THE UNIVERSITY OF NEW HAVEN

BIOLOGY & ENVIRONMENTAL SCIENCES DEPARTMENT

MARINE BIOLOGY PROGRAM

ARM REGENERATION OF *ASTERIAS FORBESI* IN HYPOXIC CONDITIONS

A THESIS

submitted in partial fulfillment

of the requirements for the degree of

BACHELOR OF SCIENCE

in

MARINE BIOLOGY

BY

Sam Gurr

University of New Haven
West Haven, Connecticut
2014

ARM REGENERATION OF *ASTERIAS FORBESI*

IN HYPOXIC CONDITIONS

APPROVED BY

Jean-Paul Simjouw, Ph.D.
Senior Thesis Advisor

Gail Hartnett, Ph.D.
Senior Thesis Co-Advisor

Carmela Cuomo, Ph.D.
Director, Marine Biology Program

ACKNOWLEDGEMENTS

I genuinely thank my advisors Dr. Carmela Cuomo, Dr. Jean-Paul Simjouw and Dr. Gail Hartnett for all of their advice, direction and support. I thank the University of New Haven Department of Biology and Environmental Science for the funds to make this research possible. I also acknowledge Dr. John Kelly, Jennifer Passaretti, members of the invertebrates thesis group, the marine biology senior class, friends and family for all of their assistance.

ABSTRACT

Hypoxia impacts the physiology, survival and behavior of organisms throughout the water column (Altieri 2006; Cuomo 2005; Chabot 2008; Nilsson and Skold 1996; Parker and O'Reilly 1991; Simpson 1994; Domenici 2007). The ophiuroid echinoderm species *Amphiura filiformis* suffers decreased regeneration rate when exposed to hypoxia (Nilsson and Skold 1996). *Asterias forbesi* is the dominant asteroid echinoderm species of the Long Island Sound (LIS), a temperate marine estuary that exhibits annual hypoxic events (Parker and O'Reilly 1991). *A. forbesi* were stimulated to expel a single arm (autotomy) and were exposed to oxic (normoxia) and hypoxic conditions. *A. forbesi* specimen were measured at 20-day intervals to determine whether hypoxia affects their rate of regeneration. Evidence from survival and regeneration revealed that *A. forbesi* is impacted most from exposure to hypoxia during the initial days after autotomy. *A. forbesi* also expressed a decreased feeding rate when exposed to hypoxia. Implications to regeneration, survival and feeding behavior from this study provide evidence that the stress of hypoxia can reduce populations of *A. forbesi* in native estuarine habitats such as LIS that express annual hypoxic conditions.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Hypoxia (defined as dissolved oxygen concentration < 3.50 mg/l) is a common occurrence in many temperate marine estuaries due to the combined seasonal effects of surface temperature rise with increased primary productivity (Parker and O'Reilly 1991; Goebel et al. 2006). Hypoxia occurs when biological oxygen demand outweighs available dissolved oxygen (DO) concentrations (Anderson 2001). The extent and variation of the effects of hypoxia on organisms ranges throughout the water column (Altieri 2006; Chabot 2008; Simpson 1994; Domenici 2007).

Hypoxic events are associated with a decline in the abundance of epifauna and infauna organisms such as arthropods, mollusks and echinoderms (Altieri 2006; Cuomo 2005; Parker and O'Reilly 1991; Simpson 1994). Cuomo et al. (2005) correlated the lobster die-off in Long Island Sound (LIS) with ammonia and sulfides released from anoxic sediments. Hypoxia in the Neuse River (North Carolina) estuarine ecosystem was related to a reduction in trophic energy transfer because of the increased mortality of benthic invertebrates such as bivalves and polychaetes (Baird 2004).

Hypoxia not only causes stress to marine epifauna and infauna, but also detrimentally impacts highly mobile species throughout the water column (Howell and Simpson 1994). Populations of Atlantic croaker (*Micropogonias undulates*) off the coast of Florida declined due to impaired gonadal growth when exposed to hypoxic conditions (Thomas 2007). Hypoxia has also been found to reduce locomotion (Chabot 2008) and alter fish antipredator behaviors such as schooling and frequency of aquatic surface respiration, increasing potential for fish exposure to predation (Domenici 2007). In addition to the hypoxic effects in LIS studied by Cuomo et al. (2005), water column hypoxia in the LIS is related to decreased abundance and diversity of mobile species such as squid, bluefish and butterfish (Howell and Simpson 1994). Since the effects of hypoxia on marine life are widespread and varied, it is important to study individual species and their specific responses.

Autotomy is the ability of echinoderm species to deliberately expel an entire appendage when injured or stressed. Most species of the classes ophiuroidea (brittle stars) and asteroidea (sea stars) can expel and regenerate lost appendages. Frequencies of natural arm injury for the two classes of stellate echinoderms are predicted to be 56-62% for ophiuroids and 17-44% for asteroids (Lindsay 2010). Higher potential for arm injury of ophiuroids is likely caused by their means of suspension feeding by protruding arms from benthic substrate (Sköld and Rosenberg 1996). This involves a high risk of injury from predators and pressure upon arm autotomy (Sköld and Rosenberg 1996).

Since ophiuroids commonly autotomize appendages, studies have addressed their regeneration rate in hypoxic conditions. Nilsson and Sköld (1996) found that the brittle star *Amphiura filiformis* suffers decreased single arm regeneration length and area from both mild (2.7 mg l^{-1}DO) and moderate (1.8 mg l^{-1}DO) hypoxic conditions. Compared to the regeneration of specimens in normoxia, *A. filiformis* experienced a 14% reduction in length and 21% in area in mild hypoxia, and 25% length and 36% area in moderate hypoxia (Nilsson and Sköld 1996).

Any injury leading to limb loss in a regenerative echinoderm species is a loss of energetic investment, however asteroid species have a higher cost from arm injury and autotomy than ophiuroids (Lawrence 2010). Since asteroids are active predators of bivalves and barnacles, their arms are essential for both locomotion and prying apart the shells of prey items (MacKenzie 1969; Loosanoff 1964; Altieri 2006). In addition, an autotomized arm for asteroids is a loss of gonads, nutrient reserves, digestive tract (pyloric caeca) and body wall (Lawrence 2010; Anderson 1953). Energetic costs to arm injury are most significant for asteroid echinoderms, posing potential for variations in energy allocation and feeding behavior within oxygen-deprived waters (Lawrence 2010). The arm regeneration of asteroids under hypoxia has yet to be studied.

The ability of *Asterias rubens* to heal under hypoxic conditions was studied by Holm (2008). Heat shock protein HSP-70 and coelomocytes were monitored on the incision site after wounding. Coelomocytes are sources of tissue regenerative cells induced by stress that are largely comprised of phagocytes (Pinsino 2007). Holm's (2008) work demonstrated total coelomocytes counts doubled in *A. rubens* at six hours after wounding under hypoxic conditions. This reveals that asteroid species can endure significant stress responses from arm loss under hypoxic conditions. This result is comparable to findings by Nilsson and Skold (1996) on the reduced arm regeneration of brittle stars exposed to hypoxia. However, Holm's conclusions do not reveal the regeneration rate or survival consequences of long-term hypoxia exposure to asteroid species (Holm 2008).

Asteroid sea star species demonstrate varied survival to hypoxic conditions. *Ctenodiscus crispatus*, *Asterias vulgaris* and *Asterias forbesi* were studied in an LT_{50} test for survival in 3.0 mm Hg (3.0 mg 1^{-1} dissolved oxygen) and resulted in 248 h (*C. crispatus*), 105 h (*A. vulgaris*) and 140 h (*A. forbesi*) (Shick 1976). During exposure to hypoxia, *A. forbesi* individuals lost both tube foot function and muscle tone in 48 to 84 h, but recovered fully when returned to normoxic conditions (Shick 1976).

A. forbesi is the predominant asteroid echinoderm inhabiting LIS (Loosanoff, 1964). LIS exhibits annual summer depletion of oxygen ranging within hypoxic (<3.0 mg 1^{-1}) and anoxic (0 mg 1^{-1}) intensities (Parker and O'Reilly 1991), posing a potential stress to *A. forbesi* regeneration. *A. forbesi* demonstrates increased foraging behavior during the months of June-July that precede lower feeding during July-September months due to temperature indicators and spawning (MacKenzie 1969). This change in activity occurs simultaneously with annual August peaks of hypoxia in LIS (Parker and O'Reilly 1991). Hypoxic conditions reduce the growth and abundance of *Mytilus edulis* (blue mussel), the main prey item for *A. forbesi*. *A. forbesi* emigrates from hypoxic hot spots due to optimal foraging for *M. edulis* and decreased survival in hypoxic exposure (Altieri 2006; Shick 1976). Although they actively move from hypoxic conditions, *A. forbesi* may need access to hypoxic areas and anoxic sediment to produce forbesin, a sulfated gycolipid unique to *A. forbesi* (Findlay 1990). Since concentrations of sulfides in LIS hypoxic zones are found within the epifaunal habitats of sea stars (5cm above substrate) (Cuomo 2005), *A. forbesi* may rely on seasonally declined bottom oxygen levels to synthesize this compound. Considering differences between the energetic investment and functions of the arms of ophiuroids and asteroids, there remains the possibility that *A. forbesi*'s arm regeneration rate is a distinctive adaptation to its native LIS habitat.

MATERIALS AND METHODS

Set-up of Experimental Conditions

Two 67.1L Rubbermaid™ Clever Store containers were filled with 27 ppt Instant Ocean™ artificial seawater. Both tanks had a Tetra™ Whisper 40 output to one end of a Petco™ undergravel filter and an additional air stone fed by a Tetra™ Whisper 20. Tubing was passed through holes in the container lids and openings were sealed with epoxy. The lids were sealed with rubber tubing and mini ¾" spring clamps to keep the tanks airtight. Tanks contained an Eheim™ compact 300-water pump that flowed through a bottle carbon/bio filter. Cleaned whole oyster shells were used as substrate in the tanks. A YSI ProODO Professional Series™ handheld optical sensor was used to take daily measurements of dissolved oxygen (ppm) and temperature (°C).

Pre-autotomy

A total of 14 *Asterias forbesi* specimens (<5.0" diameter) were obtained from the Marine Biological Laboratory in Woods Hole, Massachusetts. Sea stars were held in a 30-gallon holding tank filled with Instant Ocean™ seawater (27 ppt) and maintained at 20°C. The tank was divided into two sections: five individuals were assigned to the control group and five for the experimental group. All sea stars were patted dry and weighed prior to initiation of the experiment. Photos were taken of the aboral (top) and oral (bottom) sides of each sea star prior to excision of the arm, post-excision, and every 20 days thereafter.

Autotomy

After one-day acclimation in the holding tank, autotomy was induced in the arm opposite the madreporite (target arm). *A. forbesi* were provoked to release the target arm by simulating predation with a 2" rubber-tipped spring clamp halfway down the arm. Pinches from the clamp were held for 1-3 minutes before the appendages were released. This was recorded at "Day 0" of regeneration.

Arm Measurements

Image J software (http://imagej.nih.gov/ij/) was used to measure arm lengths (mm) of oral side photos. Arms were measured from the central-most tube feet to the arm tip. Since the entire tube feet arm structure was not lost from autotomy, lengths measured after autotomy represented 0 mm of regeneration and were subtracted from later measurements.

Percent arm regeneration was calculated from length measurements at both 20 and 40 days after autotomy. Distinguishing size and color characteristics allowed for identification of *A. forbesi* specimen in photos throughout the study. Percent target arm regeneration was calculated specific to individual *A. forbesi*.

Regeneration Experiment #1 (RE#1): Hypoxia vs. Normoxia

Day 0 of this test started on 1 February 2014 after sea stars were autotomized. Nitrogen gas was introduced into the hypoxic tank until the desired condition of ~3.50 ppm DO was reached. Daily DO levels were recorded and adjustments were made with nitrogen gas. Mean hypoxic conditions were 3.51 ± 0.16 ppm DO. All animals were fed live *M. edulis*. Unexpected mortality occurred on Day 4 in the hypoxic tank (72 h of hypoxia); the remaining specimens in the hypoxic tank displayed characteristics of stress (ie. lack of skeletal rigidity, loss of tube feet function, lack of feeding behavior, complete lack of mobility). Given the behavior of the hypoxic organisms, the experiment was terminated and oxygen was introduced into the hypoxic tank to bring the levels up to normoxia. An additional two sea stars died in this tank three days later despite the oxygenated conditions. This cause of mortality was avoided in the second regeneration experiment (RE#2) by maintaining stars in normoxia for 72 h before partial hypoxia treatments began.

Regeneration Experiment #2 (RE#2): Partial Hypoxia vs. Normoxia

Three *A. forebesi* were autotomized to replace sea stars that did not survive the first regeneration experiment (RE#1). Initial wet weights and photos were taken for each sea star. New *A. forbesi* were placed into the experimental tank to start the 20-day regeneration period on 20 February 2014 (Table 1).

Table 1. Displays the periods of regeneration for all the *A. forbesi* in the second regeneration experiment (RE#2).

		Days Since Autotomy		
Condition	*A. forbesi* count	31-Jan	20-Feb	12-Mar
Control	5	0	20	40
Partial Hypoxia	2	0	20	40
	3	-	0	20

The experimental tank remained oxygenated for three days to ensure acclimation before the first partial hypoxia treatment. Hypoxic conditions were achieved by pumping nitrogen gas for about a half hour. The experimental tank was deoxygenated to an initial hypoxic condition of 3.58 ± 0.03 ppm. Partial hypoxia was maintained for three hours daily for 16 days (24 February 2014 – 11 March 2014). After three hours, the DO was measured and air pump feeds were reconnected to oxygenate the tank. The experimental tank arrived at normoxia after an hour of oxygenation (Table 2.).

Table 2. Water conditions of the tanks in RE#2. The 'hypoxic' mean ± standard error DO was from measurements taken at the beginning and end of daily partial hypoxia treatments.

	Control Tank Normoxia	**Experimental Tank Partial Hypoxia**	
Condition	Normoxic	Normoxic	Hypoxic
ppm DO	7.07 ± 0.04	6.94 ± 0.06	3.48 ± 0.05
°C Temperature	23.27 ± 0.28	23.36 ± 0.22	

A. forbesi were fed live *Mytilus edulis* between 29 - 45 mm. Stars were given two hours daily to feed before all *M. edulis* not held were removed. Eaten *M. edulis* were counted the next day to calculate feeding rate.

RESULTS

Regeneration Experiment #1: Hypoxia vs. Normoxia

Hypoxia was maintained in the experimental tank for three days (~ 72 h) until 4 February because one *A. forbesi* had died and signs of stress were prevalent in all living sea stars. Although the tank was oxygenated on 4 February, two additional sea stars died on 7 Feb (>50% ~144 h).

Regeneration Experiment #2: Partial Hypoxia vs. Normoxia

A. forbesi in normoxia survived the full 40-days in RE#1 and RE#2. Normoxia sea stars grew 5.0 ± 1.2 mm of their target arm after 20-days post-autotomy and an additional 10.5 ± 1.0 mm between 20 - 40-days post-autotomy (15.5 ± 0.4 mm total). The two sea stars that survived both hypoxia treatments of RE#1 and RE#2 regenerated 9.3 ± 3.1 mm of their target arm at 40-days post-autotomy. The other three sea stars in the partial hypoxia treatment of RE#2 grew 3.4 ± 1.0 mm after 20 days post-autotomy. Percent target arm regeneration demonstrates regeneration specific to the *A. forbesi* individual to remove discrepancies between sea star sizes (Table 3). Over the course of RE#2, each *A. forbesi* in normoxia ate 4.6 *M. edulis* and sea stars in partial hypoxia ate 2.0 (Fig. 1).

Table 3. Percent target arm regeneration (mean ± standard error). Asterisk (*) signifies a post-autonomy period that occurred 31-Jan – 20 Feb (Table 1). All other data occurred simultaneously 20-Feb – 12-March. There were no significant differences found with ANOVA tests ($P > 0.05$).

	0 - 20 days	20 - 40 days
Normoxia	* 8.9 ± 2.1 n = 5	18.8 ± 2.0 n = 5
Partial Hypoxia	6.0 ± 1.4 n = 3	14.6 ± 2.9 n = 2

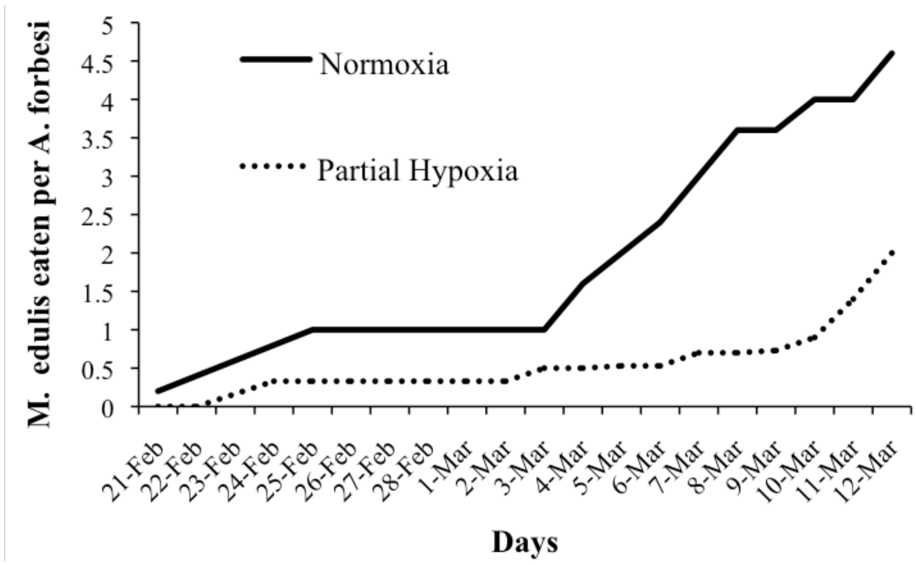

Figure 1. *M. edulis* eaten per *A. forbesi* throughout RE#2.

DISCUSSION

A. forbesi displayed severe signs of stress after 72 h of hypoxia exposure post-autotomy in RE#1. Additional mortalities during optimal DO conditions show that *A. forbesi* were unable to recover after hypoxia. *A. forbesi* without autotomy have been found to endure around twice the exposure of hypoxia (140 h) before reaching >50% mortality (Shick 1976). Evidence from RE#1 demonstrated that the physical stress of autotomy increases the severity of hypoxia for *A. forbesi.*

Hypoxia also affected *A. forbesi*'s behavior. Stars were consistently observed aggregating at the surface of the tank water during periods of partial hypoxia. Similar behaviors in *A. forebesi* have been suggested to be an escape response from the stress of hypoxia (Shick 1976). The physiological stress and escape behaviors found in this study reveal that *A. forbesi* likely emigrate from hypoxic conditions.

Evidence from both survival (RE#1) and regeneration rate (RE#2) show that *A. forbesi* were affected most from immediate hypoxia after autotomy. *A. forbesi* had a slower initial growth rate in hypoxia. At 0-20 days post-autotomy, stars in normoxia grew an average of 2.9% more of their target arm than stars in partial hypoxia (Table 3). Although sample sizes were small and there is an overlap of standard error, data suggest that hypoxia disrupts the ability for *A. forbesi* to regenerate lost appendages (Table 3). The initial decrease of regeneration in hypoxia has also been found in a related asteroid species *Asterias rubens*. Exposure to hypoxia during the initial wound healing response reduced coelomocyte counts and heat-shock protein in *A. rubens* (Holm 2008). Impacts of hypoxia to *A. forbesi* and *A. rubens* could be universal throughout the genus *Asterias.*

After the initial decrease in rate of regeneration, *A. forbesi* arm growth was unaffected by hypoxia. *A. forbesi* in both treatments doubled in regeneration rate at 20-40 days post-autotomy (Table 3). Growth during the 20-40 day period supports a physiological consistency of the species that was unchanged by the variables addressed in this study.

A. forbesi in normoxia fed over twice the rate of stars in partial hypoxia during RE#2 (Fig. 1). Although feeding periods occurred while tanks were oxygenated, *A. forbesi* in partial hypoxia often did not pursue prey and remained dormant on tank walls. This is evidence that the return of normoxia conditions does not result in immediate recovery from hypoxia. Altieri (2006) suggested that the low density of *M. edulis* beds in hypoxia hotspots influences the movement of *A. forbesi* from hypoxia. The difference in feeding rates and escape behaviors in RE#2 suggest that optimal foraging is not the only factor influencing *A. forbesi*'s movements from hypoxia in natural environments.

CONCLUSION

This study supports that asteroids have decreased regeneration rates in hypoxia as do ophiuroids. Hypoxic conditions impact the post-autotomy regeneration rate of *A. forbesi*. Implications to survival and feeding behavior from this study provide further evidence that the stress of hypoxia can reduce populations of *A. forbesi* in estuarine habitats that express frequent hypoxia. Future research should address the distributions of *A. forbesi* in hypoxic waters and the regenerative ability of other *Asterias* species in hypoxia to support the results of this study.

REFERENCES

Altieri AH 2006. Local extinction of a foundation species in a hypoxic estuary: Integrating individuals to ecosystem. Ecology. 87(3): 717-730.

Anderson JM 1953. Structure and function in the pyloric caeca of *Asterias forbesi*. Biological Bulletin. 105(1): 47-61.

Anderson TH 2001. Nutrient pulses, plankton blooms, and seasonal hypoxia in western Long Island Sound. Estuaries. 24(2): 228-243.

Baird D 2004. Consequences of hypoxia on estuarine ecosystem function: Energy diversion from consumers to microbes. Ecological Applications. 14(3): 805-822.

Budington RA 1942. The ciliary transport-system of *Asterias forbesi*. Biological Bulletin. 83(3): 438-450.

Chabot D 2008. Environmental hypoxia as a metabolic constraint on fish: The case of Atlantic cod, Gadus morhua. Marine Pollution Bulletin. 57: 287-294.

Cole and Burggren 1981. The contribution of respiratory papulae and tube feet to oxygen-uptake in the sea star Asterias forbesi (Desor). Marine Biology Letters. 2: 279-287.

Cuomo C 2005. Seasonal variations in sediment and bottom water chemistry of western Long Island Sound: Implications For Lobster Mortality. Journal of Shellfish Research. 24(3): 805-814.

Domenici P 2007. Hypoxia and the antipredator behaviors of fishes. Philosophical Transactions: Biological Sciences. 362(1487): 2105-2121.

Findlay JA 1990. Forbesin: A novel sulfated glycolipid from the starfish Asterias forbesi. Journal of Natural Products. 53(4): 1015-1018.

Goebel NL 2006. Primary production in Long Island Sound. Estuaries and Coasts. 29(2): 232-245.

Holm K 2008. Haematopoietic and proteomic responses to wounding stress in the common sea star Asterias rubens. [dissertation]. [Plymouth, (UK)]: University of Gothenburg.

Howell and Simpson PH 1994. Abundance of marine resources in relation to dissolved oxygen in Long Island Sound. Estuaries. 17(2): 394-402.

Nilsson HC, Sköld M. 1996. Arm regeneration and spawning in the brittle star Amphiura filiformis (O.F. Müller) during hypoxia. Journal of Experimental Marine Biology and Ecology 199: 193-206.

Lawrence JM 2010. Energetic costs of loss and regeneration of arms in stellate echinoderms. Integrative and Comparative Biology. 1-9.

Lindsay SM 2010. Frequency of injury and the ecology of regeneration in marine benthic invertebrates. Integrative and Comparative Biology. 50(4): 479-493.

Loosanoff VL 1964. Variations in time and intensity of setting of the starfish, *Asterias forbesi*, in Long Island Sound. Biological Bulletin. 126(3): 423-439.

MacKenzie CL Jr 1999. A decline in starfish, *Asterias forbesi*, abundance and a concurrent increase in Northern Quahog, *Mercenaria mercenaria* abundance and landings in the northeastern United States. Marine Fisheries Review. 61(2): 66-71.

MacKenzie CL Jr 1969. Feeding rates of starfish, *Asterias forbesi* (Desor) at controlled water temperature and during different seasons of the year. Fishery Bulletin. 68(1): 67-72.

Maginnis TL 2006. The cost of autotomy and regeneration in animals: a review and framework for future research. Behavioral Ecology. 17: 857-872.

Marrs J 2000. Size-related spects of arm damage, tissue mecanics, and autotomy in the starfish Asterias rubens. Marine Biology.137(1): 59-70.

Parker and O'Reilly 1992. Oxygen depletion in Long Island Sound: A historical perpective. Estuaries. 14(3): 248.

Pinsino A 2007. Coelomocytes and post-traumatic responce in the common sea star Asterias rubens. Cell Stress Chaperones. 12(4): 331-341.

Ramsay K and Richardson CA 2001. Invest in arms: Behavioral and energetic implications of multiple autotomy in starfish (Asterias rubens). Behavioral Ecology and Sociobiology. 50(4): 360-365.

Shick J 1976. Physiological and behavioral responses to hypoxia and hydrogen sulfide in the infaunal asteroid Ctenodiscus crispatus. Marine Biology. 37(3): 279-289.

Simpson J 1990. Tidal straining, density currents, and stirring in the control of estuarine stratification. Estuaries. 13(2).

Theede H 1969. Studies on the resistance of marine bottom invertebrates to oxygen-deficiency and hydrogen sulphide. Marine Biology. 2(4): 325-337.

Thomas P 2007. Widespread endocrine disruption and reproductive impairment in an estuarine fish population exposed to seasonal hypoxia. Proceedings: Biological Sciences. 274(1626): 2693-2701.

Vahl O 1984. The relationship between specific dynamic action (SDA) and growth in the common starfish, *Asterias rubens* L. Oecologia. 61(1): 122-125.