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Arm Loss and Regenerative Capacity of the Common Soft - Bottom Sea Star Luidia Clathrata Exposed to Near -Future Conditions of Ocean Acidification

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BIOLOGY

ABSTRACT

Recent rapid increases in ocean acidification (OA) are triggered by the absorption of excess anthropogenically–derived atmospheric CO_2 that reacts in seawater to elevate free H^+ concentrations. This process can cause a wide range of impacts on marine organisms. To date no studies have focused on the sub-lethal effects of predicted near-future (year 2100) conditions of OA on aspects of regeneration in sea stars, including aspects of growth, nutrient resource allocation, or behavior. As sea stars, including *Luidia clathrata*, exhibit high regenerative capacity (see page 70), arm loss provides an excellent model to evaluate the effects of ocean acidification on regenerative processes. In the present study, individuals of *L. clathrata* had two arms excised and were then maintained in seawater either bubbled with air alone (pH = 8.2, control treatment) or with a mixture of air/CO_2 (pH 7.8, experimental treatment) for 14 weeks. Individuals in both treatments were fed a sub-satiation diet formulated for sea stars. Arm regeneration (length), total body growth (wet weight), feeding-associated behaviors, and righting responses were measured regularly over the experiment. At the end of 14 weeks, a pyloric caecal index was calculated for all individuals. Soluble and insoluble protein, soluble carbohydrate, lipid, and ash were determined for the body wall and pyloric caecal tissues of intact and regenerating arms of individuals held under the two pH treatments. Reduced pH did not inhibit whole-body growth or arm regeneration rate, yet final length of regenerated arms

of individuals in the experimental treatment were shorter than those in the control treatment. There were no significant differences in the levels of any of the organic constituents or ash in the body wall of intact and regenerating arms of individuals held in either pH treatment. Pyloric caecal indices were low under both pH conditions and showed no changes from time zero individuals. There were no pH effects on organic constituents of the pyloric caeca. There were also no significant seawater pH effects on righting times measured immediately post-feeding or during weekly non-feeding related observation periods. This indicates that at least at a reduced seawater pH of 7.8 there was no evidence of physiological stress that was manifested in reduced behavioral righting time.

Key words: Ocean acidification, regeneration, sea star, *Luidia clathrata*

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INTRODUCTION

The current phenomenon known as ocean acidification is the outcome of increasing levels of anthropogenic carbon dioxide (CO_2) being released into the atmosphere (Feely et al. 2004; Kleypas et al. 2006; Guinotte et al. 2008; Zeebe and Wolf-Gladrow 2008). This is causing an increase in the amount of CO_2 absorbed by oceanic waters, which in turn has led to a decrease in surface water pH. It is estimated that on average seawater pH has already decreased by about 0.1 units since the beginning of the industrial revolution, and it is projected to continue to decrease a maximum of another 0.5 pH units by 2100 (Bindoff et al. 2007). A rapid change in seawater chemistry such as that projected to occur in the next several hundred years as a result of the absorption of higher concentrations of atmospheric CO_2 has a wide range of potential impacts on marine organisms, as well as the people and animals that depend on marine organisms and the marine system (Raven et al. 2005). There are a variety of avenues through which CO_2 enriched seawater may influence marine organisms. Two of the most elemental are how increases in CO_2 concentrations in seawater bathing marine organisms may impact their ability to produce shells or calcified skeletal elements, and the other is how decreases in seawater pH may increasingly impact an organism's net growth, metabolism, and reproduction (Pörtner et al. 2004; Widdicombe and Spicer 2008). Emerging research indicates that the manner in which these changes in seawater chemistry influence a particular marine organism may depend on its level of activity and metabolism as well as other aspects of its life history. The present study investigates the

prospective impacts that predicted decreases in seawater pH may have on the ability of *Luidia clathrata*, a common and ecologically important species of soft bottom sea star, to regenerate autotomized arms, allocate materials to somatic body components, and function behaviorally (exhibit post-feeding stimulus behaviors and self-right) during a period of active regeneration. Importantly, this study employs a future pH level estimated to occur by the end of the century or before (Caldiera and Wickett 2003).

To understand the significance of increasing atmospheric CO_2, and therefore the potential impacts of the predicted flux in seawater CO_2 levels on *Luidia clathrata* during regeneration, it is important to review the basic water chemistry involved in the ocean acidification process. The oceanic inorganic carbon species are impacted by the CO_2 absorbed by seawater from the atmosphere at the seawater-atmosphere interface as well as the different inorganic carbon species in seawater [aqueous carbon dioxide ($CO_{2(aq)}$), bicarbonate ions (HCO_3^-), and free carbonate ions (CO_3^{2-})]. The following equation demonstrates how the inorganic carbon species are balanced by and related to each other in the oceanic system.

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+$$

Changes in CO_2 absorbed by sea surface waters affect the total alkalinity (TA) and dissolved inorganic carbon (DIC) of the marine carbonate system (Zeebe and Wolf-Gladrow 2001). When CO_2 is absorbed by seawater it first binds with water molecules to form carbonic acid (H_2CO_3), a weak acid that quickly dissociates into its more stable ionic forms. Carbonic acid dissociation subsequently results in the formation of bicarbonate (HCO_3^-) and hydrogen (H^+) ions, increasing the H^+ concentration of seawater. Bicarbonate ions can then further dissociate to form carbonate (CO_3^{2-}) and

another free proton (H^+), further increasing acidity (Zeebe and Wolf-Gladrow 2001). There is a natural balance between all of these carbon forms to accommodate fluxes in CO_2, usually acting as a pH buffering system within marine systems, which when coupled with the total alkalinity, results in a stable global average pH (Feely et al. 2004). To maintain this chemical balance, the bulk of the inorganic carbon absorbed from the atmosphere by the ocean spontaneously forms HCO_3^- at the water and air interface. This is the most abundant carbon species and results in an accumulation of free hydrogen ions and consequently a decrease in pH. However, the carbonate ions can now bind the freed H^+ ions to form HCO_3^-, this additional elimination of free hydrogen ions also acts to push pH levels back towards their original levels. This process maintains the naturally occurring carbonate balance. Currently, changes in CO_2 concentration in the atmosphere are occurring more quickly than this buffering system can compensate, leading to decreases in CO_3^{2-} concentration below optimal levels as CO_2 partial pressure increases (Zeebe et al. 2008). Unfortunately, the HCO_3^- form does not readily lend itself to biogenic calcification, unlike CO_3^{2-}, the carbon form favored for calcification (Feely et al. 2004). These suboptimal concentrations of carbonate ions may over time lead to declines in biogenic calcification of skeletal elements and shells in marine invertebrates (Langdon et al. 2000; Leclercq et al. 2000; Feely et al. 2004; Orr et al. 2005)

Increases in seawater absorption of anthropogenic CO_2 are directly causing increases in dissolved inorganic carbon (DIC) of surface waters, resulting in a decrease in surface seawater pH. On the other hand the total alkalinity (TA), which is essentially the measure of balance the of excess cations and dissociated anions that act to neutralize acids to an equivalency point, has experienced smaller indirect effects resulting from

increased absorption of atmospheric CO_2, (Zeebe and Wolf-Gladrow 2001; Feely et al. 2009). In terms of calcification and OA, the changes of the highest concern in relation to TA is the dynamic balance of dissociations of weak acids in seawater, such as carbonic acid, and the subsequent changes in the saturation states of cations, like Ca^{2+} (Feely et al. 2009). Despite increased levels of carbon in the water column, the form of carbon favored for maintenance and development of skeletal elements remains unavailable to the majority of calcifying marine organisms.

On the other hand, TA can be impacted when the rate of dissolution and formation of calcified $CaCO_3$ structures changes. For instance, as biogenic calcification rates decrease, both DIC and TA increase. DIC is impacted because some of the inorganic carbon that formerly was integrated in crystalline structures utilized in calcified skeletal elements of marine organisms has dissolved in solution, and TA changes because newly freed cations and anions have been introduced into solution when crystalline solids are dissolved (Zeebe and Wolf-Gladrow 2001; Feely et al. 2004). It is important to understand how oceanic DIC and TA change as a result of CO_2 absorption, because both can be measured directly and used to calculate the CO_3^{2-} concentrations in seawater while Ca^{2+} concentrations can be calculated from salinity measurements (Zeebe and Wolf-Gladrow 2001; Feely et al. 2004). This is an essential step in understanding the availability of CO_3^{2-} and Ca^{2+} for production of crystalline structures that form many calcified skeletal elements and shells.

Calcite and aragonite are two common polymorphs of $CaCO_3$, differing only in the crystalline structural conformations that comprise $CaCO_3$ structural elements in shells and skeletal elements of sponges, molluscs, corals, echinoderms, barnacles and other

calcified marine invertebrates (Fabry et al. 2008). These polymorphs are formed by marine organisms through their utilization of Ca^{2+} and CO_3^{2-} dissolved in seawater. Most $CaCO_3$ in marine systems has been secreted biogenically via the formation of aragonitic or calcitic shells and skeletons (Zeebe and Wolf-Gladrow 2001). Of the two calcium carbonate polymorphs, many shelled planktonic species produce calcitic structures while some benthic species such as hard corals utilize aragonite to produce their skeletons. Other benthic species, exploit calcite for their shells or skeletons. For example, echinoderms are known to produce their skeletal components out of magnesium-rich calcite, a form of calcite that is particularly vulnerable to the impacts of ocean acidification (Gayathri et al. 2007). The levels of calcification involved in the formation and maintenance of skeletal elements depend on the saturation levels of aragonite and calcite in the seawater in which organisms live (Gattuso et al. 1998).

The degree to which organisms will be affected by these changes can be assessed stoichiometrically by calculating the saturation states of calcite and aragonite (Fabry et al. 2008). Currently, oceanic surface waters are super-saturated with respect to both aragonite and calcite, with saturation values ranging from two-four for aragonite (Ω_{Ar} = 2-4) and four-six for calcite (Ω_{Ca} = 4-6) with the cutoff value for being considered saturated being a value of one ($\Omega \geq 1$), creating layers of seawater with saturation values greater than termed a "saturation horizon" (Feely et al 2009). Areas with seawater having saturation levels below this horizon make biogenic calcification more challenging. As the partial pressure of CO_2 of atmospheric and seawater CO_2 increases, the saturation horizon of aragonite or calcite polymorphs becomes shallower (Bindoff et al. 2007).

Scientific evidence for increasing levels of CO_2 in the earth's atmosphere has been mounting since the beginning of the industrial revolution. Examples include measurements of trace isotope levels in corals as a proxy for past oceanic pH levels in shallow tropical and deep-sea corals (Smith et al. 1997; Pelejero et al. 2005), as well as within deep ice cores removed from the Antarctic ice sheet (Petit et al. 1999; Siegenthaler et al. 2005). The recent spike in CO_2 concentrations in the atmosphere are largely attributed to increased industrial activities, deforestation, and the burning of coal and fossil fuels (Kleypas et al. 2006). Some estimates suggest that approximately one third of the CO_2 emissions produced through the use of fossil fuels and by-products from the production of materials such as cement, have been absorbed by the world's oceans over the past 200 years (Raven et al. 2005). As humans have changed the terrestrial environment, so have we changed the composition of the atmosphere and chemistry of oceans (Raven et al. 2005; Bindoff et al. 2007).

Currently, the greatest concentration of anthropogenic CO_2 is found in the subtropical waters of the Atlantic Ocean (Sabine et al. 2004). This is the result of this region being characterized by high rates of evaporation, resulting in an increase in seawater salinity and density that causes water masses to sink along with the CO_2 absorbed at the sea surface (Sabine et al. 2004). The sinking water is then circulated along isopycnal layers distributing anthropogenic CO_2 beyond surface layers (Sabine et al. 2004). To truly illustrate just how much CO_2 gets absorbed by the ocean, consider that the ocean has already absorbed approximately one third of the total CO_2 released by human activities over the past 200 years (Sabine et al. 2004). Some of the resultant acidified seawater has seasonally begun upwelling onto the continental shelf over vast

coastal regions stretching from central Canada to northern Mexico. The upwelled seawater is undersaturated in terms of aragonite at relatively shallow depths (~40-120 m) along the coast of California (Feely et al. 2008).

One of the important topical areas of ocean acidification (OA) that has garnered great interest is biogenic calcification of marine organisms that comprise coral reef systems (Gattuso et al. 1998; Kleypas et al. 1999; Kleypas et al. 2006; Guinotte 2008). The biologically-derived $CaCO_3$ structure of coral reefs support a very high biodiversity of organisms, some of considerable economic value. Coral reefs and their dependent organisms currently are in imminent danger due to the dual stresses of rapid increases in the atmospheric CO_2 concentrations causing ocean acidification and as well as increasing global seawater temperatures that stress the symbiotic zooxanthellae that characterize hermatypic hard corals (Hoegh-Guldberg et al. 2007). It is predicted that as ocean acidification increases corals will suffer from lower accretion rates of calcium carbonate skeletal materials and consequently increasing their vulnerability to erosion and storm damage (Kleypas et al. 1999). In one experimental study, complete dissolution of carbonate skeletal structures occurred in scleractinian corals collected from five Mediterranean colonies of the reef building corals *Oculina patagonica* (an encrusting species) and *Madracis pharencis* (a bulbous species) that were maintained at a seawater pH of 7.3 to 7.6 (Doney et al. 2007; Fine & Tchernov 2007). This indicates the potential for a significant decline in the structure of coral reefs and raw materials for coral skeletal structures. Doney et al. (2007) point out that the combination of ocean acidification in addition to other anthropogenic sources of toxins and pollutants are likely to dramatically reduce biodiversity in coral reef systems.

Corals are not the only marine invertebrates with life histories whose larval or juvenile stages produce calcium carbonate skeletal structures. Studies have been conducted to evaluate the impacts of OA on a variety of adult stages of marine macroinvertebrates. Among these are molluscs (Michaelidis et al. 2005, Bibby et al. 2008), gastropods (Bibby et al. 2007), barnacles (McDonald et al. 2009), polychaetes (Batten and Bamber 1996; Widdicombe and Spicer 2007) and echinoderms (Wood et al. 2008; Gooding et al 2009; Wood et al. 2009; Dupont et al 2010). These studies have broadly focused on how calcifying marine organisms handle their routine activities, including burrowing activities, defense, reproduction, and foraging with the added stress of living in seawater under conditions of CO_2-induced decreased pH. These studies revealed a diverse assemblage of responses including, but not limited to, the depression of metabolic activities (Batten and Bamber 1996; Michaelidis et al. 2005; Bibby et al. 2007), compromises in immune responses (Bibby et al. 2008) and fundamental alterations in community structure (Widdicombe and Spicer 2007; Martin et al. 2008). One important finding to date is that the degree to which a marine organism is impacted by decreases in pH due to increased CO_2 concentrations varies by species (Fabry et al. 2008).

In addition to the studies above that have focused on the impacts of ocean acidification on adult stages of marine benthic macroinvertebrates, others have focused their work on more sensitive larval stages of benthic species and small planktonic calcified species (Fabry et al. 2008; Kurihara 2008; Kurihara and Ishimatsu 2008). Some ecologically dominant planktonic organisms such as coccolithophores may thrive in an environment with increased CO_2, while others such as shelled pteropods appear to be

unable to maintain their calcified elements (Fabry et al. 2008). One study has found the organismal calcification response decreased in the coccolithophore *Emiliania huxley* when exposed to CO_2-dependent seawater acidification in mesocosm and laboratory experiments (Riebesell et al.2000), while another study (Igelsias-Rodriguez et al. 2008) found an increase in calcification in laboratory experiments. This may reflect variability even within a species of marine plankton (Fabry et al. 2008). This makes the evaluation of ocean acidification response across phyla, orders, classes, genera, and especially species critical in a meaningful impact assessment process.

Other researchers have found neutral or negative impacts of ocean acidification on developmental stages of echinoderm (Kurihara and Shirayama 2004; Dupont et al. 2008; Byrne et al. 2009a; Byrne et al. 2009b), crustacean (Kurihara and Ishimatsu 2008), and molluscan (Kurihara et al. 2007) larvae. Kurihara and Shirayama (2004) found that fertilization and cleavage rates, developmental rate, and pluteus larval size decreased as CO_2 concentrations increased, suggesting delayed development that may negatively impact survival. In contrast, sperm of *Heliocidaris erythrogrammaI,* a sea urchin inhabiting a climate-change hotspot on the eastern coast of Australia, was more impacted by changes in seawater temperature than CO_2 induced changes in pH (Byrne et al. 2009a,b).

Few studies have focused on adult, benthic life stages of echinoderms. A study that bridges the gap between lecithotropic larval stage and juveniles of the sea star *Crossaster papposus* discovered that CO_2 induced acidification decreased larval development time (Dupont et al. 2010). The ensuing increase in growth rates of the larvae in reduced pH treatments may be associated with a reduction in time spent in the

pelagic planktonic life stage and consequently reduce larval exposure to this more vulnerable life history stage. This is potentially the first positive effect of OA observed in larval marine invertebrates to date (Dupont et al. 2010).

A study that examined some of the effects of OA and temperature on Asteroids focused on the growth of juveniles of a temperate species of sea star, the keystone predator *Pisaster ochraceous,* over a 70 day experimental period (Gooding et al 2009). Similar to the results in the previous study discussed, *P. ochraceous* juveniles experienced an increase in growth with elevated concentrations of CO_2 despite a decline in calcareous material. Gooding et al (2009) suggest that this result may be attributed to the lack of a continuous calcified test, shell, or endoskeleton which encompasses a large proportion of soft tissue limiting growth rate when the concentration of the raw materials for creating new calcified structures (CO_3^{2-}) are decreased. Echinoderms are an optimal species to study the effects of changing CO_3^{2-}concentrations on growth and calcification without calcification rates limiting body size because their skeletal ossicles are each produced sub-epidermally and composed of a single crystal of porous magnesium-rich calcite (Gayathri et al. 2007). With respect to *P. ochraceous,* OA may elicit a stimulating response similar to that observed with low level exposure to toxins or stressors, termed hormesis (Calabrese et al. 2002), opening up another aspect of the effects OA on marine invertebrates.

When considering the impacts of ocean acidification across marine invertebrate life history, it is important to consider the role of regeneration. A number of marine invertebrates display the capacity for regeneration (Maginnis 2006), but perhaps the group that is best known for its regenerative abilities are members of the asteroids,

crinoids, and ophiuriods (Emson and Wilikie 1980). There are several theories as to why these echinoderms have evolved the ability to regenerate body components and why regenerative capacity is so prevalent across all five taxonomic classes. One recurring theme that could account for this capacity is related to predation. Echinoderms are subject to a high incidence of sub-lethal predation by which predators nip off body components. Additionally, some echinoderms are capable of casting off (autotomizing) body parts or regurgitating internal organs when encountering a predator (Woodin 1984, Harris 1989; Oji 1996; Garcia-Arraras et al. 1999; Diaz-Guisado et al. 2006). Perhaps one of the most well known and thoroughly studied classes of echinoderms, notorious for its considerable regenerative capacity, is Asteroidea. Most sea stars are capable of regenerating either the tips of their arms, or an entire autotomized arm. Some sea stars even reproduce asexually, as a result of fission (splitting across the central disc), a subject that has been extensively researched and reviewed (Emson and Wilkie 1980; Lawrence 1992). An important field of research has developed that exploits regenerative processes to evaluate the energetic costs and allocation of materials and energy to somatic and reproductive body components in echinoderms undergoing regeneration (Lawrence 1986). When a sea star loses an entire arm, it essentially loses 20 percent of its energy storage capacity (the nutrient storage organ, or pyloric caeca, extending into each arm), and reproductive potential (the gonads extend into each arm) (Jangoux and Lawrence 1982; Pomory and Lares 2000; Lawrence 2010). Moreover, arm loss comes at the additional price of a potentially compromised ability to right, capture, and manipulate prey (Ramsay et al. 2001).

Pomory and Lares (2000) conducted a field study that focused on the costs of regenerating arms and the incidence of autotomy and regeneration in the common soft bottom sea star *Luidia clathrata* in Tampa Bay, Florida. They found that approximately 12 percent of the population were in the process of regenerating arms after suffering very significant (defined as regenerating four or five arms) bodily damage. Nonetheless, no difference was detected in the dry mass of any component of a whole arm of an intact individual when compared to the whole arm of a regenerating individual (Pomory and Lares 2000), indicating no differences occurred between nutrient storage and reproductive potential of intact arms when compared between regenerating and intact individuals. They proposed that the amount of energy required for an individual to regenerate two of its arms is offset by its ability to capture enough food to regenerate two arms, and that the loss of two arms was not sufficient to slow and significantly inhibit feeding. They also point out that at the time of their field study there may have simply been insufficient gonad and pyloric caeca to allow a representative evaluation of energy allocation. They detected a curvilinear trend in arm regeneration rate in *L. clathrata* characterized by the initial regeneration of approximately half of the arm being relatively rapid and then by a dramatic reduction in regeneration and growth. This pattern was attributed to a strategy of an individual first increasing its arm length and subsequently allocating energy to the regeneration of the pyloric caeca (Pomory and Lares 2000).

To date, the only study to examine sub-lethal effects of ocean acidification on regeneration in an adult echinoderm is that of Wood et al. (2008) who examined arm regeneration in the brittle star *Amphiura filiformis.* These investigators quantified changes in metabolism (via oxygen uptake), degree of arm regeneration (changes in

length of regenerated arms), and total calcium content of established and regenerated arms, as well as potential impacts on arm musculature and egg size. Four different experimental seawater pH treatments were employed (pH 8.0 - control, pH 7.7, pH 7.3, and pH 6.8) and brittle stars had either one or two arms removed prior to the initiation of the experiment. Individuals in the reduced pH treatments actually experienced an increase in regenerated arm length, calcium content, and metabolism indicating that reduced pH and CO_3^{2-} saturation stimulated growth for *A. filiformis*. The increase in metabolic activity indicates that brittle stars at reduced pH with arms removed may have had to work harder to regenerate lost arm segments. Moreover, it appears that this increase in growth resulted from the breakdown of arm muscle tissues as an energy source, resulting in muscle wastage. Thus, Wood et al. (2008) conclude that *A. filiformis* may be able to survive lowered seawater pH conditions but at the expense of their muscle tissue, potentially reducing their own survival and fitness as well as decreasing the nutritional value of their tissues for predators, including the commercial flatfish dab *Limanda limanda* (Bowmer and Keegan 1983).

As indicated above, to date there have been no studies that have focused on the sub-lethal effects of ocean acidification on aspects of sea star physiology, behavior, or ecology. Consequently this entire field of enquiry is essentially wide open and uncharted. Sea stars are well established to be ecologically important (Menge 1983) and in some cases even keystone predatory species (Paine 1974). Therefore, it is critical to determine the impacts of ocean acidification on the ability of sea stars to maintain and regenerate their somatic and reproductive body components as well as sustain the calcification of their magnesium-rich calcite skeletal elements (ossicles). As sea stars

commonly exhibit regeneration, experimental arm removal provides an excellent model to evaluate whole organ as well as biochemical and energetic consequences of ocean acidification during regenerative processes. Regeneration is an exceptional model for this type of study due to the rapidity of growth and resultant increase in sensitivity to abiotic changes in surrounding conditions.

The present study examines the impacts of near-future ocean acidification on rates of arm regeneration, regenerated arm length, total body growth during arm regeneration, nutrient storage indices of intact and regenerated arms, allocation of organic and inorganic constituents in body wall and pyloric caecal tissues of intact and regenerated arms, behaviors stimulated by feeding, and organismal righting responses (a measure of stress) over a 14 week(97 day) period in the sea star *Luidia clathrata*.

MATERIALS AND METHODS

Adult individuals of the sea star *Luidia clathrata* (mean arm radius (R) ± SD, R = 73.5 ± 4.4 mm, N = 36) were collected by hand from soft bottom sand habitat in June 2009. The collecting site was located south of the Courtney Campbell Causeway in Tampa Bay, Florida (27^{o}58’16”N, 82^{o}36’33”W). Sea stars are very common at this location approximately 100-150 meters from shore at shallow depths of 1 to 3 m. After collection, intact and visually healthy non-regenerating sea stars were immediately placed into 38-L coolers; all others were returned to the bay. Each cooler was equipped with a Penn-Plax Silent Air B10^{TM} battery-powered air pump that provided sufficient aeration for the duration of the 11 hour trip to the University of Alabama at Birmingham (UAB).

Upon arrival at UAB, salinity and seawater temperature were measured, and then half of the seawater was replaced with artificial sea water (Instant Ocean®, manufactured by Aquarium Systems, Inc; a Marineland Company, Mentor, Ohio USA) of the identical salinity and temperature. Following a two day adjustment period, sea stars were placed into one of two compartments of one of twelve 38- L aquaria that had been divided into two equally sized compartments (25.4 cm x 12.7 cm x 30.5 cm) using a central Plexiglas plate cemented firmly in place with Perfecto's 100 percent clear silicone rubber aquarium sealant. These divisions provided a complete separation of the two compartments of each aquarium. The lack of interchange of seawater between the two compartments of each 38-L aquarium ensured that each compartment represented an individual experimental unit so as to avoid pseudoreplication. Sea stars were held for five days without food prior to the initiation of the experiment to standardize their nutritional condition and to further acclimate them to the artificial sea water. Initially, 24 of the 36 sea stars collected were placed individually in one of the 24 aquarium compartments. Each aquarium compartment contained a 2-cm layer of clean fine grain sand (Nature's Ocean Substrate, Marine White Sand) and was fitted with a recirculating aquarium filter pump (Aqueon Power Filter 10). Due to viability concerns for nine of the original sea stars, these eight were removed from the experiment and the remaining twenty-four healthy adult sea stars were randomly divided into three groups: a time zero group that was immediately sacrificed for body component analysis (N = 17), a control group maintained at an ambient seawater pH of 8.2 (N = 8), and an experimental treatment group that was maintained at reduced seawater pH of 7.8 (N = 8).

For all sea stars in the control treatment (pH 8.2), air was delivered to each aquarium compartment using standard aquarium airline tubing (5 mm diameter) fitted with a with a one mL glass pipette so as to deliver a fine steam of bubbles. Air supply to each compartment was provided by a CORALIFE® Super Luft SL-65 high pressure aquarium air pump. For all sea stars in the experimental seawater pH treatment (pH 7.8), a mixture of air and CO_2 was delivered to each compartment through Tygon® tubing (6.35 mm inner diameter, 9.525 mm outer diameter) and bubbled by fitting each tube with a standard one mL glass pipette. The air/CO_2 mixture was attained via use of an Omega Gas Proportioner plumbed to a CORALIFE® Super Luft SL-65 high pressure aquarium air pump and to a 22.7 kg cylinder of pressurized USP grade CO_2 gas.

Experiments were conducted in a temperature controlled room with a temperature range of 25 to 26°C, and a 12 hr Light: 12 hr Dark photoperiod. Seawater quality was maintained over the course of the experiment by performing exchanges as necessary with freshly prepared artificial seawater to maintain ammonia, nitrite, and nitrate at acceptable levels (as determined by the standards established in conjunction with the protocols for water quality testing with API™, Aquarium Pharmaceuticals liquid test kits) with one large volume change (one third of the aquarium compartment volume) every 28-36 days. For the duration of the experiment, pH levels were monitored daily and adjusted so as to not vary more than ± 0.05 pH units. The pH of the sea water of both the control and experimental pH treatments were monitored daily with an ACCUMET BASIC Model AB15 pH meter and ACCUTU pH probe (Cat. # 13-620-183) calibrated with Fisherbrand pH 4, 7, and 10 buffers (NBS standards as per accepted standards at the time this study was performed). Measurements of the pH were made at least once a day and appropriate

adjustments were made to maintain pH levels within 0.05 pH units of designated pH levels, for the duration of the 14 week experimental period the pH only varied by ± 0.11 pH units of the designated pH for the control and experimental treatment groups. Seawater chemical parameters (see below) were monitored weekly. A chemical carbonate profile of the artificial seawater was conducted at the completion of the experiment to establish the baseline parameters of the seawater. Total alkalinity (as determined by titration) was 2767 μmol kg^{-1} (control pH) and 2241 μmol kg^{-1} (experimental pH), and saturation states of aragonite were Ω_{Ar} = 5.25 (control) and Ω_{Ar} = 1.83 (experimental) and calcite, where Ω_{Ca} = 8.04 (control) and Ω_{Ca} = 2.81 as calculated using the Microsoft Excel spreadsheet 'co2sys.xls' v 14 based on work by Lewis and Wallace (1998) (provided by Pelletier et al., available at www.ecy.wa.gov/programs/eap/models.html), and the National Institute of Standards and Technology (NIST) pH scale.

All artificial seawater salts (Instant Ocean®) were mixed using deionized water that was filtered from tap water using a Four Stage Barracuda RO/DI three part filter system (AQUAfx). Water quality parameters including ammonia, nitrate, and nitrite were monitored weekly using standard liquid test kits designed for saltwater aquaria (API™, Aquarium Pharmaceuticals). If concentration levels of ammonia or nitrite were above zero, a water change was performed (maximum value for either ammonia or nitrite for the duration of the experimental period was 0.25 ppm, where a level of 1 ppm was considered unhealthy for ammonia and nitrite). Water changes were also performed if nitrate levels exceeded 40 ppm (concentrations exceeding 160 ppm were considered unhealthy as per API™ standards). Sea stars in both the control and experimental

treatment were each fed a sub-satiation diet of 0.2 g formulated sea star feed (provided by Dr. Addison Lawrence, Texas A&M University) every other day over the entire course of the experiment. This ration of sea star feed was targeted because in preliminary observations this was determined to be just below satiation level, where all individuals consumed all food placed in each aquarium, yet consumed enough food to support growth. This avoided complications of feeding sea stars ad libitum and having some individuals consume more than others. The food ration selected provided adequate nutrition to support maintenance and regenerative growth.

At the outset of the experiment, all sea stars, including those for initial dissection, were weighed (wet weight) on a top loading balance following a 30 s blotting period on a paper towel. Arm lengths (distance from center of oral disc to tip of each arm) of all five arms of each individual were measured to the nearest millimeter using a 10 cm ruler. Arms were number coded by using the distinct pigmented "marker line", a distinguishing characteristic of *Luidia clathrata*, on the surface of the aboral body wall, beginning at the arm tip and bisecting the length of the each arm. On one of the arms, the marker line bisects not only the length of the arm, but also the central disc, terminating at the intersection of two arms. This arm was designated a reference arm and given the designation of R1. Moving in a clockwise direction, each subsequent arm was designated R2, R3, R4 and R5 (Figure 1), in this way metrics for each individual arm could be monitored for the duration of the experimental period and for biochemical analyses.

In order to establish the metrics of mass and dimension, as well as the biochemical condition of individuals at the beginning of the experiment, a time zero group of sea stars (N = 17) was dissected into their discrete component body parts (body

wall and pyloric caeca). There was insufficient gonadal material for biochemical analyses in any of the sea stars either pre- or post-experimental manipulation. However, prior to biochemical analysis the reference arm (R1) was removed at its base with a pair of scissors, its length measured, and then weighed. The aboral body wall of the excised arm was then cut along its length and the pyloric caeca removed using a fine forceps. The pyloric caeca was weighed wet along with the resultant body wall of the dissected arm. This procedure was repeated for each of the remaining arms (R2-R5). The body wall and pyloric caeca of a given individual were each placed into a plastic 100 ml weigh boat, sealed in a zip lock bag, labeled and immediately frozen at -20 °C for subsequent biochemical analysis (see techniques below). At the initiation of the experiment, sea stars in both the control and experimental pH treatments (N = 8 individuals in each treatment) had two arms removed at their base using scissors. Each removed arm was then processed as described above.

Each week, the radius length (R, the distance from center of oral disc to arm tip) of the two regenerating arms were measured by hand to the nearest mm using a 10 cm ruler. At the same time, R was also measured for the remaining three intact arms of each individual. Total wet body weight was determined for each individual on a weekly basis following a 30 s blotting on a paper towel. At the completion of the 97-day experimental period, all individuals were subject to one final measure of arm length. The final wet body weight was recorded, and then each individual was dissected so as to allow for determinations of organ indices, and the biochemical composition of the body component tissues. In addition, mean individual arm pyloric caecal indices were determined for intact and regenerated arms of every individual in the pH 8.2 and pH 7.8 treatment

groups by weighing each arm (wet wt) and the associated pyloric caeca (wet wt). Indices were calculated as the mass of the pyloric caeca of a given arm divided by the mass of the intact arm (body wall and pyloric caeca) X 100.

Biochemical analysis was performed by freeze drying each tissue in a lyophilizer, then determining the dry weight of the body wall and pyloric caecal tissues of each individual by weighing tissue samples on a microbalance (Mettler AM 100, Mettler-Toledo International Inc., Heights Town, New Jersey USA) before and after lyophylization. The body wall was ground using a Wiley Mill, while the pyloric caeca was ground using a mortar and pestle. Sodium hydroxide (NaOH)-soluble protein, Tricloroacetic acid (TCA)-soluble carbohydrate, and nonpolar lipids were analyzed using standard protocols described in Bradford (1976), DuBois et al. (1956) and Folch et al. (1957), respectively. Insoluble protein was determined by subtraction (Lawrence et al. 1984; McClintock and Pearse 1987a). Ash weight was determined by combusting tissues in a Muffle Furnace at 500°C for 4 hours (Paine 1971).

Measurements of post-feeding behaviors were recorded for each individual every other day over the 97-day experimental period. Behaviors were recorded for ten minutes immediately following the presentation of artificial sea star food to a given sea star. Behaviors recorded included whether an individual was: 1) 'active climbing,' where an individual attempted to climb the side of the aquarium, 2) remained 'buried/immobile,' 3) substrate active - moving around the on the substrate, and 4) feeding, to document that all food was consumed.

An individual was considered to be exhibiting 'active climbing' behavior if it climbed the sides of the aquarium before or after ingesting the food, and a climbing

attempt was recorded when an individual had three or more of its arms off of the substrate and against the sides of the aquarium. Individuals classified as buried/immobile were either partially or completely buried under the substrate, or lying on top of the substrate not moving for 30 s or more of the 10 min observation period. 'Substrate active' behavior was considered applicable if the sea star repeatedly flipped over and righted itself, or moved around the aquarium for 30 s or more of the 10 min observation period before or after ingesting the food. 'Feeding' behavior was recorded when an individual was in the act of manipulating a food pellet and directing it to its oral opening at any point during the 10 min observation period. Feeding behavior was recorded to ensure that all individuals were consuming the same quantity and quality of formulated food over the course of the experiment; no statistics were run on food consumption during the 10 min post-feeding observation period. Regardless of behaviors recorded during the 10 minute post feeding period, all food was consumed within 24 hours of when the food was placed in the aquaria.

An additional post-feeding measure of behavior was termed "post-feeding stimulation." This was predicated by the observation that sea stars would often flip themselves over repeatedly as they attempted to climb the sides of the aquarium. When a sea star flipped itself over, the subsequent length of time necessary to right was measured and recorded (Figure 2). Righting time, was measured as the time required for an over-turned individual to turn itself upright to the point where its central disc was perpendicular to the substrate (Figure 2). Righting times have been used previous studies as a measure of activity level (or physiological stress) in *Luida clathrata* (Diehl 1979; Stickle and Diehl 1986; Watts and Lawrence 1986).

In a separate measure, righting response times were measured for individuals that had not been stimulated by recent feeding in a standardized evaluation of physiological stress. Here, sea stars were turned by hand on to their aboral surface and the amount of time to right (Figure 2) was recorded. These measurements were conducted once a week on days when feeding was not conducted.

Differences in means of whole animal growth, arm length, levels of carbohydrate, lipid and protein, and calcification (ash content) of regenerating and non-regenerating arms, as well as righting times, were statistically compared between time zero, control pH (8.2) and experimental pH (7.8) treatments by using a series of Student's t-tests (for variables comparing just the two pH treatments, such as regenerated arm lengths) or using a one way ANOVA (for tests comparing the two pH treatments as well as that for the time zero individuals). All percent body weight of soluble and insoluble protein, soluble carbohydrate, and lipid of body components were arcsine transformed for analysis by ANOVA and post-hoc Tukey analysis, conducted using SigmaPlot10.2 (SYSTAT Software, Inc., Richmond, CA, USA). Post-transformed data that were still determined to not be normally distributed by a Shapiro-Wilk test were analyzed using a Kruskal-Wallis test. Post-hoc analyses were conducted using a Dunn's Method statistical analysis following the Kruskal-Wallis test. For clarity, means are presented in figures where the results of the Kruskal-Wallis analyses are presented.

The rate of regeneration was modeled using a two parameter exponential rise to maximum funtion: $Y = (a + zda)(1 - e^{-(b+zdb)x})$. To test for differences in the rate of regenerated arm lengths (length grown in mm over time (days) a coded dummy variable (z) was employed. For the control treatment (pH 8.2) group, $z = 0$, and for

experimental treatment (pH 7.8) $z = 1$. Subsequently, a nonlinear least squares regression (in SYSTAT11, SYSTAT Software, Inc., Richmond, CA, USA) was employed with extra "difference" parameters *da* and *db* to allow the parameter estimates to take on different values for the 8.2 and 7.8 pH treatments. The two treatment groups were considered significantly different at $\alpha = 0.05$ if the 95% confidence interval estimated by SYSTAT for the *da* or *db* estimates for *a* or *b* did not contain zero.

For post-feeding behavior observations, both the time spent buried/immobile and the number of climbing attempts (if an individual displayed climbing active behavior) were compared between the two pH treatments using a Student's t-test for each 10 min post-feeding observation period and then graphed to determine the development of any trends in the levels of post-feeding activity of the two pH treatments in relation to each other. We also performed a one by two contingency table analysis for each 10 min post-feeding observation period to obtain a Chi^2 statistic which was graphed for each observational period to determine developing trends in the relative presence or absence of the substrate active behavior and climbing active behavior over the course of the experiment. To analyze the post-feeding related righting times we used a rank based Mann-Whitney analysis to compare the ranked mean righting times (in seconds) for individuals in the two pH treatments. This non-parametric test was selected because of the presence of one severe outlier in the lower pH treatment group. A nonparametric Sign Test was also run to compare the frequency of longer mean righting times over the entire course of the experiment (a total of 39 comparisons) between the two pH treatments.

For the non-feeding related comparison of righting times, a one way Repeated Measures ANOVA was used to analyze the weekly righting times for individuals in both pH treatments.

RESULTS

The mean final regenerated lengths of arms (total new growth in arm length after arm removal at time zero) for individuals held at pH 8.2 and 7.8 was significantly different (Student's t test, $t = 1.70$, $P = 0.02$) (Figure 3). Individuals held under ambient (pH 8.2) conditions had longer regenerated arms at the end of the 97 day experimental period than those in the reduced pH treatment. However, the mean rate of arm regeneration (represented as the arm length regenerated per unit time; Figure 4) of sea stars in the pH 8.2 and pH 7.8 treatment groups, was not significantly different from one another over the course of the experiment (a Nonlinear Regression Model (SYSTAT analysis) indicated that the 95% confidence intervals for parameters in both pH treatment groups passed through zero, thus the two curves were not significantly different from one another).

Mean whole animal wet weights (mass) at beginning of the experimental period were not significantly different (Student's t test, $t = -0.993$, $P = 0.338$) and following the 97 day period of the experiment had significantly increased (one way ANOVA, $F = 12.650$, $P < 0.001$) in both pH treatments when compared with the time zero group. However, despite there being a small trend of decreased total body mass in the reduced

pH treatment at the termination of the experiment, this was not significant (Student's t test, t = 1.771, P = 0.30) (Figure 5). The rates of growth based on entire individuals (change in total wet weight per unit time) in the two pH treatments (Figure 6) indicated no significant difference (one way Repeated Measures ANOVA, F = 0.128, P = 0.726) between the two pH treatment groups.

There was no significant difference in the mean pyloric caecal indices (one way ANOVA; F = 1.934, P =0.107) of individual arms of sea stars at time zero and after 97 days in the two pH treatments (Figure 7). This was the case regardless of whether arms were regenerating or intact (Figure 7). However, in both pH treatments, it was observed during dissections that regenerating arms had no pyloric caeca in the newly regenerating portion of the arm. Thus, the pyloric caecal mass recovered from regenerating arms was localized almost exclusively in the section of the arm proximal to the point of initial arm removal at time zero.

The levels of protein, carbohydrate, and lipid in the body wall and pyloric caecal tissues for individuals at time zero and in the two pH treatments were determined as a percentage of total organic tissue. The levels of soluble protein in the body wall did not differ significantly (Kruskal-Wallis test, H = 4.631, p = 0.327) between any of the treatment groups (time zero, pH 8.2, pH 7.8) (Figure 8). This pattern was not affected in either pH treatment by the state of the arm (regenerative versus intact). Similarly, there were no significant differences (Kruskal-Wallis test, H = 7.230, P = 0.124) in levels of insoluble protein in the body wall tissues of individuals from any of the treatments (Figure 9), the soluble carbohydrate in the body wall tissues (Kruskal-Wallis test, H = 7.521, P =0.111; Figure 10), nor in levels of lipids (Kruskal-Wallis test, H = 7.490,

P = 0.112; Figure 11), or in levels of ash (inorganic material) (Kruskal-Wallis test, H = 9.440, P = 0.051, Figure 12). All figures associated with the body wall represent means for ease of interpretation.

The mean levels of soluble and insoluble protein in the pyloric caeca at time zero were significantly lower (soluble protein one-way ANOVA, F = 22.840, P <0.001) than levels in either the control or experimental pH treatment regardless of whether the arms were intact or regenerating (Figures 13). Insoluble protein levels of the time zero individuals were significantly different from the intact and regenerating arms of the control treatment group (Kruskal-Wallis test, H = 7.230, P = 0.124, Figure 14). A post-hoc Dunn's Method analysis revealed no significant difference in insoluble protein content of the intact or regenerating arms of either pH treatment groups (Figure 14). The time zero sea stars had significantly lower (one way ANOVA, F = 7.283, P <0.001, Figure 15) levels of soluble carbohydrates in the pyloric caeca, than that observed in the pyloric caeca of the intact arms of individuals maintained in the pH 8.2 seawater treatment (Tukey test, q = 6.812, P < 0.001) and the intact and regenerating arms of individuals maintained in pH 7.8 (Tukey test, q = 5.145, P = 0.006 for intact arms and q = 4.218, P = 0.036 for regenerating arms). There was no significant difference (Tukey test, q = 3.117, P = 0.198) in the soluble carbohydrate levels of the pyloric caeca between the time zero individuals and the pyloric caeca from regenerating arms of the individuals maintained in the pH 8.2 seawater treatment. There was no significant effect (Tukey test, q ranged from 0.795 to 3.169 and P ranged from 0.184 to 0.980).

The lipid levels of the pyloric caeca were significantly higher (Kruskal-Wallis,

H = 34.823, P < 0.001) in individuals at time zero than that found in the pyloric caeca of intact and regenerating arms individuals held in either the control (pH 8.2) or experimental (pH 7.8) treatment (Figure 16). Levels of lipid in the pyloric caeca from intact and regenerating arms of individuals in both pH treatments were similar (Dunn's Method, Q ranged from 1.382 to 4.999, P = 0.05).

The mean ash levels in the pyloric caeca were significantly different (one way ANOVA, F = 3.483, P = 0.015) with individuals at time zero having significantly lower levels (Tukey test, q = 4.228, P = 0.035) than those observed in either the intact or regenerating arms of individuals maintained in pH 8.2 or pH 7.8 seawater (Figure 17).

Discrete behavioral observations of sea stars over a ten minute post-feeding increment during the course of the 97 day experiment included the mean number of aquarium active climbing attempts by active individuals, mean time spent immobile/buried in the sand, the number of individuals that made aquarium climbing attempts, and the frequency of substrate active versus non-substrate active individuals. The pH of the seawater had no significant impact on the any of these behaviors (Figure 18). Analysis revealed no pH treatment dependent patterns or trends.

Overall, mean righting times for individuals that were stimulated to overturn themselves during the ten minute post feeding observation period in pH 8.2 and pH 7.8 seawater treatments are presented in Figure 19. Individuals held in the pH 7.8 treatment did not have significantly different (Mann-Whitney test, U = 47.000, P = 0.115) righting times when compared to the individuals held in the pH 8.2 control treatment and considered collectively over the course of the 97 day experimental period. When examined over time (measurements were made every other day), the mean righting times

for individuals in the 7.8 pH treatment group were higher than those at pH 8.2 on a significantly greater number of the 39 observation days (37 of 39 observation days; Sign test, C = 12, P = 0.034, Figure 20).

Weekly measurements of righting times recorded for individuals that were conducted by hand (each individual turned onto their aboral surface once per observational period) indicated that sea stars held in the pH 7.8 seawater treatment did not have significantly increased mean righting times (one way Repeated Measures ANOVA, F = 0.010, P = 0.922) when compared to those held in the pH 8.2 seawater treatment (Figure 21). Over the 14 week (97 day) experimental period (where non-feeding related stimulation measures were made weekly), the mean righting times for the pH 7.8 experimental group were slower to self right than or the same as those for the pH 8.2 control group (Figure 22) 71% of the 14 times that RAC measurements were taken over the course of the experiment (Figure 22).

DISCUSSION

The present study provides the first ever examination of the physiological and behavioral impacts of ocean acidification on a sea star undergoing regenerative processes. The importance of understanding the prospective impacts of ocean acidification in marine organisms such as sea stars cannot be understated given the unprecedented rapidity of the chemical changes occurring in the world's oceans. This ongoing reduction in the pH of the world's oceans is the direct result of elevated levels of atmospheric CO_2 brought

about by increased amounts of anthropogenically produced green house gases that are subsequently absorbed by seawater (Caldeira and Wickett 2003). This absorption results in a change in the balance of the carbon species that in turn cause an increase in the free hydrogen ion concentrations (and thus a decline in seawater pH) while also altering the saturation states of calcite and aragonite, two polymorphs of calcium carbonate. In some coastal regions these chemical changes are already seasonally creating potentially corrosive sea water environment (Feely et al. 2008) for many marine organisms with external calcium carbonate ($CaCO_3$) structures (Feely et al. 2008).

The current changes in the carbon chemistry of the world's oceans set the stage for the present study that examined arm regeneration in the ecologically important soft-bottom sea star *Luidia clathrata* under ocean acidification. This model system was selected because this sea star is known to commonly autotomize and then regenerate its arms under conditions of sub-lethal predation (Lawrence 2010). In order to encompass a broad suite of prospective sub-lethal impacts of ocean acidification on *L. clathrata* undergoing regeneration, the effects of seawater acidification on rates of arm regeneration, the ultimate length of regenerated arms, whole body growth, and both the organic and inorganic composition of the regenerated and intact arms of regenerating individuals were quantified. Moreover, few studies to date have measured aspects of behavior that might be impacted by near-future ocean acidification conditions.

Luidia clathrata had two intact arms removed at the initiation of the 14-week (97 day) experimental period and were subsequently maintained on a formulated sea star diet to support rapid regeneration of their arms. By the end of the experiment, arm length of the regenerating arms for individuals held in the control and experimental pH treatments

was approximately 81% of the length of the intact arms of regenerating individuals. Despite the rapid regeneration of both excised arms, which represents a physiological process involving considerable allocation of materials and energy, there was no significant difference between the rate of arm regeneration between individuals held in the ambient pH (8.2) treatment and the experimental pH (7.8) treatment. At the termination of the experiment, individuals maintained in the control pH treatment had significantly longer regenerated arms than did individuals maintained in the experimental pH treatment. Regenerated arms of individuals maintained in reduced pH seawater were approximately 90% of the length of regenerating arms of individuals held in ambient pH seawater. This relatively small but significant difference in regenerated arm length has functional consequences for individuals in terms of the efficiency of feeding or locomotion seems unlikely, but needs to be tested. If this pH-effect on arm length in regenerating arms were to continue over complete arm regeneration it may have functional consequences.

The rates of arm regeneration in *Luidia clathrata* in the present experiment appeared to exceed those observed previously in this species. Complete regeneration of two arms amputated at the central disc was estimated to require a 380 day period in a field study of regeneration of *L. clathrata* in Tampa Bay, Florida (Pomory and Lares 2000). These investigators considered full arm regeneration to be complete when the newly regenerated arm length attained that of the intact arms, and there was mature development of the pyloric caecal and gonadal tissues in the regenerated arms. In the present study, the regenerating arms *L. clathrata* maintained in either pH seawater treatment were within 1-2 cm of attaining the full length of intact arms after the 14 week

(97 day) study period. This suggests that at the measured rate of growth, full arm length would have been attained in about one more month, followed by development of pyloric caecal and gonadal tissues. The rapid arm regeneration in the present experiment could be the result of a dependable, nutritionally superior, formulated sea star diet rather than the natural field diets of individuals in the study by Pomory and Lares (2000). In addition to the use of a controlled formulated diet that allowed for direct comparisons between pH treatments, the present study employed a regular feeding regime that provided each individual with a sub-satiation level diet, where all of the formulated food placed in each aquarium was consumed by each individual within 24 hours. As such, one is confident that any changes observed between budgets of tissue regeneration are attributable to pH treatment rather than the quantity or quality of food consumed (Lawrence et al. 1986; Lawrence 1991; 2010).

New tissue production requires anabolism of the raw materials composing body tissues, typically utilizing energy secured from the catabolism of lipids and carbohydrates (Lawrence 2010). Previous observations by Lawrence (2010) have noted that total amounts of gonadal and pyloric caecal tissues increase in *L. clathrata* when they are maintained on formulated diets such as the one that was used in the present study (formulated by Addison Lawrence, Texas A&M University). Another aspect of the pattern of arm regeneration observed in the present study that could be related to the consumption of a high quality formulated diet is that arm regrowth in both pH treatments displayed initial almost linear growth with little evidence of asymptotic slowing as the regenerated arms approached the lengths of intact arms. This is consistent with the curvilinear arm growth pattern typical of arm regeneration growth curves previously

observed for *L. clathrata* on diets that are not formulated to the specific nutritional requirements of sea stars (Lawrence and Ellwood, 1991; Lawrence 1992; Pomory and Lares 2000). In the present study, it is possible that as arm regeneration continued to full maturity, rates may have proportionally declined to yield a more traditional asymptotic growth pattern.

The only previous study to date to examine the effects of ocean acidification on arm regenerative capacity in an echinoderm was conducted with the common temperate soft bottom brittle star *Amphiura filaformis* (Wood et al. 2008). When maintained under a reduced seawater pH (7.7, 7.3, or 6.8 pH) arm regeneration rates were accelerated in individuals that had one or two arms excised at the beginning of a 40 day regeneration period and compared with regeneration rates of individuals maintained in an ambient pH of 8.0. The mechanism for this increase in growth and metabolism is not clear.

The results of the Wood et al. (2008) study contrast with the present study where sea stars held in reduced pH seawater had shorter arms by the end of the 97 day experimental period. Similar to other studies of the effects of CO_2-driven ocean acidification within the same genus or class (Riebesell et al. 2000; Iglesias-Rodriguez et al. 2008), the present study reinforces the assertion that representative species of different classes of echinoderms may display different patterns of arm regeneration under ocean acidification. However, additional studies involving more echinoderm species are needed before making conclusions about patterns of response in higher taxonomic groups, and experiments must be devised that employ standardized nutritional conditions. For example, in the present study individual sea stars in both pH treatment groups were fed equal quantities of a high quality formulated food. In contrast, Wood et al. (2008) do

not provide any information about the type or amount of food provided to brittle stars over their 40 day regeneration study period. Consequently, it is difficult to know if differences they detected in arm regeneration rates were due in part to differences in diet or pH.

Despite the rapid growth rates of regenerating arms of individuals maintained in both pH treatments, only relatively small pyloric caeca, a nutrient storage organ (Lawrence 1984), were present in the regenerating arms of individuals maintained in either pH treatment by the end of the 14 week period of the experiment. Indeed, pyloric caecal indices had not risen in either treatment beyond the small indices observed in sea stars at the beginning of the experiment. A statistical comparison of the pyloric caecal indices of regenerating and intact arms with those of individuals held under ambient and reduced seawater pH revealed no significant differences in these respective indices. The relatively large variation in the sizes of the pyloric caeca, even within a given treatment, may have masked pH effects but are consistent with previous observations that the size of the pyloric caeca in *L. clathrata* may vary within and among sea stars despite good nutritional condition (Lawrence 1984). Thus, there was no detected effect in the present study of reduced pH inhibiting or stimulating the allocation of materials to the nutrient storage organ. This could also be due in part to the fact that the initial rapid regeneration of the arms of individuals in both treatments appeared to be devoted to the regeneration of the body wall, rather than the nutrient storage organ. This pattern is consistent with the conclusion that in an adaptive context it is necessary for arm length to increase rapidly concurrently with differentiation to attain functional recovery before development of new pyloric caecal tissues (Lawrence 2010). Had sea stars completed arm

regeneration (approximately an additional month of growth), it is likely it may have been possible to better characterize how ocean acidification influences the allocation of materials to the pyloric caeca in *L. clathrata*. There were insufficient gonads present in the arms of individuals at the initiation or the completion of the experiment for analysis. This lack of gonad development is likely the result of the experiment being conducted during the time of year when individuals are not in their reproductive phase (Lawrence 1973).

While regenerating *Luidia clathrata* maintained in both control (pH 8.2) and experimental (pH 7.8) treatments increased 37% in total body wet mass over the experimental period, there was no significant effect of pH on the whole body growth. Only two other studies have measured growth of sea stars under near-future conditions of ocean acidification, and both detected a stimulatory effect of reduced pH on whole body growth. Gooding et al. (2009) measured the effects of increased temperature and sea water acidity on the sea star *Pisaster ochraceous*. They observed a 67 % increase in total wet body mass for five individuals maintained either at pCO_2 levels of 380 ppm (consistent with present ambient seawater of pH of 8.25) and five individuals maintained in seawater with a pCO_2 of 780 ppm (consistent with pH levels predicted for 2100 of approximately 7.85), over a 10 week period. In contrast, *L. clathrata* in the present study were subject to the added stress of arm regeneration in addition to CO_2-driven seawater acidification. Provided there is a stimulatory effect of reduced pH on sea star growth, this change in pH may have inhibited differential growth under conditions of ocean acidification. It is also possible that compensatory or stimulatory growth under reduced seawater pH may come at some expense to the individual. For example, Wood et al.

(2008) found that the brittle star *Amphiura filiformis* displayed compensatory growth and calcification in regenerating arms under reduced seawater pH, but after histological examination there appeared to be a degeneration of the muscle tissue in regenerated arms of brittle stars held in the reduced pH treatments (7.7, 7.3, and 6.8 pH).

To determine the degree of sensitivity of organic constituent (soluble and insoluble protein, soluble carbohydrate, and lipid) and skeletal (ash) levels of somatic body components of *Luidia clathrata* to the impacts of ocean acidification, an examination of the proximate composition of the body wall and pyloric caeca was conducted on the regenerating and intact arms of regenerating individuals. At the end of the three month experiment, the biochemical constituents of the body wall tissue of the intact and regenerating arms of individuals in both the pH 8.2 and pH 7.8 treatments were not significantly different from one another nor the constituents of the body wall of arms of individuals analyzed at the initiation of the experiment. This indicates that reduced seawater pH had no effect on the organic or inorganic composition of the body wall of regenerating or intact arms. It appears that despite the combined stress of regeneration and exposure to CO_2-driven seawater acidification, *L. clathrata* in this study were able to consume enough food to support functional regeneration of the lost arms. Lawrence et al. (1986) observed that lack of adequate nutrition resulted in preferential allocation of energy to the development of the pyloric caeca of intact arms in *L. clathrata*, with subsequent energy secondarily allocated to arm growth. In contrast, preferential allocation of energy to regeneration of arm length was favored over an allocation of materials and energy to the pyloric caeca when sufficient quantities of food were consumed (Lawrence et al. 1986).

Most relevant to ocean acidification is the lack of any significant changes in the inorganic content (ash levels) of the body wall under conditions of reduced seawater pH, which indicates that there were no detectable effects of CO_2-acidified seawater on the ability of *Luidia clathrata* to produce skeletal ossicles. Nor is it likely that the magnesium-calcite ossicles of individuals at pH 7.8 suffered dissolution, although no microscopic studies were made in the present study for confirmation. The ossicles of sea stars are generally imbedded in the tissue or covered by a thin epithelial layer, and as such have no direct contact with seawater (Dubois and Chen 1989). This contrasts with the spines of some sea urchins that are exposed to sea water. The tissues surrounding the ossicles in *L. clathrata* are likely to exert buffering against the effects of exposure to seawater at reduced pH. However, this absence of a negative impact of reduced seawater pH (7.8) on skeletal ossicles in *L. clathrata* does not appear to be universal among sea stars. For example, Gooding et al. (2009) observed a significant decrease in calcified mass accompanied by a significant increase in growth in the temperate sea star *Pisaster ochraceous* reared for 70 days at a reduced seawater pH consistent with pH 7.85. Gooding et al. (2009) suggests that unlike many of the previously studied organisms exposed to near-future levels of ocean acidification, it is possible that since body growth was not limited by external skeletal structures, growth was stimulated but calcification rates did not increase at the same rate as soft tissue growth. This possibility may have arisen as a result of hormesis, where the stress of decreased pH and subsequent carbonate ion solubility increase triggered a positive response as a relatively low level stressor (Gooding et al. 2009).

In contrast to the static nutrient and skeletal composition of the body wall tissues of regenerating and intact arms of *Luidia clathrata* there were some significant changes in the relative percentages of organic components of the organic and inorganic constituents comprising the pyloric caeca in the arms of individuals maintained in the two pH treatment groups when compared to that of the pyloric caeca of individuals at the initiation of the experiment. Levels of soluble protein and soluble carbohydrate in the pyloric caeca removed from regenerating or intact arms of regenerating individuals held in either pH treatment were modestly higher than that removed from arms of individuals at the initiation of the experiment. Levels of inorganic material (ash) also increased in the pyloric caeca of arms of all individuals over the course of the experiment. In contrast, lipid levels declined from approximately 37% in the pyloric caeca of individuals at the initiation of the experiment to 20 to 25 % in the pyloric caeca of the regenerating and intact arms of regenerating individuals in both pH treatments by the end of the three month experiment. Thus, despite the slight increase in pyloric caecal soluble carbohydrate and protein, it would appear that the nutrient storage capacity (e.g., higher lipid levels) of the pyloric caeca had been depleted and not replaced over the course of the experiment in both pH treatments. The pattern observed here is the likely outcome of the individuals in the present study being held on a sub-satiation diet and some of the resources from the pyloric caeca being primarily reserved while some of the nutrients formerly comprising the pyloric caeca may have been allocated to maintaining rapid regrowth of the body wall of the regenerating arms. It is also important to note that benthic species, including *L. clathrata*, often utilize stored lipids for gamete production (Lawrence 1976). As indicated above, the current study was performed during the

summer when there is essentially no gamete production in *L. clathrata* (Lawrence 1973), making lipid storage for gamete production a lower priority than more immediate energy requirements related to arm regeneration.

Few studies of the impacts of ocean acidification on marine invertebrates have taken into consideration prospective consequences on behavior. Behavior represents the ultimate integration of physiological and sensory processes and is an important measure of whole animal response to CO_2-driven seawater acidification. In the present study the examination of prospective behavioral effects of exposure to reduced seawater pH was divided into two discrete observation periods. One observation period consisted of the 10 minute post-feeding observation period. The post-feeding time period was selected because *Luidia clathrata* displayed a discrete set of measurable behaviors immediately after food was introduced into each aquarium. The behaviors quantified included the average number of attempts to climb the aquarium wall for individuals exhibiting active climbing behavior, the average time spent inactive (buried), the total number of individuals that attempted to climb the aquarium wall, and the frequency of active (moving about on the substrate) versus inactive (buried) individuals in each pH treatment group. Additionally, a righting activity (used here to measure organismal activity level) was recorded for individuals that turned themselves over during this 10 minute post-feeding period.

The second observation period was divorced from any feeding stimulation and also consisted of determining righting responses of individuals under the two pH treatments as a measure of organismal activity indicative of stress (Watts and Lawrence 1990). Here, sea stars were turned over by hand to provide a measure of stress in the

absence of feeding stimuli. Behaviors during both of these two observation periods were followed over the entire course of the 14 week experiment; all post-feeding behavior observations were made every other day and manipulated righting behavior observations were made weekly.

When discrete behaviors displayed by post-fed individuals maintained under the two pH treatments were examined no pH effects were detected for any of the behaviors related to climbing the aquarium walls (mean attempts or total number of individuals making climbing attempts), active versus inactive patterns (presence of active behavior or time buried) or righting times. Nonetheless, the frequency of righting activity of individuals measured during the 10 minute post-feeding stimulation period were consistently lower (as indicated by longer time to right) for individuals in the pH 7.8 seawater treatment than those held in pH 8.2 control seawater treatment. This reduced organismal activity could compromise foraging efficiency. Post-feeding righting behaviors of individual sea stars were very common in both pH treatments over the course of the experiment, in many cases occurring multiple times during the 10 min period.

Similarly, the righting times of individuals in the two pH treatments measured during weekly observation periods that were divorced from feeding stimulation were not significantly different from one another. However, it should be noted there was an extremely low power (SigmaPlot 10.2, power = 0.050, P = 0.05, with desired power = 0.800) of the one way Repeated Measures ANOVA conducted for these data. This low power indicates it is unlikely to detect a difference when one is exists, and that negative results should be interpreted cautiously (SigmaPlot 10.2, Zar 1999). A relatively low

number of observations with relatively high variability contributed to this low power. Given that despite a low number of observations righting times were consistently the same as or lower for individuals exposed to reduced pH 7.8 seawater in 71% of the 14 weekly measurements, a larger sample size of individuals may have indicated reduced righting time with exposure to reduced seawater pH.

Righting is a measure of the level of organismal activity, and has long been considered an appropriate means to evaluate organismal stress in sea stars and other echinoderms (Diehl et al. 1979; Stickle and Diehl 1986; Watts and Lawrence 1990). As such, exposure to a reduced ocean acidification level of 7.8, a level expected to occur by the end of the century or earlier (Bindoff et al. 2007), may impose some impact on behavior in *Luidia clathrata*. Whether this trend towards an increase in righting response time is symptomatic of longer-term impediments to organismal functions such as locomotion and foraging efficiency is unknown. While no other studies have examined righting activity of sea stars under conditions of ocean acidification, a similar measure of whole organism activity was measured in the temperate brittle star *Ophiura ophiura* by Wood et al. (2010). In their study, the righting times of brittle stars were found to be significantly depressed in individuals held for a 40 day period in CO_2 acidified seawater at a pH of 7.7 or 7.3 when compared to those in ambient pH 8.0 seawater. However, caution must be employed in interpreting these findings as no information about the feeding regime of these brittle stars under experimental conditions was provided.

In summary, *Luidia clathrata* exposed to conditions of ocean acidification representative of levels predicted for the end of the century are unimpaired in their ability to regenerate somatic components of their arms (body wall and pyloric caeca) during the

regenerative process. Nonetheless, despite similar rates of arm regeneration, individuals held in reduced pH seawater by the termination of the 14 week experimental period developed significantly shorter arms than those held in ambient pH seawater. While chronic exposure to reduced seawater pH did not significantly inhibit righting behavior time, there was a trend over the course of the experiment for individuals to require greater times to right under reduced seawater pH. This indicates that a further reduction in seawater pH may lead to an inhibited ability to right, but that individuals chronically exposed to a seawater pH of 7.8 are able to function normally at pH levels predicted to occur by the end of the century.

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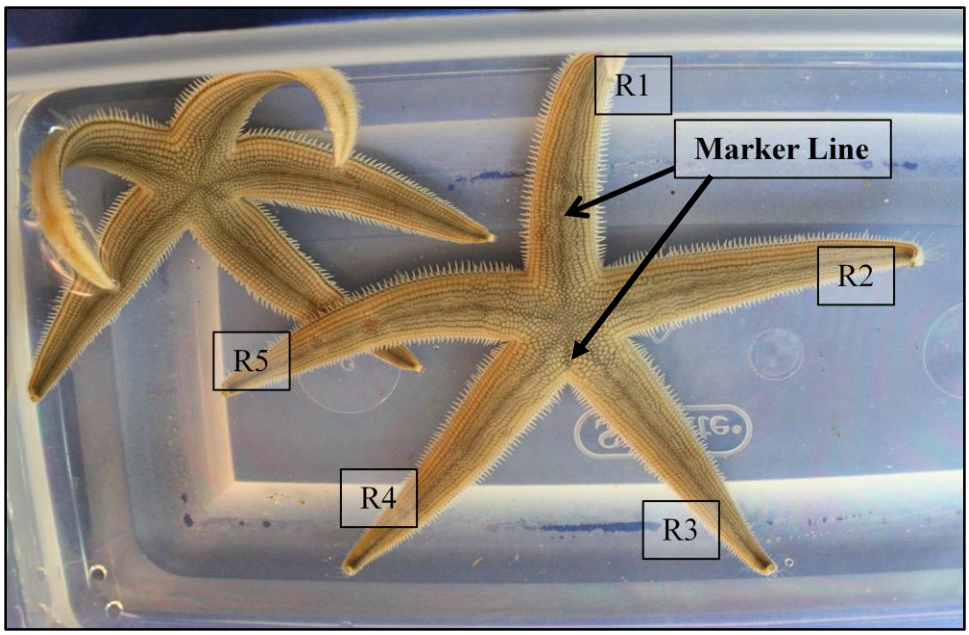

Figure 1. Photograph of a representative individual of *Luidia clathrata* depicting the arm coding system employed. The arrow designates the marker line on the lead arm used for orienting the arm code pattern. The individual pictured with its arms demarcated has an R of approximately 60 mm.

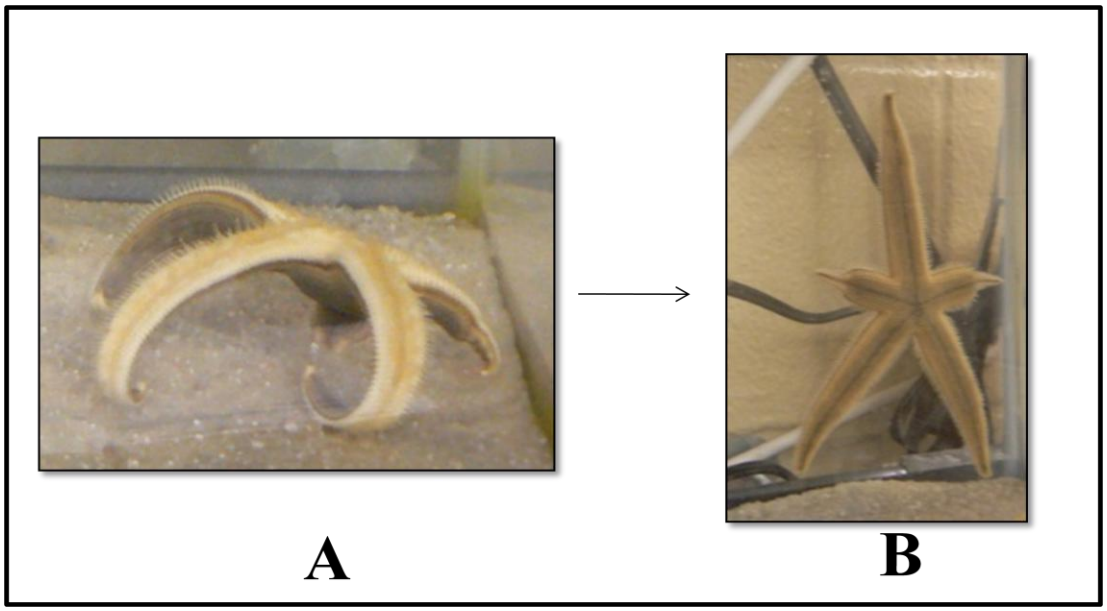

Figure 2. Photographs depicting the behavioral righting response of *Luidia clathrata.* The time required to right was measured from the moment an individual was positioned on its aboral side with its disc parallel to the substrate (see A above) to the moment when the sea star's central disc was perpendicular to the substrate (see B above). The sea stars in the photographs above have an R of approximately 60 mm.

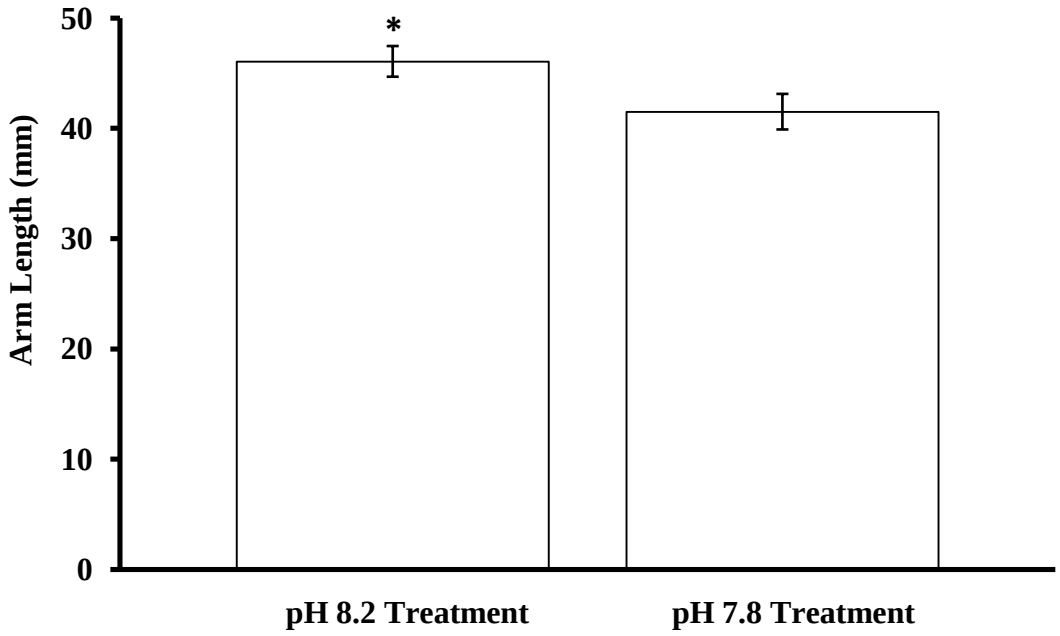

Figure 3. Mean ± 1 SE final regenerated length (mm) (distance from base of arm to regenerated arm tip) of regenerated arms of individuals of *Luidia clathrata* maintained in pH 8.2 and pH 7.8 seawater over a 97 day period. Two arms were removed from each individual sea star on day zero. The asterisk indicates statistical significance at the $P < 0.05$ level. $N = 8$ for each bar.

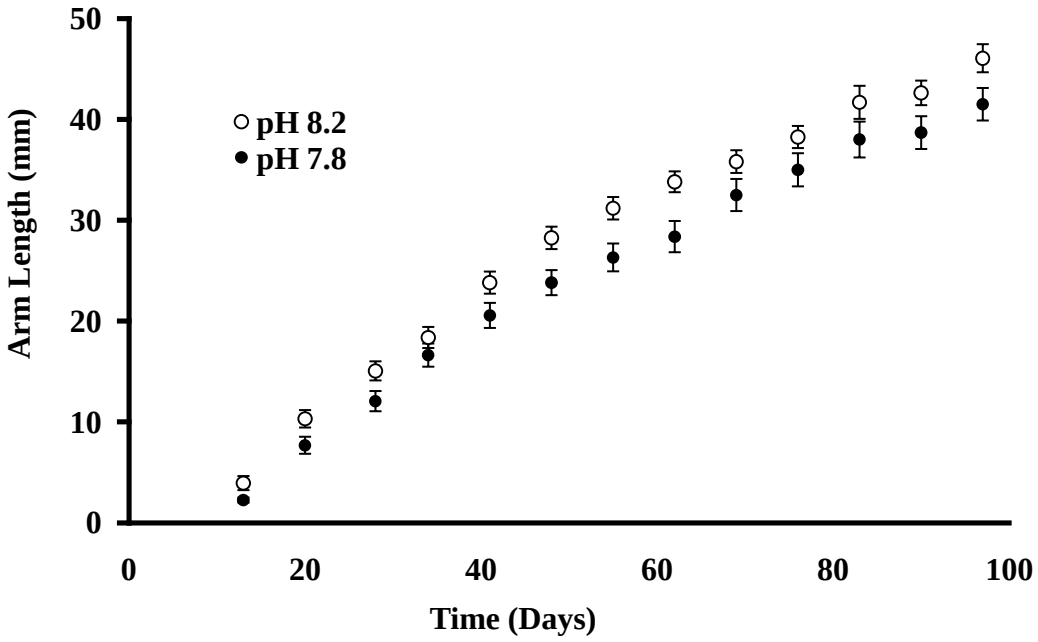

Figure 4. Mean ± 1 SE regenerated arm length (mm) of *Luidia clathrata* maintained in pH 8.2 and pH 7.8 seawater over a period of 97 days. Arm length measurements were made weekly beginning the second week of the experiment. N = 8 for each open or closed circle.

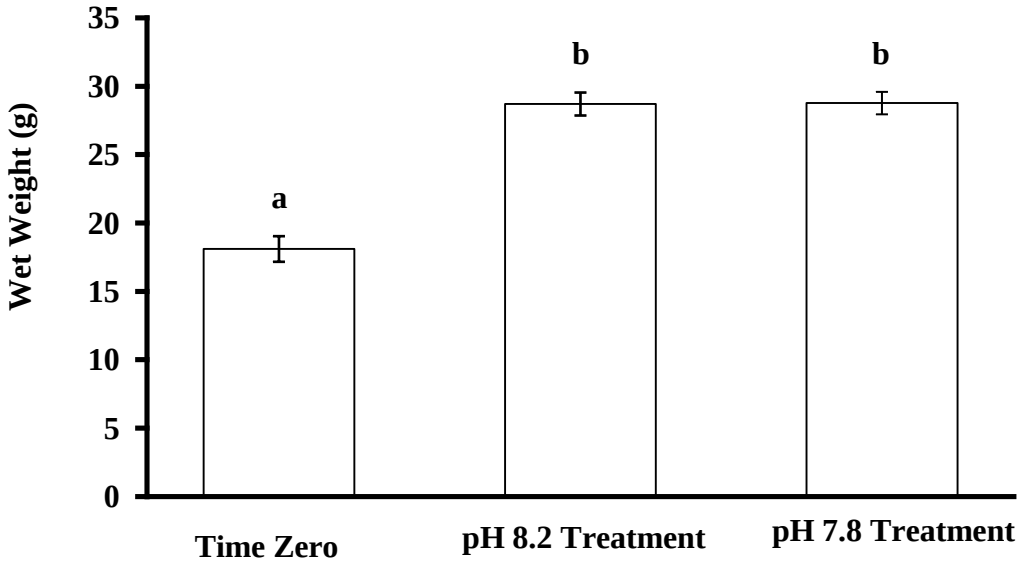

Figure 5. Mean ± 1 SE total body mass (g wet wt) of regenerating adult *Luidia clathrata* at time zero and after being maintained in pH 8.2 and pH 7.8 seawater over a 97 day period. The asterisk indicates statistical significance at the P = 0.006 level. N = 17 for time zero. N = 8 for control (pH 8.2) and experimental (pH 7.8) treatments. Bars with the same letter are not significantly different (P = 0.05).

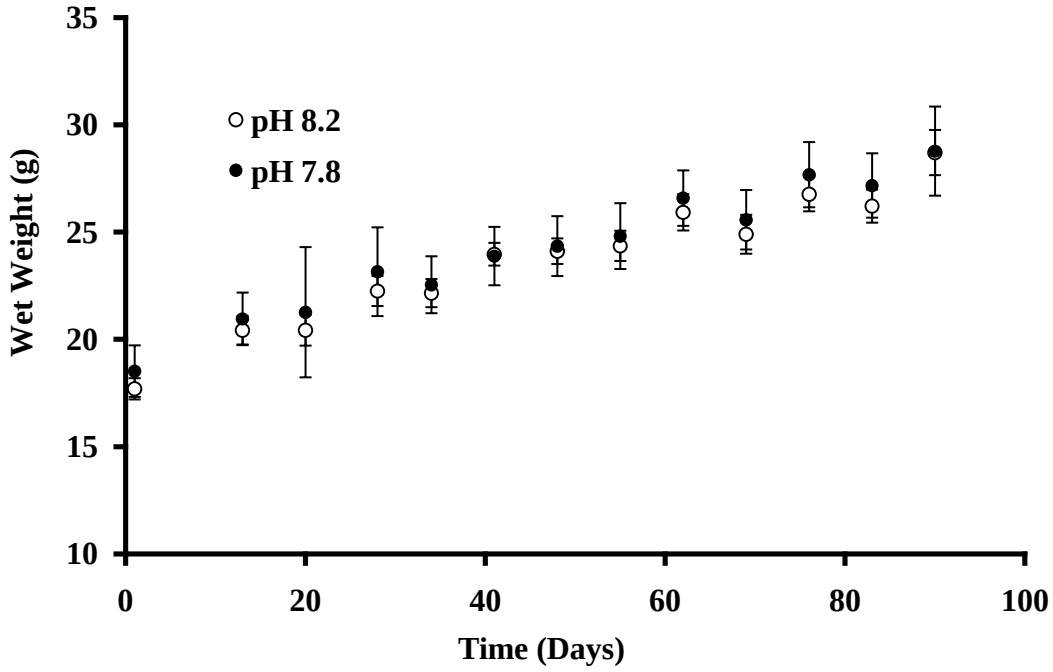

Figure 6. Mean ± 1 SE growth [change in total body weight (wet g) over time] in regenerating *Luidia clathrata* maintained in pH 8.2 and pH 7.8 seawater for 97 days. Each individual had two arms removed at time zero. N = 8 for each open or closed circles.

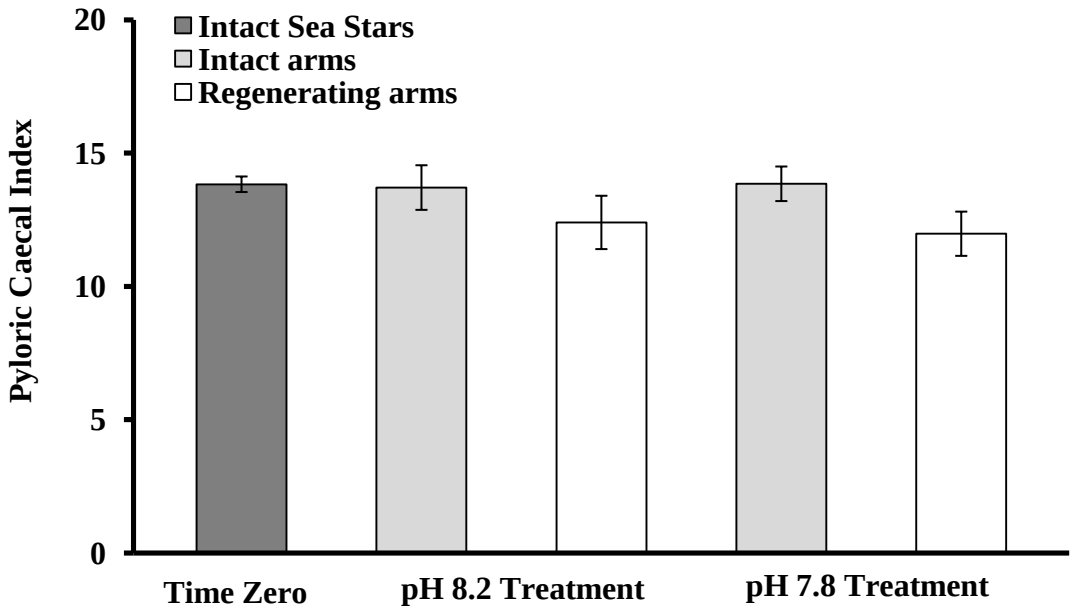

Figure 7. Mean ± 1 SE pyloric caecal index values for individual arms [wet wt of pyloric caecal tissue removed from a given arm divided by its intact wet wt x 100] for *Luidia clathrata* maintained in pH 8.2 and pH7.8 seawater for 97 days. Shown are pyloric caecal index values for individuals at time zero and for the intact and regenerating arms from individuals examined at the end of the control and experimental pH treatments. N = 17 for time zero. N = 8 for control (pH 8.2) and experimental (pH 7.8) treatments. There were no significant differences between any of the treatments ($P > 0.05$).

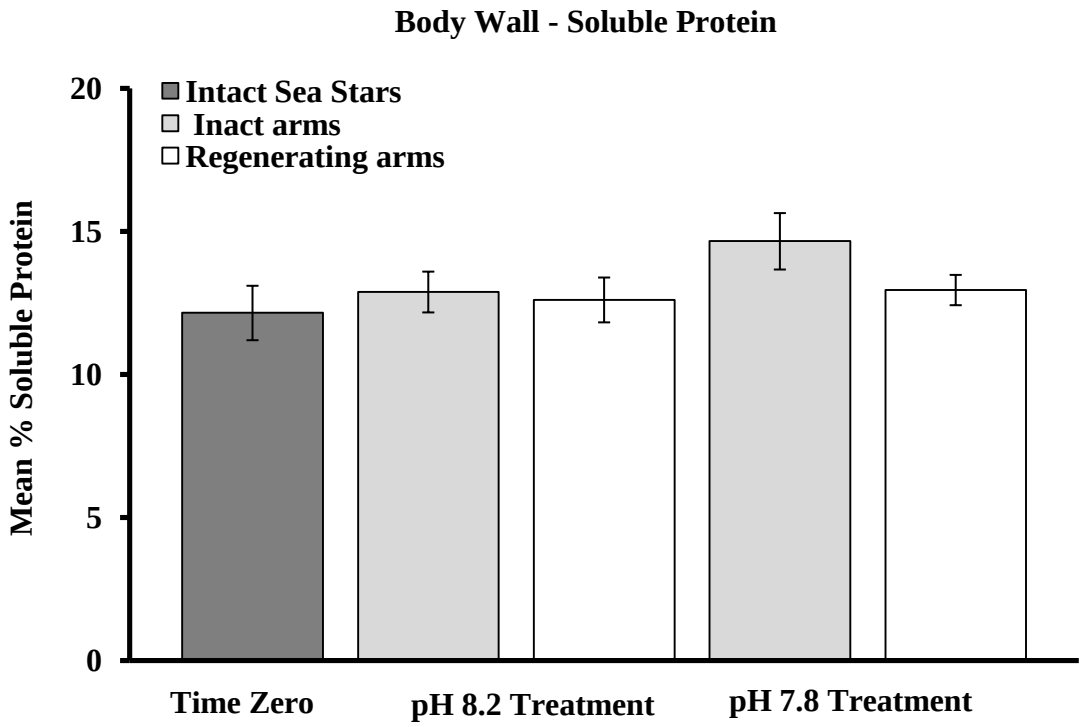

Figure 8. Mean ± 1 SE percent soluble protein for body wall tissues from *Luidia clathrata* at time zero and for intact and regenerating arms of individuals maintained in pH 8.2 and pH 7.8 seawater for a period of 97 days. N = 17 for time zero. N = 8 for control (pH 8.2) and experimental (pH = 7.8) treatments. There were no significant differences between any of the treatments ($P > 0.05$).

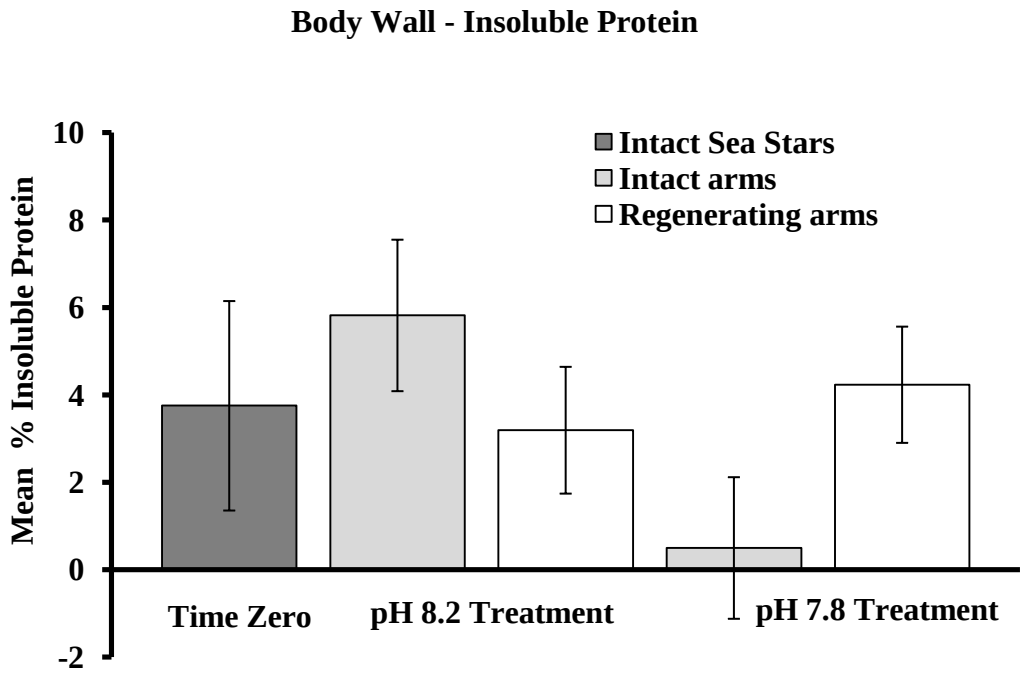

Figure 9. Mean ± 1 SE percent insoluble protein for body wall tissues from *Luidia clathrata* at time zero and for intact and regenerating arms of individuals maintained in pH 8.2 and pH 7.8 seawater for a period of 97 days. N = 17 for time zero. N = 8 for control (pH 8.2) and experimental (pH = 7.8) treatments. There were no significant differences between any of the treatments ($P > 0.05$).

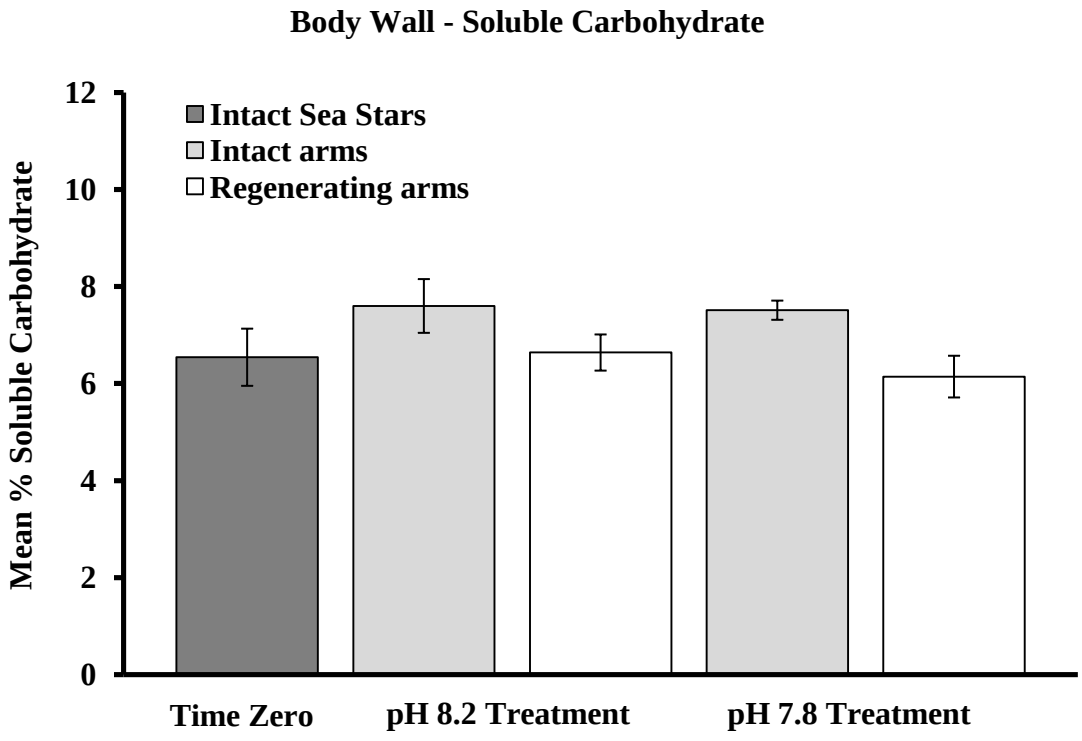

Figure 10. Mean ± 1 SE percent soluble carbohydrate for body wall tissues from *Luidia clathrata* at time zero and for intact and regenerating arms of individuals maintained in pH 8.2 and pH 7.8 seawater for a period of 97 days. N = 17 for time zero. N = 8 for control (pH 8.2) and experimental (pH = 7.8) treatments. There were no significant differences between any of the treatments ($P > 0.05$).

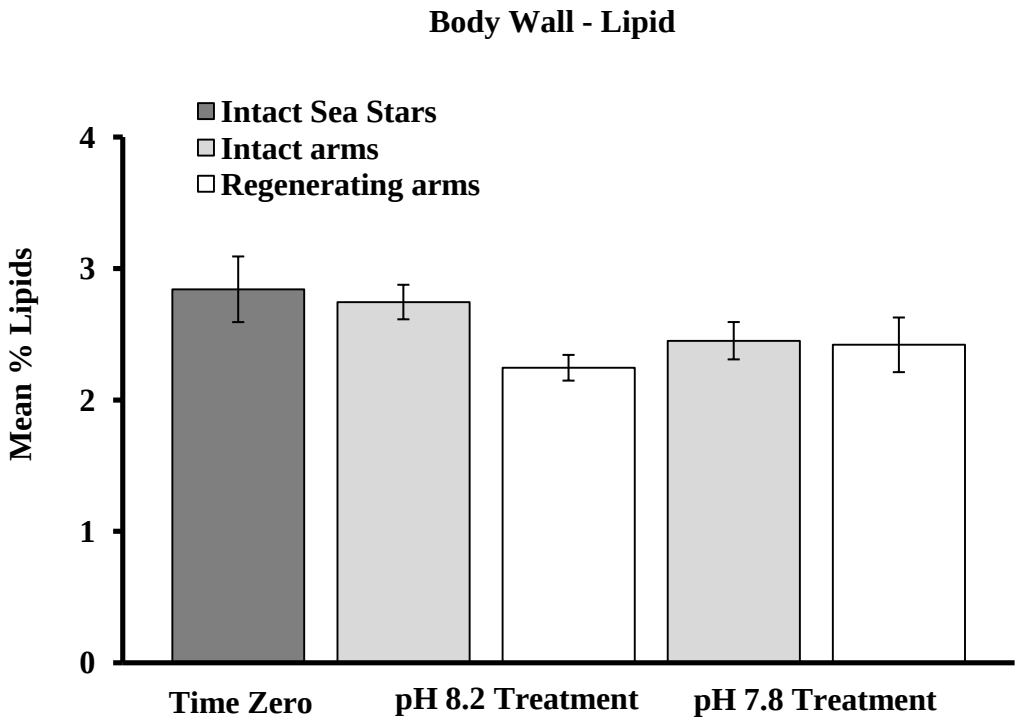

Figure 11. Mean ± 1 SE percent lipid for body wall tissues from *Luidia clathrata* at time zero and for intact and regenerating arms of individuals maintained in pH 8.2 and pH 7.8 seawater for a period of 97 days. N = 17 for time zero. N = 8 for control (pH 8.2) and experimental (pH = 7.8) treatments. There were no significant differences between any of the treatments ($P > 0.05$).

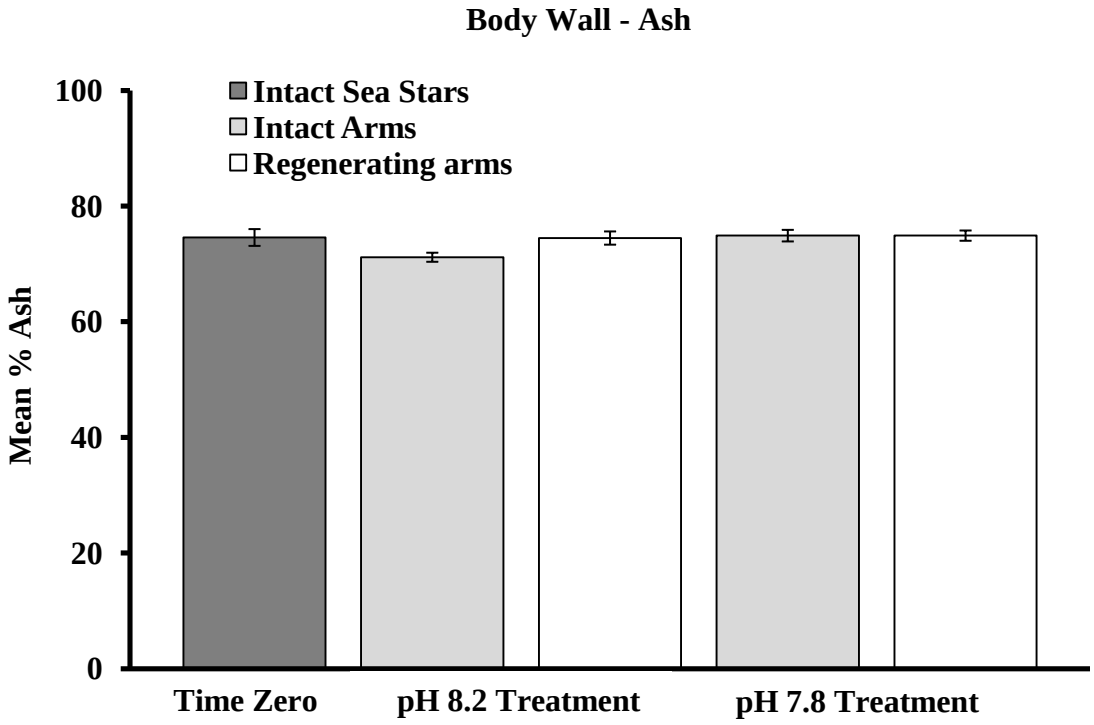

Figure 12. Mean ± 1 SE percent ash (insoluble material) for body wall tissues from *Luidia clathrata* at time zero and for intact and regenerating arms of individuals maintained in pH 8.2 and pH 7.8 seawater for a period of 97 days. N = 17 for time zero. N = 8 for control (pH 8.2) and experimental (pH = 7.8) treatments. There were no significant differences between any of the treatments ($P > 0.05$).

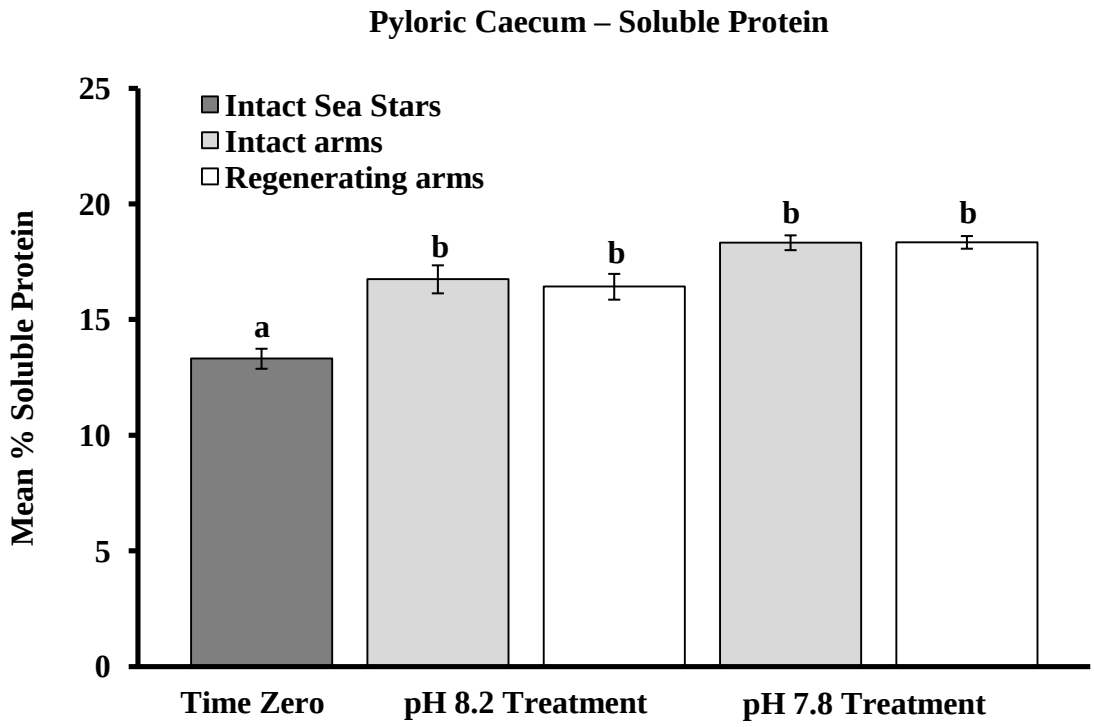

Figure 13. Mean ± 1 SE percent soluble protein for pyloric caecal tissues from *Luidia clathrata* at time zero and for intact and regenerating arms of individuals maintained in pH 8.2 and pH 7.8 seawater for a period of 97 days. N = 17 for time zero. N = 8 for control (pH 8.2) and experimental (pH = 7.8) treatments. Bars with the same letter are not significantly different ($P < 0.05$).

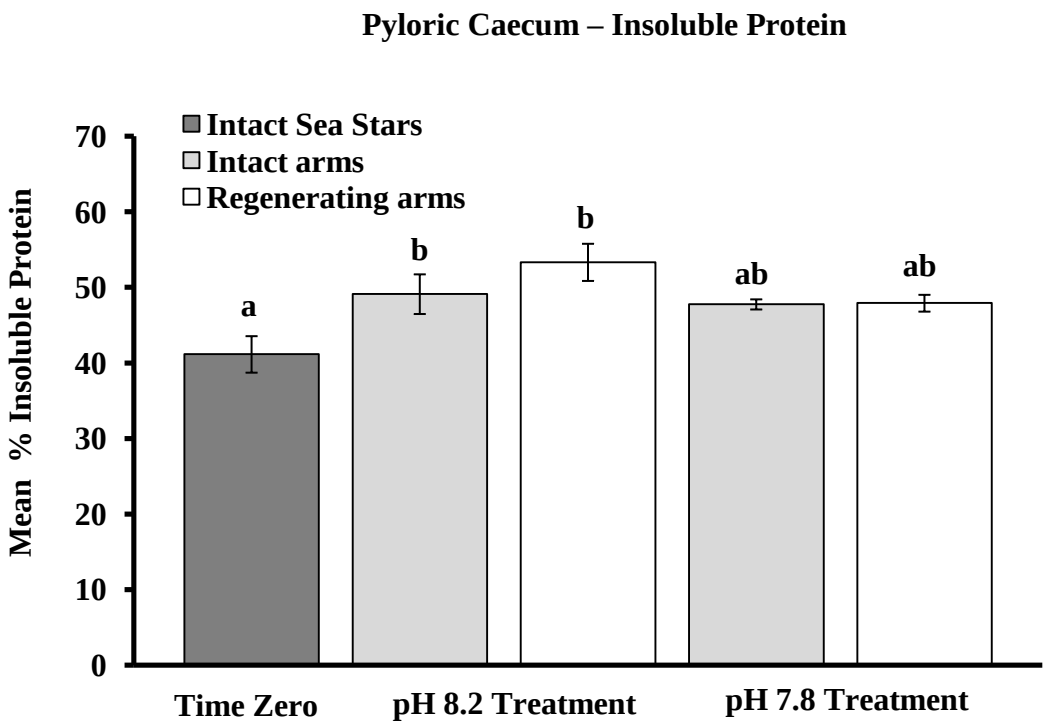

Figure 14. Mean ± 1 SE percent insoluble protein for pyloric caecal tissues from *Luidia clathrata* at time zero and for intact and regenerating arms of individuals maintained in pH 8.2 and pH 7.8 seawater for a period of 97 days. N = 17 for time zero. N = 8 for control (pH 8.2) and experimental (pH = 7.8) treatments. Bars with the same letter are not significantly different ($P < 0.05$).

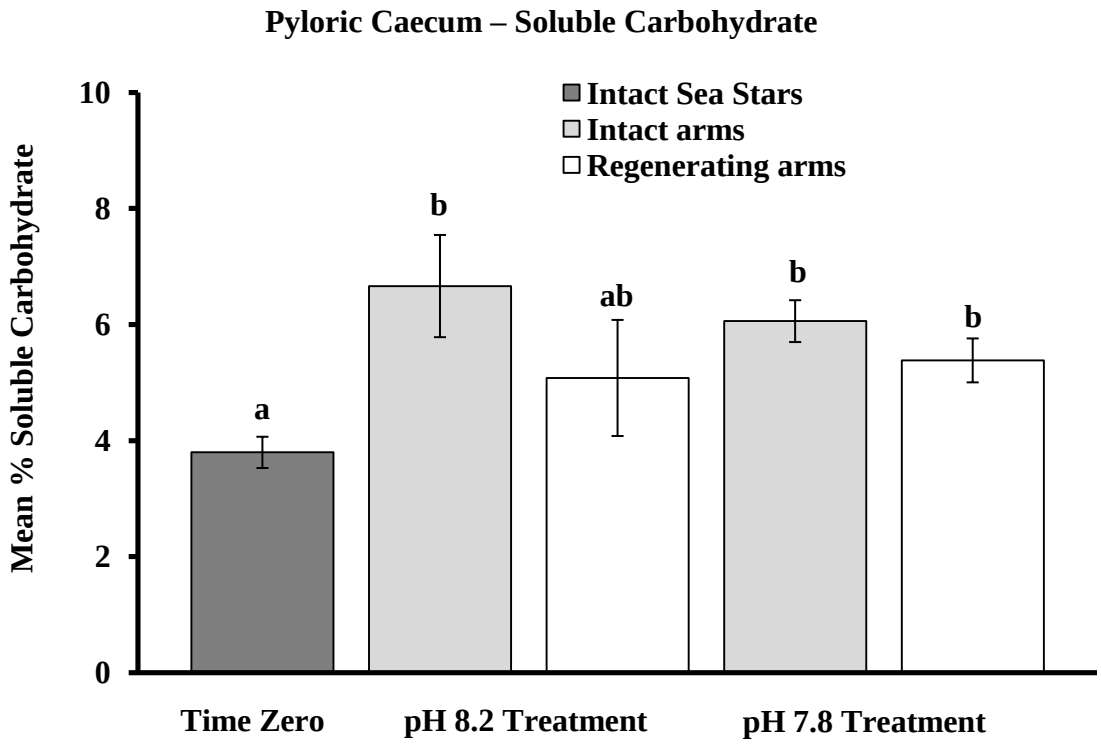

Figure 15. Mean ± 1 SE percent soluble carbohydrate for pyloric caecal tissues from *Luidia clathrata* at time zero and for intact and regenerating arms of individuals maintained in pH 8.2 and pH 7.8 seawater for a period of 97 days. N = 17 for time zero. N = 8 for control (pH 8.2) and experimental (pH = 7.8) treatments. Bars with the same letter are not significantly different ($P < 0.05$).

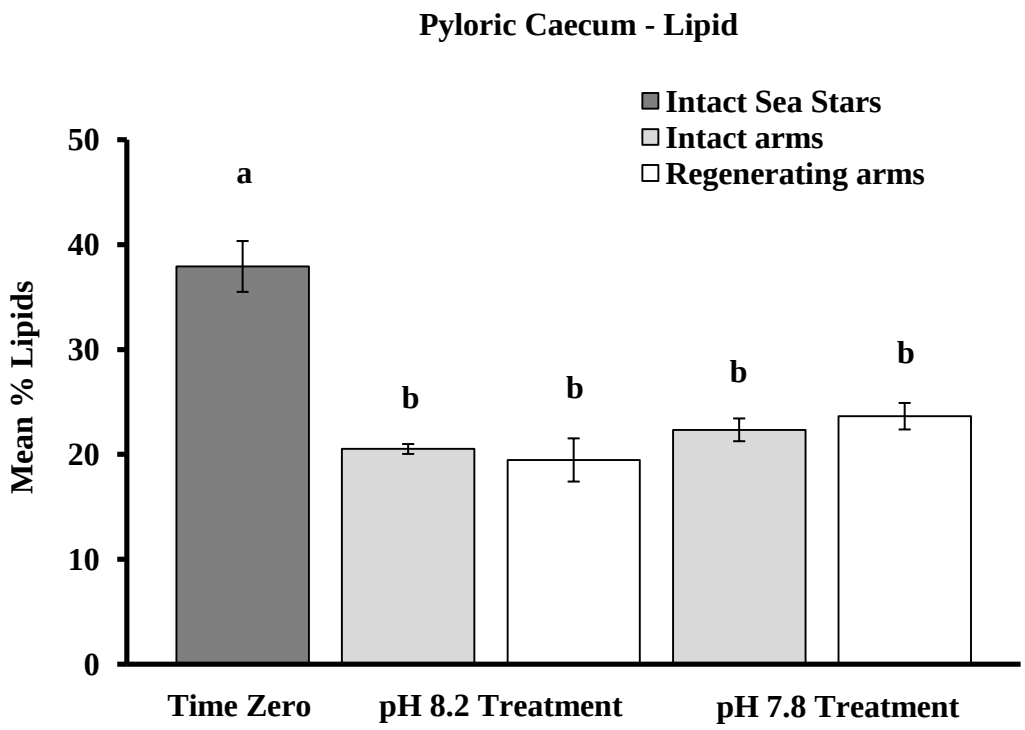

Figure 16. Mean ± 1 SE percent lipid for pyloric caecal tissues from *Luidia clathrata* at time zero and for intact and regenerating arms of individuals maintained in pH 8.2 and pH 7.8 seawater for a period of 97 days. N = 17 for time zero. N = 8 for control (pH 8.2) and experimental (pH = 7.8) treatments. Bars with the same letter are not significantly different ($P < 0.05$).

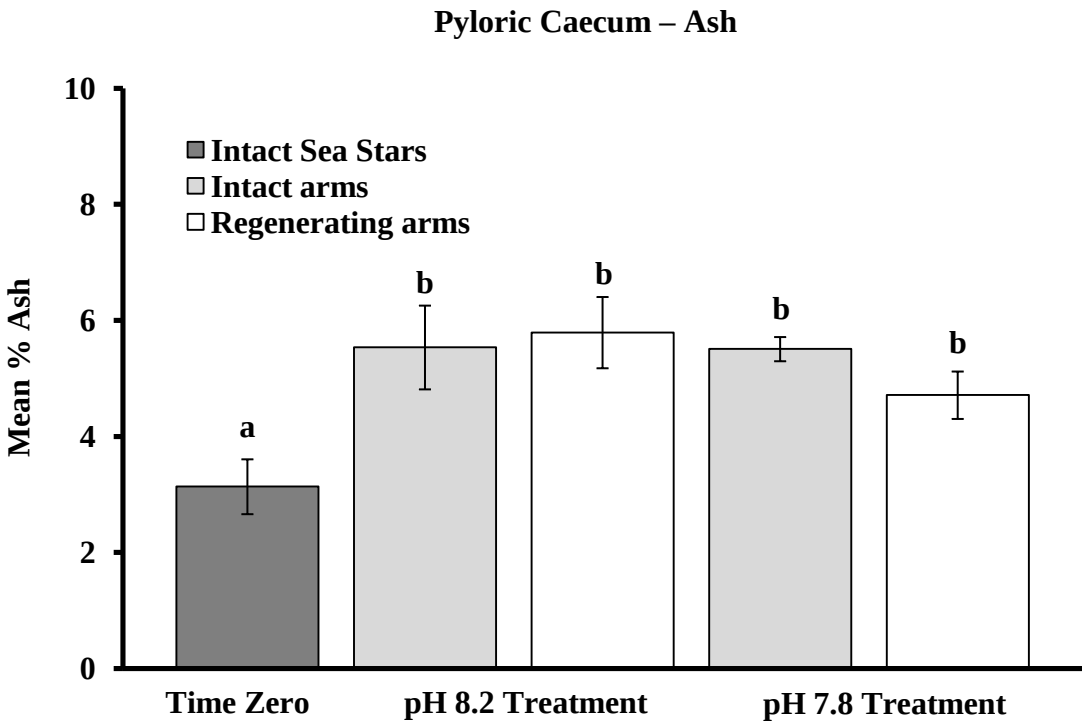

Figure 17. Mean ± 1 SE percent ash (insoluble material) for pyloric caecal tissues from *Luidia clathrata* at time zero and for intact and regenerating arms of individuals maintained in pH 8.2 and pH 7.8 seawater for a period of 97 days. N = 17 for time zero. N = 8 for control (pH 8.2) and experimental (pH = 7.8) treatments. Bars with the same letter are not significantly different ($P < 0.05$).

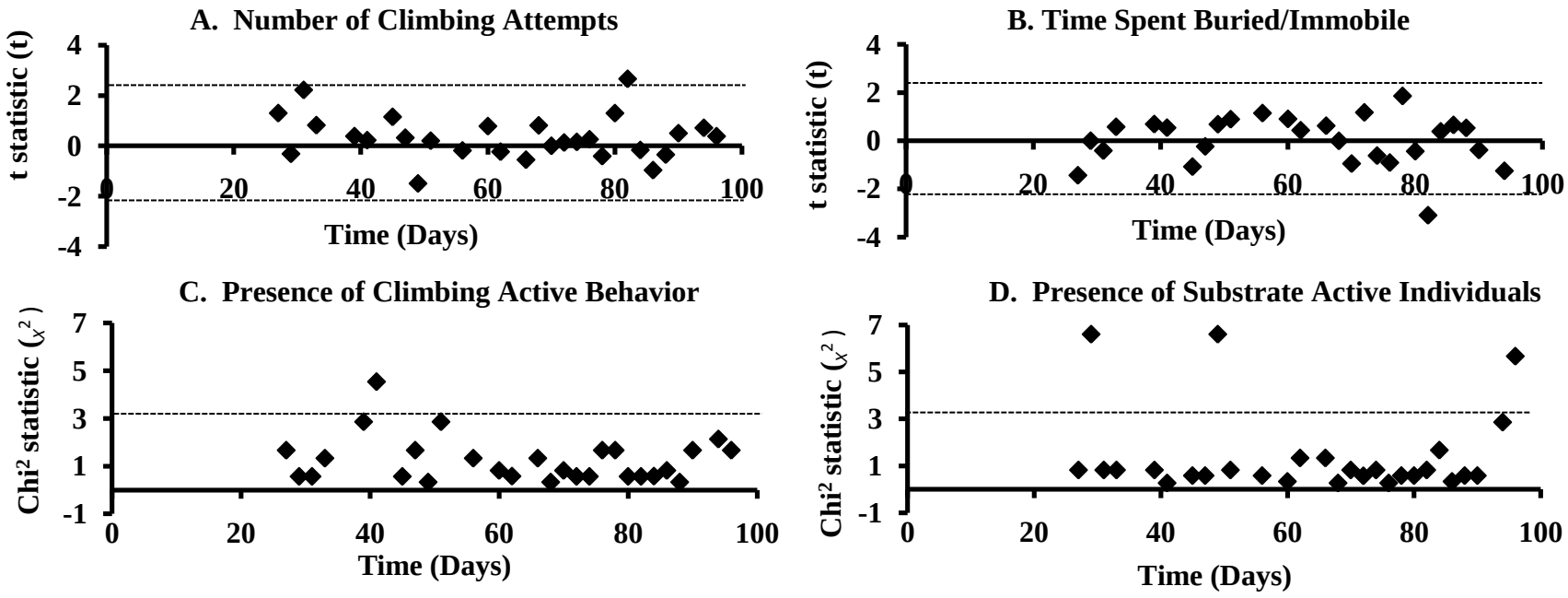

Figure 18. Ten-minute post-feeding behavioral responses for *Luidia clathrata* maintained in pH 8.2 and pH 7.8 seawater for a period of 97 days. For all figures any points above or below the dashed line indicate a significant pH effect at P = 0.05 level. A. Student's t test values for each observation period, comparing the frequency of aquarium climbing attempts (critical value t = ± 2.145; P = 0.05). B. Student's t test values for time spent buried by individuals (critical value t = ± 2.145; P = 0.05). C. Chi^2 values obtained from a one by two contingency table for the proportion of individuals displaying aquarium climbing (critical chi square value $_X{}^2 = 3.841$; P = 0.05). D. Chi^2 values for the number of active (not buried) individuals (Chi square value $_X{}^2 = 3.841$; P = 0.05).

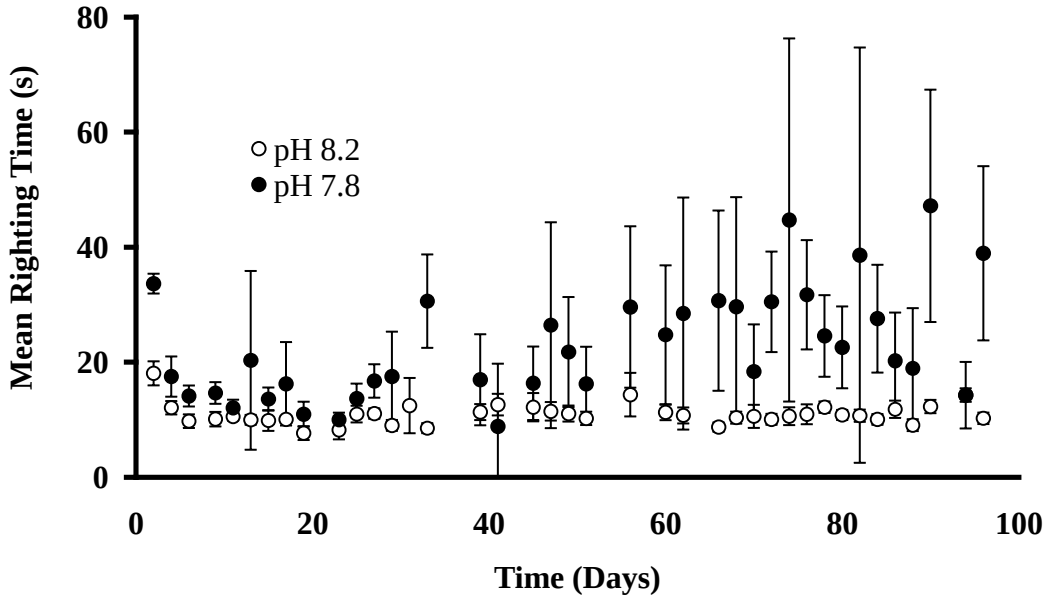

Figure 19. Mean ± 1 SE post-feeding righting times for *Luidia clathrata* maintained in pH 8.2 (control) or pH 7.8 (experimental) seawater plotted over the 97 day experimental period. Measurements shown are for every other day and are for individuals that turned themselves over of their own accord over during a 10-min post-feeding period. Open and closed circles each represent an N of 8.

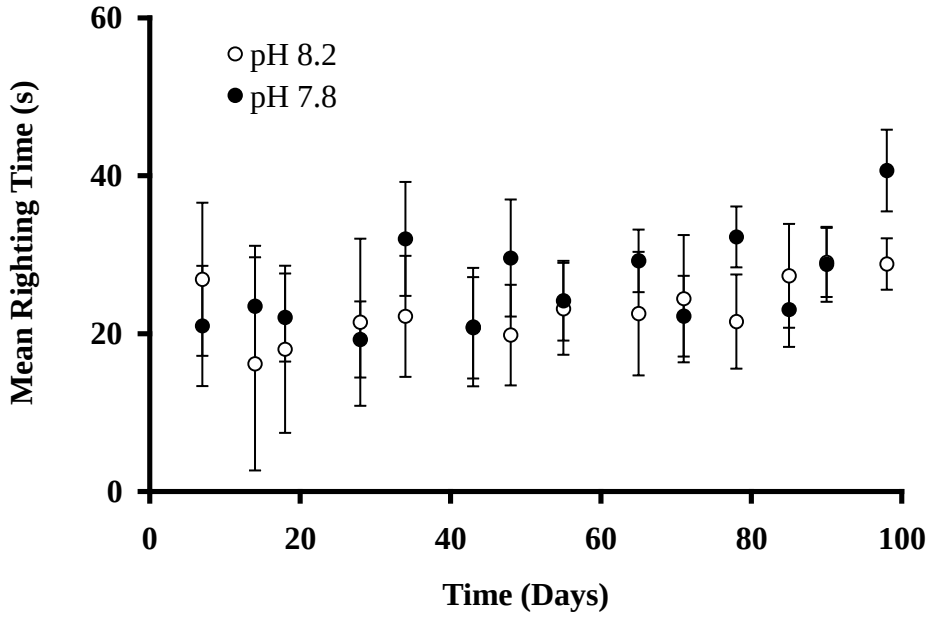

Figure 20. Mean ± 1 SE righting times for *Luidia clathrata* maintained in pH 8.2 (control) or pH 7.8 (experimental) seawater plotted over the 97 day experimental period. Measurements shown are for weekly increments. Individuals were placed on to their aboral surface and time measured until their oral disc was perpendicular to the substrate. Open and closed circles each represent an N of 8.

RAPID REGENERATION OF THE BODY WALL OF THE ABORAL CENTRAL DISC IN THE SEA STAR *LUIDIA CLATHRATA*

JULIE B. SCHRAM AND JAMES B. MCCLINTOCK

Submitted to Gulf of Mexico Science

Format adapted for thesis

CHAPTER 2: RAPID REGENERATION OF THE BODY WALL OF THE ABORALCENTRAL DISC IN THE SEA STAR *Luidia clathrata*

Regeneration is a general process that is displayed in many representatives of the phylum Echinodermata (Wilkie 2001). However, to date the regeneration of the body wall associated with the aboral disc has been associated strictly with the class Ophiuroidea (brittle stars) (Wilkie 2001 and references within). On June 16, 2009, adult individuals of the sea star *Luidia clathrata* (Say) (mean R = 7.4 cm; n = 60) were collected by hand from shallow (1-2 m depth) sandy substrates along the Courtney Campbell Causeway of Tampa Bay, Florida (27°58′N 82°39′W). Sea stars were immediately transported in coolers in ambient seawater to the University of Alabama at Birmingham and placed individually into 10 gallon aquaria divided into two separate 5 gallon sections containing a 2-cm layer of sand. Presumably due to stress associated with high ambient seawater temperatures in Tampa Bay at the time of collection (J. Lawrence, pers. comm.), a number of the individuals autotomized distal portions of their arms the day following their collection. Moreover, one of these individuals (R = 6.0 cm; mean g wet wt = 13.75) also spontaneously discarded a circular segment of the body wall that covered a large portion of the aboral region of disc (Fig. 1, A). The autotomization of the body wall over the central aboral disc (with autotomy defined in this case as strictly the spontaneous loss of healthy tissue) exposed the intact pyloric stomach and several pouches of the stomach extended slightly above the central disc. Despite the loss of the body wall over the central disc and a portion of two of its arms, the autotomized *L. clathrata* appeared healthy. This individual was subsequently followed over a one month

period to document regeneration of the body wall over the region of the disc. During this time period the individual was maintained in artificial seawater (Instant Ocean®) that was filtered by an aquarium pump (Aqueon Power Filter 10) and provided a constant supply of air via an air stone powered by an air pump (CORALIFE® SL-65). Light was maintained on a regular 12:12 L:D cycle and the seawater temperature and salinity held at 24°C and 32 ppt, respectively. A food ration of 0.2 g dry weight of a formulated feed developed specifically for *L. clathrata* (A. Lawrence) was presented to the individual on alternating days. The regenerating individual was periodically photographed with a digital camera (Panasonic Lumix DMC-FZ7) so as to facilitate a description of regeneration of the body wall in the disc region.

Despite the regenerating sea star initially remaining buried in the sand and lacking a feeding response to daily food rations, repair of the body wall over the central disc proceeded rapidly. After a period of 10 days, the circular hole in the aboral body wall tissue over the central disc had elongated and the pouches of the pyloric stomach had become internalized (Fig. 1, B). By day 14, the individual began to move about the aquarium and initiated normal feeding behaviors. After 18 days, the oblong hole in the body wall tissue over the central disc had narrowed sufficiently so as to seal itself completely closed (Fig. 1, C). By day 29, the body wall tissue over the central disc had completely regenerated, likely through a combination of tissue re-growth combined with a muscularly-induced cinching closed of the existing body wall (Fig. 1, D).

To the best of our knowledge this represents the first documentation of the loss and regeneration of the body wall in the aboral region of the central disc in a sea star. *Luidia clathrata* commonly exhibits partial or complete autotomization of its arms in the

field which is considered either due to sub-lethal predation or a response to an environmental stress (Lawrence, 1990; Lawrence and Ellwood, 1991; Lawrence and Vasquez, 1996; Pomory and Lares, 2000). There are significant consequences of regeneration of the arms on the allocation of materials and energy to somatic and gonadal growth in *L. clathrata* (Lawrence et al., 1986; Lawrence and Ellwood, 1991). Similarly, regeneration of the body wall in the central disc region in *L. clathrata* could be expected to have consequences on bioenergetics. This may particularly be the case if feeding ceases for some period of time during regeneration, as noted during our observation period. The rate of arm regeneration in *L. clathrata* is measured on the order of 6-12 months in the field and is rapid compared to other sea stars (Pomory and Lares, 2000). However, the regeneration of the body wall covering the aboral disc is an order of magnitude more rapid than the arms (essentially only 18 days), perhaps because it requires only the regeneration of the body wall tissue and not ossicles, the pyloric ceca, or gonads. Rapid regeneration of the body wall over the aboral disc in *L. clathrata* would be advantageous so as to protect vulnerable internal organs.

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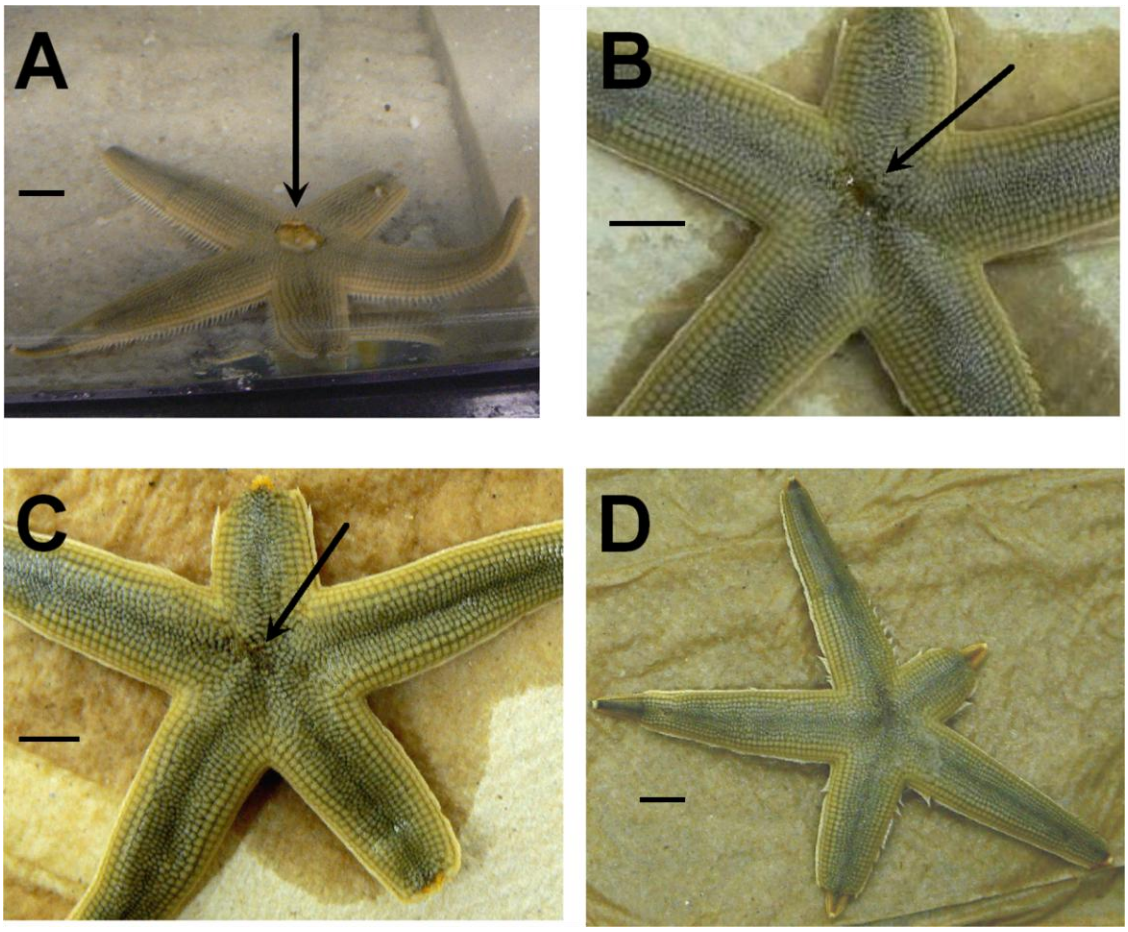

Figure 1. Photographs depicting the loss and regeneration of the body wall over the central aboral disc in an adult individual of the sea star *Luidia clathrata* (R = 6.0 cm). A. 12-hr post loss of body wall over central region of disc. B. Day 10: Elongation and narrowing of body wall surrounding region of disc autotomization. C. Day 18: Body wall of aboral disc resealed; irregular pattern of body wall ossicles at zone of regeneration. D. Day 29: Regeneration of body over aboral disc complete. All scale bars represent 1 cm.

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APPENDIX

INSTITUIONAL ANIMAL CARE AND USE COMMITTEE

APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: December 18, 2008

TO: James B. McClintock, Ph.D.
CH-368 1170
FAX: 975-6097

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: An Investigation of the Effects of Ocean Acidification on Regeneration in the Sea Star Luidia Clathrata
Sponsor: Internal
Animal Project Number: 081208656

On December 18, 2008, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Invertebrates	A	70

Animal use is scheduled for review one year from December 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 081208656 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
B10 Volker Hall
1670 University Boulevard
205.934.7692
FAX 205.934.1188

Mailing Address:
VH B10
1530 3RD AVE S
BIRMINGHAM AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: December 18, 2008

TO: James B. McClintock, Ph.D.
CH-368 1170
FAX: 975-6097

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on December 18, 2008.

Title of Application: An Investigation of the Effects of Ocean Acidification on Regeneration in the Sea Star Luidia Clathrata

Fund Source: Internal

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW) (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

Institutional Animal Care and Use Committee
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