out the tissues in addition to measuring the thicknesses with the calipers.

Conclusion

Sea cucumbers harbor some of the most energetically defense mechanisms known in the animal kingdom. However, how much these defense mechanisms compromise their survivability and their capacities to regenerate organs become important to their life histories and evolution as a species. My results show that *H. leucospilota* eviscerate crucial organs that compromise their feeding abilities and abilities to defend against predators, which only restart with the regeneration of the tissues that support the growth of a completely new digestive tract. The energy for regeneration does not seem to come from the body wall or muscle tissues, though further investigation may provide additional insight into where the sea cucumber obtains its energy for regeneration.

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Moorea has 3 different black-colored sea cucumber species that difficult to identify, but be easily differentiated by habitat preference, size, and evisceration behaviors.

The following chart was created to help identify these species in Moorea:

Picture of Species	Species Name	Habitat Preference/Sites	Unique Species Traits
	Holothuria leucospilota	Low currents, low wave action Prefers hiding under rock or algae cover Popular Sites: Around rocky shores in Cook/Opunohu Bays	Has soft black spikes around its soft body 20-30 cm when extended Has Cuverian tubules
	Holothuria whitmaei	High currents, high wave action Found under coral heads, sometimes found with no cover Popular Sites: Temae Public Beach, back reef area of Cook's Bay	Smooth, but very firm to the touch 20-30 cm when extended Found solitary
	Holothuria atra	Low currents, low wave action Prefers extremely shallow waters (0-0.5 m) Very exposed, no rock cover Popular Sites: Haapiti Mangroves, Tetiaroa, any motu	Smooth, but very firm to the touch 10-20 cm when extended Does not eviscerate Cuverian tubules Found in groups of hundreds/thousands.