Anthropogenic Effects on Queen Conch Reproductive Development in South Florida

A Final Report

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Submitted by: Robert Glazer[1], Nancy Denslow[2], Nancy Brown-Peterson[3], Patricia, McClellan-Green[4], David Barber[2], Nancy Szabo[2], Gabriel Delgado[1], Kevin Kroll[2], Iris Knoebl[2], Daniel Spade[2]

[1]Florida Fish and Wildlife Conservation Commission
Fish and Wildlife Research Institute
2796 Overseas Highway, Ste. 119
Marathon, Florida 33050
305-289-2330 x106; fax: 305-289-2334
bob.glazer@myfwc.com

[2]University of Florida
Center for Environmental & Human Toxicology
Bld 471-Room 52
471 Mowry Rd
Gainseville, FL 32611
352-392-2243 ext 5563; fax: 352-392-4707
ndenslow@ufl.edu

[3]University of Southern Mississippi
Institute of Marine Sciences
Gulf Coast Research Laboratory
703 East Beach Drive
Ocean Springs, MS 39564
228-872-4288; fax: 228-872-4204
nancy.brown-peterson@usm.edu

[4]North Carolina State University
Department of Environmental and Molecular Toxicology
Center for Marine Sciences and Toxicology
303 College Circle
Morehead City, NC 28557
252-222-6367; fax: 252-222-6311
pdmcclel@ncsu.edu

Executive Summary

From spring 2004 through March 2008, a team of scientists from the Florida Fish and Wildlife Conservation Commission, The University of Florida, The University of Southern Mississippi, and North Carolina State University were engaged in a project to determine the cause(s) of reproductive failure in the sub-population of Florida queen conch (*Strombus gigas*) that is found closest to shore. The primary focus of this project was to isolate the protein biomarker vitellin, which is the major egg yolk protein associated with reproduction in queen conch and to develop antibodies against it to use in analyzing reproductive failure in the nearshore animals. Isolating and identifying vitellin was problematic, but was eventually achieved from gonads. In the process, alternative approaches were investigated to identify the stressor(s) responsible for the deficiencies as well as to determine the underlying impacted physiological processes. The project was multidisciplinary in scope and incorporated histological examinations of the tissues, biomarker development for proteins known to be indicators of stress in organisms, neuropeptide assessments, and genomics. Concentrations of metals and select organic compounds in the environment and in the tissues of conch were also examined. Although we did not ascertain the exact stressor(s) causing the reproductive deficiencies, we were able to make considerable gains in a number of areas that are critical for a comprehensive understanding of conch reproductive impairment. The most significant results of this study are detailed below.

Morphology

- **Verge length is significantly larger in offshore male conch than male conch found nearshore.** An analysis of the penis length of male conch found nearshore indicates that the nearshore conch have deficiencies which may ultimately lead to inefficient or ineffective fertilization.
- **Lip thickness was greater in conch collected offshore** probably due to higher mortality nearshore (*i.e.*, conch do not live long nearshore).
- **There were two instances of imposex in nearshore conch.** One conch had external male sex organs, but female gonadal tissue after histological inspection. The other conch had external female sexual characteristics, but male gonadal tissue after histological inspection.

Histology

- **Neural ganglia tissues are structurally impaired in nearshore conch of both sexes, suggesting the neuropeptides involved in reproduction may be impaired.**
- **Gonadal tissues in nearshore conch have serious deficiencies relative to offshore conch.** This confirms the results of our previous studies.

Chemistry

- **Zinc concntrations in conch tissues were significantly higher in conch found nearshore than offshore.** The differences in tissue concentrations of zinc

between nearshore and offshore conch, the identification of genes coding for zinc exposure, and the literature on reproductive impairments associated with zinc argue for expanded and targeted investigations of the effects of zinc on reproductive impairment in conch.

- **Other metals in the environment that were examined do not appear to be causing the reproductive deficits** because concentrations were higher in tissues of offshore animals.
- **Chlorinated compounds (OCP, PAH, and PCB) in the environment do not appear to be causing the reproductive deficits.**
- **Naled and DDVP (Dichlorvos) pesticides do not appear to be causing the reproductive deficits.** These chemicals were undetectable in sediments and water samples.
- **Estradiol and ethynylestradiol (EE2) were found in measurable quantities both offshore and nearshore.** This implies that sewage enters the offshore zone because EE2 is a synthetic estrogenic prescribed for birth control in humans.

Protein Biomarkers

- **The utility of vitellin (Vtg) as a biomarker is questionable at this point.** Although we isolated Vtg from gonadal tissue, Vtg has questionable utility because it is not found in the hemolymph and because nearshore female conch have no gonadal tissue. Additionally, Vtg is not inducible using 17-β estradiol; however, we do not know if other estrogenics may induce Vtg synthesis.
- **Heat Shock Protein 60 was higher in the neuroganglia of conch nearshore suggesting cellular disruption.** However, other Hsps and protein biomarkers were higher in conch collected offshore than nearshore.

Neuropeptides

- **Egg-laying hormone was higher in offshore female *and* male conch than in both female and male conch found nearshore in a variety of tissues.** This suggests that hormone synthesis is impaired nearshore and is affecting both sexes.
- **AGPWamide was higher in offshore conch of both sexes than those found nearshore.** AGPWamide is a proposed penis morphogenic factor and may be related to the smaller penis sizes found in nearshore conch.

Genomics

- **A library of Expressed Sequence Tags was developed** with over 8,000 well-annotated genes. This library was used to examine gene expression between nearshore and offshore conch.
- **A number of genes coding for basic organismal function were identified** that were differentially expressed between conch in the nearshore and offshore zones.

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Chapter 1 – The History and Background of Conch Reproductive Failure in the Florida Keys with a Review of Xenobiotics in the Environment

Background

The queen conch, *Strombus gigas*, is a marine gastropod that inhabits the tropical western Atlantic. In south Florida, it once comprised significant commercial and recreational fisheries (Stevely and Warner, 1978). In the mid-1980s, the stock declined precipitously resulting in a moratorium on harvest in 1985 in state waters; this ban was extended to federal waters in 1986. Until very recently, the population showed no sign of recovering (Berg and Glazer, 1995; Glazer and Berg, 1994; Glazer *et al*., in review).

In south Florida, queen conch exist in two spatially distinct regions: nearshore (i.e., immediately adjacent to the islands and north of Hawk Channel) and offshore (i.e., beyond Hawk Channel) (Glazer and Berg, 1994; Fig. 1). Recent research has demonstrated that there has been a complete cessation of spawning in adult queen conch from nearshore waters (Glazer and Quintero, 1998; McCarthy *et al*., 2002; Delgado *et al.*, 2004); although, anecdotal reports from as late as the mid-1980's indicate that conch used to spawn nearshore (B. Lapointe, Harbor Branch Oceanographic Institution, personal communication). Histological examinations of gonadal tissues from male and female conch found nearshore showed serious deficits in gonadal condition when compared with their offshore counterparts (Delgado *et al.*, 2004). Reciprocal transplant studies demonstrated that the gonads of conch transplanted from offshore to nearshore degraded, whereas those transplanted from nearshore to offshore regenerated their gonads after about six months (McCarthy *et al.*, 2002; Fig. 1). The greatest impacts were observed in female conch.

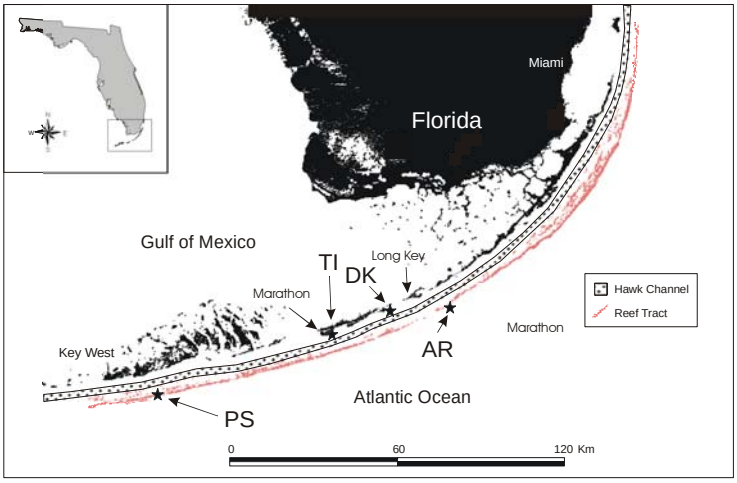

Figure 1. Offshore (PS, AR) and nearshore (DK, TI) sites from tranplant experiments

A variety of xenobiotics introduced into the environment have estrogenic qualities and are suspected of exerting endocrine disrupting effects within biological systems (Arcand-Hoy *et al*., 1998), especially in wildlife. While some of the causal agents are known, their biological effects are poorly understood. There is widespread belief, however, that these agents damage reproduction, reducing fertility, hatchability, and viability of offspring. They reportedly may impair hormone activity and alter sexual behavior, and there is a possibility that exposure of embryos to these chemicals causes inalterable developmental damage and that larval conch may receive a neurological stimulus to undergo metamorphosis from these compounds prior to competence (Delgado *et al*, 2007).

Special interest has been focused on estrogen mimics because of the critical role that estrogen plays in reproduction and development. Estrogenicity, by its nature, disrupts normal endocrine function (Safe *et al*., 2000) in-part by activating the gene that codes for vitellogenin production in males; in females, vitellogenin production is retarded.

Among the xenobiotics implicated in endocrine disruption are the alkylphenol ethoxylates (APEs) which have been identified as estrogenic endocrine disrupters (see Gronen *et al*., 1999 for a review), butyltins implicated as the causative agent in molluscan imposex (see Matthiessen and Gibbs, 1998 for a review), polycyclic aromatic hydrocarbons (PAHs) which depress both female and male reproductive development (Spies and Rice, 1988), current use and banned organochlorine pesticides, a number of which may impact the endocrine system (Celius and Walther, 1998; Cross and Hose, 1988), and human use pharmaceuticals. Additionally, perfluorooctane sulfonate (PFOS), which is used as a stain resistant coating for fabrics, and polybrominated diphenyl ethers (PBDEs), used as flame retardants in products from electronics to textiles, have been implicated in mammalian thyroid dysfunction (Fowles *et al.*, 1994; Meerts *et al.*, 2000).

It is likely that many of these compounds find their way into the nearshore waters of the Florida Keys via sewage discharges (Bright *et al.*, 1981, Kruczynski, 1999), surface water runoff (Heatwole, 1987) shipping discharges and oil spills (Zheng and Van Vleet, 1988), fish house discharges (Heatwole, 1987), discharges from the south Florida mainland (Jaap, 1984), and mosquito pesticide application (Pierce *et al*, 1996). Lapointe and Clark (1990), LaPointe *et al.* (1994), and Szmant and Forrester (1996) showed that point and non-point sources of nutrient discharges have contributed to eutrophication in nearshore waters and that there were nutrient gradients from nearshore to offshore as well as elevated nutrients in nearshore developments. Additionally, Snedaker *et al.* (1995) found significant levels of *n*-alkanes in Florida Keys waters with greater concentration nearshore than offshore. *n*-Alkanes are indicative of petroleum-based carbon contaminants in the environment.

Several studies have examined pesticide application and concentrations in the non-targeted nearshore waters of south Florida. The Monroe County Mosquito Control Board currently uses two mosquito adulticides both of which have been investigated extensively. The first, naled (Dibrom®), is sprayed from a fixed wing aircraft as an ultra-low volume spray (ULV). Naled is an organophosphate and, therefore, a cholinesterase inhibitor and is toxic to most aquatic life (Hartley and Kidd, 1983). It also photolyzes readily, degrading to the much more toxic 2,2-dichlorovinyl dimethyl phosphate (DDVP). The other adulticide used by The Monroe County Mosquito Control Board is Permethrin (Permanone® R.T.U.). It was administered by spray truck in a ULV protocol. Permethrin is a synthetic pyrethroid that paralyzes the nervous system and is highly toxic to fish, hence, the warning on the label to not apply this product within 100 feet (30 meters) of lakes and streams. Despite this, Permanone has been found in canal surface waters and in aerial collections over nearshore waters (Pierce, 1998). Permanone has been found to be toxic to a wide suite of marine invertebrates (Anderson, 1982; Hill, 1985). The pyrethrins have known estrogenic qualities. Because drift over, and deposit into, non-targeted areas is not uncommon (Bird *et al.*, 1996; Pierce, 1998), it is reasonable to suspect that mosquito control pesticides may affect nearshore marine communities.

In 1998, an Advisory Panel report was submitted to the Workshop Steering

Committee of the South Florida Ecosystem Restoration Task Force (Atkeson *et al.*, 1998). The report addressed the development of a strategic plan to address ecological issues in south Florida that require further action. Among those recommendations was the recognition that "pesticides, metabolites, and chemical degradation products should be screened for their endocrine disruption potential." Additionally, they recognized that a variety of other point and non-point sources of contaminants are present and their products may be harmful and should be identified and tested for toxicity.

The lack of reproductive development in nearshore conch, coupled with the long history of mosquito pesticide application, inadequate sewage treatment systems, and other sources of anthropogenic discharges in the Florida Keys suggests that there may be a linkage between reproduction and water quality. Furthermore, this relationship may have directly influenced the recent decline in conch abundance by decreasing reproductive output.

This study represents a thorough examination of many factors that may be causing the reproductive deficits identified in nearshore conch. The project made extensive use of the many tools involved in ecotoxicological research. Among these include the identification of protein biomarkers, neuropeptide expression, analysis of organic chemicals, analysis of metals, histology, immunohistology, and genomics.

During the past two decades, environmental scientists have searched intensively for biomarkers that can detect early physiological or biochemical changes in organisms exposed to anthropogenic chemicals. One of these compounds, vitellogenin (VTG) has been characterized as an ideal biomarker for measuring exposure of oviparous animals to estrogen-mimicking xenobiotics (Denslow *et al.*, 1999). VTG is not normally produced by males; however, exposure to estrogen-mimicking compounds induces them to begin VTG synthesis. VTG is found in detectable concentrations in the plasma of males exposed to estrogen mimics. The chemical structure of VTG is not conserved among species; however, there are short sections that are highly conserved. In this study, the isolation of this biomarker served as a primary focus. However, due to the difficulty in isolating it, other biomarkers related to ecotoxicity were also considered. Among the studies that were conducted were the isolation and characterization of vitellin (VTG), Heat Shock Proteins (HSP), metallothionine, egg-laying hormone (ELH), AGPWAmide, and cytochrome P450s. We coupled these results to histological examination of tissues and to chemical analyses of the water column, sediment, and tissue for organic pollutants as well as metals. Finally, we examined the genes of conch that were up-regulated and down-regulated in conch found in the two zones. Taken together, this study provides a holistic picture of the possible mechanisms for the documented reproductive failure in the nearshore conch.

Chapter 2 – The Objectives of the Study

Objectives of the Study

The overall goal of this project was to determine the cause(s) of the reproductive failure in queen conch found in nearshore waters. To meet this goal, many approaches were integrated together. Each of the approaches was designed to complement the results of the others and, taken together, to form a complete picture of the environment within which conch live, the chemicals that they are exposed to, and their response(s) to environmental conditions. The original focus of the study was to use vitellin (VTG) production in gonadal tissue to quantify the impacts to conch reproductive physiology that result from exposure to xenobiotics present in the nearshore waters of the Florida Keys. However, due to unanticipated difficulties in isolating VTG from conch, we expanded the study to examine a number of other biomarkers, such as proteins and steroids, while still continuing the isolation of VTG. These are detailed in subsequent chapters. Overall, however, the objective of the study remained the same: identification of the stressor(s) causing reproductive failure in nearshore conch.

More specifically, the original objectives of the study were to

(1) determine if there were higher concentrations of chemical contaminants (both organic and metallic) in conch tissues, water column, and sediment samples between the nearshore and offshore environments,

(2) isolate VTG,

(3) develop polyclonal and monoclonal antibodies to VTG,

(4) validate the antibodies using Western blot,

(5) test the suspected xenobiotic(s) identified from the chemistry on adult male and female queen conch to quantify Vtg production and, therefore, reproductive impairment, and

(6) couple the results from the VTG studies to histological examinations of conch gonadal tissue.

The study expanded to include the following additional objectives:

(1) test additional protein biomarkers and neurohormones that have been implicated in terrestrial snail reproductive impairment

(2) expand the scope of tissue examination to include neuroganglia,

(3) develop a library of Expressed Sequence Tags (ESTs) of nucleotide sequences and compare the ESTs to genes libraries from other species to identify genes that may be expressed in impacted versus healthy conch populations.

Whereas the isolation of VTG remained elusive until the end of the study, the other objectives were achieved and a comprehensive picture has begun to emerge on the stressors causing reproductive impairment in nearshore conch.

Chapter 3: Collections of Conch Tissue, Water, and Sediment

Gabriel Delgado and Robert Glazer
Florida Fish and Wildlife Conservation Commission
Fish and Wildlife Research Institute
2796 Overseas Highway, Ste. 119
Marathon, Florida 33050
305-289-2330 x114; fax: 305-289-2334
gabriel.delgado@myfwc.com

Estrogenizing Male Conch and Tissue Collections

On 5 April 2004, we began a series of experiments designed to elucidate the best method for injecting conch with 17-β estradiol. Introduction of 17-β estradiol is designed to induce vitellin in the hemolymph of animals and is widely used as a biomarker in ecotoxicology studies. Three adult conch that had been cultured at the Florida Fish and Wildlife Conservation Commission's queen conch hatchery were injected into the foot with 10 mg 17-β estradiol dissolved in 3 ml ethanol and 7ml corn oil as a carrier (Fig. 3.1). The injectable solution was based on a final concentration of 5 mg / kg body weight. Conch were then returned to their tanks. On 15 April, two of the conch had died and we hypothesized that it was caused by a bolus from the corn oil. An additional experiment with injections of corn oil, DMSO, and saline solution confirmed that the corn oil was indeed the cause of mortality in the first experiment.

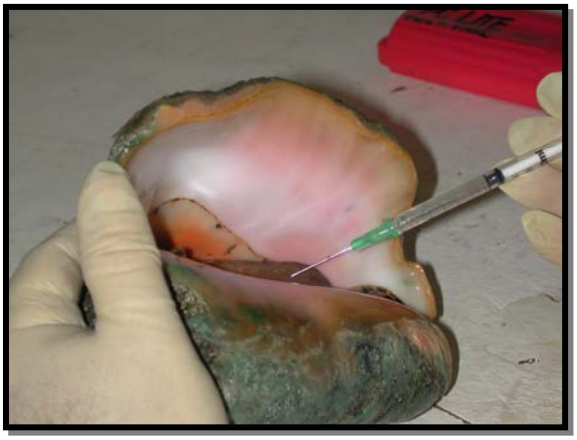

Figure 3.1. Injection of conch with a solution of 17-β estradiol for hemolymph vitellin induction.

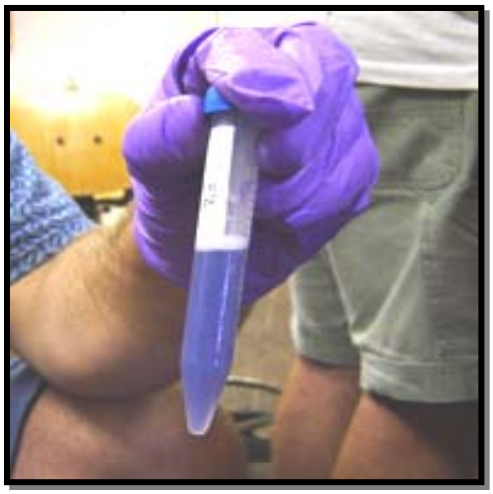

Figure 3.2. Conch blood (hemolymph) used for vitellin analysis.

Based on the previous experiments, seven adult hatchery-reared male conch were injected with a solution of 3 mg 17-β estradiol in a solution of 3 ml ethanol and 7 ml DMSO on 30 April. Injections were to a final concentration of 4 mg 17-β estradiol / kg body weight (Denslow *et al*., 1999). The body weight was based on data collected in Florida and the Turks and Caicos Islands for hatchery-reared conch. On 3 May, we injected an

additional three conch. After approximately two weeks, we gave each conch a booster injection of 2.5 mg/kg. On 8 June, we collected three wild male conch and three wild female conch from Sombrero Reef (Fig. 3.3) to be used as reference animals. On 9 June, we sacrificed the wild males and females as well as the original seven estrogenized male conch and harvested the hemolymph (Fig. 3.2). Gonadal tissue and digestive tissue were also collected for histology (Fig. 3.4). The hemolymph was shipped on dry ice to the University of Florida laboratory in Gainesville for analysis. On 25 October, the second batch of estrogenized hatchery conch were sacrificed, tissues and blood were collected, and shipped to our research partners.

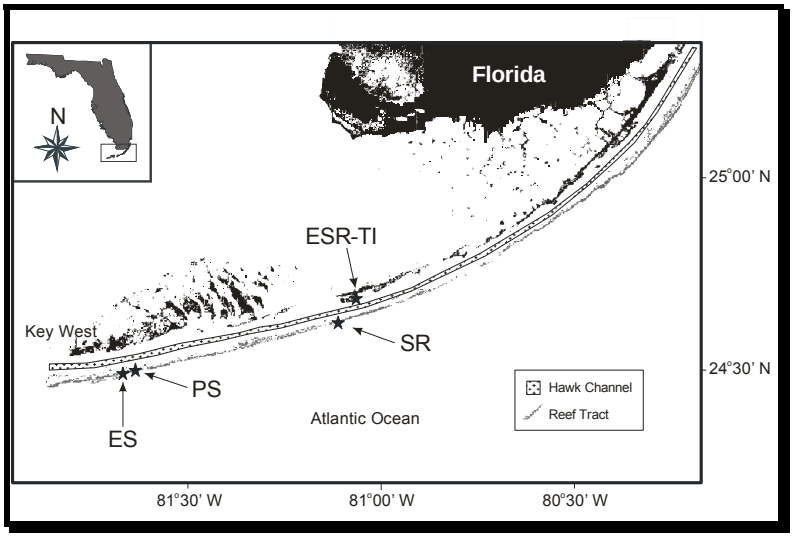

Figure 3.3. Collection sites for queen conch tissue, water, and sediment samples. Offshore sites: ES = Eastern Sambo; PS = Pelican Shoal; SR = Sombrero Reef. Nearshore sites ESR-TI = East Sister's Rock & Tingler Island.

Gonads were sent to the University of Southern Mississippi for histological examination in order to assess gonadal development. A one-cm^3 piece of tissue from the middle of the gonad of each animal was surgically removed, placed in a labeled plastic cassette, and preserved in 10% neutral buffered formalin. After 7 to 14 days in fixative, the tissue samples were rinsed overnight in freshwater. The samples were then dehydrated in a series of graded ethanols following the procedure of Delgado *et al,* (2004) and shipped to the University of Southern Mississippi. All chain of custody protocols were followed. Unfortunately, results showed that the 17-β estradiol did not induce vitellin production in conch; other biomarkers would have to be used.

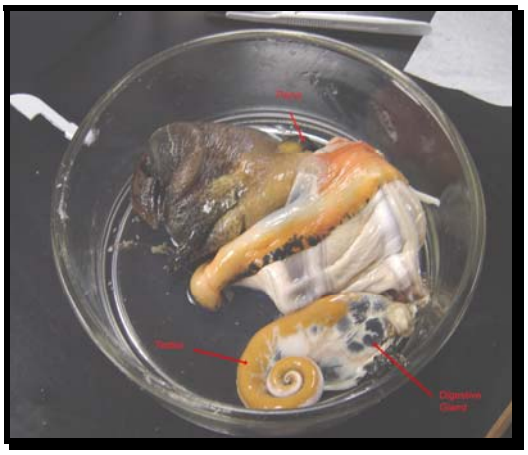

Figure 3.4. Male conch tissues collected for histology, chemistry, and genomics.

As such, we collected adult conch of both sexes from nearshore and offshore aggregations in 2005 and twice in 2007 (Table 3.1). Tissues (*i.e.*, muscle, gonad, neuroganglia, digestive gland, and hemolymph) were collected and preserved in liquid nitrogen to test for additional protein biomarkers and neuropeptides that have been implicated in reproductive impairment in other species of snails. These samples were shipped to the University of Florida for subsequent analysis and comparison between nearshore and offshore animals. Gonads were also preserved in formalin, as described above, for histological comparisons between nearshore and offshore conch. All chain of custody protocols were followed.

Table 3.1. Collection dates and the number of adult queen conch collected from nearshore and offshore sites.

		OFFSHORE			NEARSHORE	
DATE	SITE	**males**	**females**	SITE	**males**	**females**
Jun 2004	Sombrero Reef	3	3	n/a	0	0
Mar 2005	Eastern Sambo	2	3	East Sister's Rock	0	3
Feb 2007	Pelican Shoal	4	4	Tingler Island	5	3
Jun 2007	Eastern Sambo	2	5	East Sister's Rock	4	2

We also took morphometric measurements on all the conch we collected for tissue samples. We measured shell length, lip thickness (a proxy for age), total weight, shell weight, and body weight (foot and viscera). Offshore conch were significantly longer and heavier than those nearshore in all aspects (Table 3.2). Offshore conch also had significantly thicker lips (Table 3.2) which means that the offshore animals were probably older than their nearshore counterparts, partly explaining the size differences. That being said, older conch (comparable to those found offshore) are very difficult to find in the nearshore region. This may be interpreted as the nearshore environment being deficient in some manner, thereby, producing smaller conch and/or not allowing them to reach advanced ages. However, this is speculation at this point.

Table 3.2. Shell length, lip thickness, total weight, shell weight, and body weight (including viscera) of adult queen conch collected in the offshore (N = 26) and nearshore (N = 17) regions of the Florida Keys. The t-test and *P* values calculated from comparisons of the two groups of conch are given.

	Region	Mean ± 1 std. dev.	Student's t-test	$P_{2\text{-tailed}}$ (d.f. = 41)
shell length (cm)	offshore	23.49 ± 0.93	2.25	0.030
	nearshore	22.62 ± 1.60		
lip thickness (mm)	offshore	20.69 ± 6.67	5.09	<0.001
	nearshore	11.85 ± 3.17		
total weight (g)	offshore	2787 ± 316	9.53	<0.001
	nearshore	1812 ± 346		
shell weight (g)	offshore	2236 ± 295	10.25	<0.001
	nearshore	1350 ± 247		
body weight (g)	offshore	551 ± 96	2.84	0.007
	nearshore	461 ± 109		

Starting in 2007, we also began measuring penis length from male conch collected nearshore and offshore in order to determine if there were morphological differences between conch found in these two regions (Table 3.3). If the conch nearshore had smaller penises as we hypothesized, then they would have a decreased probability of fertilization success should they mate. Our results did indeed indicate that conch nearshore had significantly smaller penises (Student's t-test = 12.004, d.f. = 14, $P_{2\text{-tailed}} < 0.001$; Fig. 3.5).

Table 3.3. Penis length of male conch collected in 2007 from nearshore and offshore sites.

REGION	MONTH	SIZE (mm)
Offshore	Feb	79.0
Offshore	Feb	76.0
Offshore	Feb	66.0
Offshore	Feb	80.0
Offshore	Jun	49.50
Offshore	Jun	57.62
Nearshore	Feb	53.70
Nearshore	Feb	71.80
Nearshore	Feb	50.50
Nearshore	Feb	42.60
Nearshore	Feb	30.80
Nearshore	Jun	26.60
Nearshore	Jun	38.72
Nearshore	Jun	35.80
Nearshore	Jun	66.00

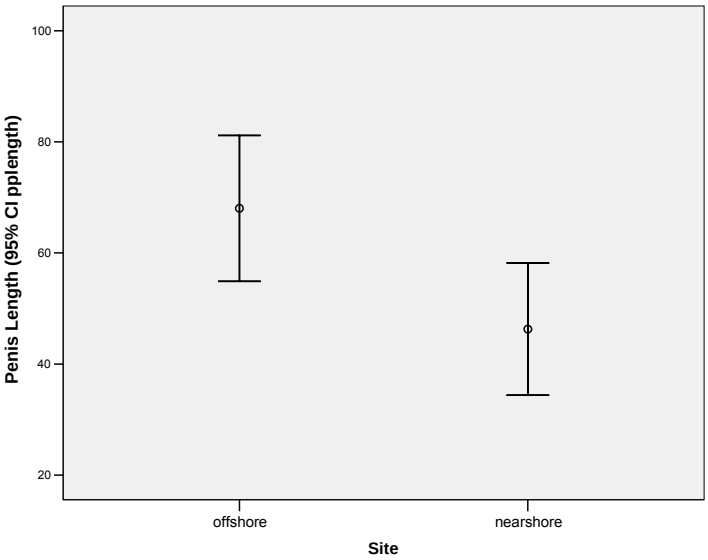

Figure 3.5. Penis length of male conch from nearshore and offshore sites.

Water and Sediment Samples

On April 27, 2007, we deployed two arrays for water column sampling, each consisting of three POCIS filter samplers and three SPMD membrane samplers (Fig. 3.6). These samplers accumulate polar organic materials and non-polar compounds, respectively. One array was deployed at Tingler Island in the nearshore environment and the other was located offshore at one of the conch aggregations at Eastern Sambo (Fig. 3.3). The POCIS samplers examine polar pesticides, prescription and OTC drugs, steroids, hormones, antibiotics, personal care products, etc. The SPMDs sample nonionic organic compounds. The following classes of compounds have been shown to concentrate in SPMDs and were sampled for this project: polycyclic aromatic hydrocarbons, polychlorinated biphenyls, polychlorinated dioxins and furans, organochlorine pesticides, pyrethroid insecticides, nonyl phenols, alkylated selenides, several herbicides, and many industrial chemicals.

Figure 3.6. POCIS and SPMD sampler attached to a stake. An acoustic transmitter was attached to the sampler to allow retrieval in turbid conditions.

The arrays were collected on June 4, 2007. At the same time that the samplers were deployed and collected, blank SPMD membranes and POCIS filters were exposed for the same amount of time that the samplers were being prepared in

order to calibrate for background environmental concentrations not associated with the specific sites. We also collected triplicate water and sediment samples from Tingler's and Eastern Sambo on June 4, 2007. Water and sediment samples were collected in 125ml amber glass jars with Teflon lined caps. All samples, membranes, and filters were put on ice for transportation to the laboratory and once there, were refrigerated at -25^{o}C. The samples were then distributed to our colleagues for analysis; chain of custody protocols were followed.

Chapter 4 – Tissue, Sediment, and Water Column Chemistry

Introduction

A variety of chemical analyses were conducted on conch tissues, sediment, and seawater from nearshore (Tingler Island) and offshore (Eastern Sambo) sites. The number and extent of the testing was limited due to funding constraints. Given these limitations, the samples that were analyzed and the tests that were preformed provide a broad scan of contamination in the environment and the accumulation and exposure to conch. As the project evolved and results were obtained from the various subprojects, it became evident that two tissues (i.e., gonad and neural ganglia) were implicated in the reproductive deficiencies. Based on this assessment, we examined both tissues with respect to metal burdens.

Metal Analysis in Sediment and Queen Conch Tissues

David Barber, PhD
Center for Environmental and Human Toxicology
University of Florida
Gainesville, FL 32611
dbarber@ufl.edu

Sample Description: Samples of queen conch gonad, muscle, neuroganglia (referred to as brain in graphs), blood, and digestive gland were collected by FWC on two separate dates. Samples in Group III were collected from Pelican (offshore) and Tingler (nearshore) sites on 2/15/07. Samples in Group IV were collected from E. Sambo (offshore) and E. Sisters sites on 6/7/07 (nearshore). In Group III, 8 gonad samples (3 M and 2 F from Pelican, 3 M from Tingler, 11 muscle samples (2 M and 4 F from Pelican and 3M and 2F from Tingler), 9 neuroganglia samples (1 M and 2 F from Pelican and 3 M and 3F from Tingler), 6 blood samples (2M from Pelican and 3M and 1 F from Tingler), and 9 digestive gland samples (3M and 2F from Pelican and 3M and 1F from Tingler) were analyzed. ***It is noted that the sample from Tingler identified as NSM3 was later determined to be female based on histology of gonad. This sample was considered as female in all analyses conducted for metals***. In Group IV, 8 gonad samples (4F and 2M from E. Sambo and 2F from E. Sisters), 9 digestive gland samples (5F and 2 M from E. Sambo and 2F from E. Sisters), 5 samples containing both gonad and digestive gland (4M and 1F from E. Sisters), 13 muscle samples (5F and 2M from E. Sambo and 4M and 2F from E. Sisters), and 13 neuroganglia samples (5F and 2M from E. Sambo and 4M and 2F from E. Sisters) were analyzed.

Four sediment samples were also analyzed: 2 from a nearshore site (Tingler Island) and 2 from offshore site (Eastern Sambo).

Sample Digestion and Analysis: Frozen tissue samples were weighed and digested with a standard nitric acid-peroxide digestion. Samples were placed into borosilicate glass tubes with 0.25 mL of concentrated ultra-pure nitric acid (Optima grade, Fisher Scientific). Samples were heated at 140°C until digest volume was reduced to ~100uL. An additional 0.25 mL of nitric acid was added and sample volume again reduced to ~100µL. Samples were cooled to 110°C and 0.25 mL of ultra-pure 30% hydrogen peroxide (Ultrex-II, JT Baker) was added to complete digestion of organic matter. Samples were maintained at 110°C for 30 minutes and then cooled to room temperature. Following digestion, samples were diluted with 5 mL with milli-Q deionized water and filtered through 0.22µm filters in preparation for analysis.

Sediment samples (2 g) were digested by adding 500µL of ultra-pure nitric acid and heating at 140°C until volume was reduced to ~500µL. This process was repeated 4 additional times. 500µL of ultra-pure hydrogen peroxide was then added to each sample and heated at 110°C for 30 minutes. Samples were diluted to 5 m with milli-Q deionized water and filtered through 0.22µm filters.

Metal content of each sample was determined by inductively coupled plasma-mass spectrometry analysis (ICP-MS). Quantitative standards of nickel, copper, zinc, strontium, silver, cadmium, tin, mercury and uranium were prepared in 2% nitric acid from certified standards. Analysis was conducted on an ThermoScientific X-series ICP-MS using indium as an internal standard. Metal concentration was determined from a 7 point standard curve of metals from 0.1ppb to 1 ppm. Nickel, copper, zinc, strontium, silver, cadmium, tin, mercury and uranium were quantified based on the m/z 58, 65, 66, 88, 107, 111, 118, 202 and 238 signals, respectively. Three measurements were performed per sample and the average used to calculate metal concentration. Standard curves with r^2 values of 0.99 or greater were obtained for all analyses.

Tissue metal data is normalized by wet weight of tissue in milligrams and presented as ng metal/mg tissue wet wt.

Statistical Analysis: Two-way ANOVA analysis was performed (JMP by SAS) for each metal using sample collection site and tissue as the variables. Analysis of data demonstrated that there were no significant differences between the metal content of male and female tissues. Therefore, sex was removed as a variable. This was necessary because there was insufficient sample size to perform three-way ANOVA models, which could include sex (See Appendix 1).

Results: Significant differences between collection site and tissue were observed for all metals (Figs. 4.1 – 4.5). However, only zinc and silver exhibited a significant difference between nearshore and offshore sites. Zinc was significantly higher in nearshore digestive gland samples (Fig. 4.1). Silver was significantly higher in offshore digestive gland (Fig. 4.1) and blood samples (Fig. 4.3).

Results of analyses are presented by tissue and collection group with male and female data separated in the figures. Results of statistical analysis and additional figures

depicting data with male and female data combined as well as those depicting the significant differences between nearshore and offshore samples are presented in Appendix 1.

Sediment analysis revealed apparent differences between nearshore and offshore sites (Table 4.1). Unfortunately, due to budget constraints only two samples from each region were collected, thus, great care must be made in interpreting the results. Because conch are likely to ingest significant amounts of sediment while grazing, it is possible that differences in sediment metals may impact data obtained for digestive gland. However, the values of zinc appear to be lower at nearshore sites than offshore which is opposite of the pattern observed in digestive gland. No significant amount of silver is observed in these sediment samples.

Table 4.1. Metal content in sediment samples (n=2). B.D. indicates that values were below detection.

Location		58 Ni ng/mg	65 Cu ng/mg	66 Zn ng/mg	88 Sr ng/mg	107 Ag ng/mg	111 Cd ng/mg	118 Sn ng/mg	202 Hg ng/mg	238 U ng/mg
Nearshore	Mean ± SEM	0.997 ± 0.203	0.903 ± 0.148	0.124 ± 0.010	2046 ± 551	0 ± .001	0.002 ± 0	B.D.	0 ± .001	1.712 ± 0.049
Offshore	Mean ± SEM	0.574 ± 0.005	0.312 ± 0.134	0.251 ± 0.26	2699 ± 1345	B.D.	0.005 ± .001	B.D.	0 ± .001	0.940 ± 0.670

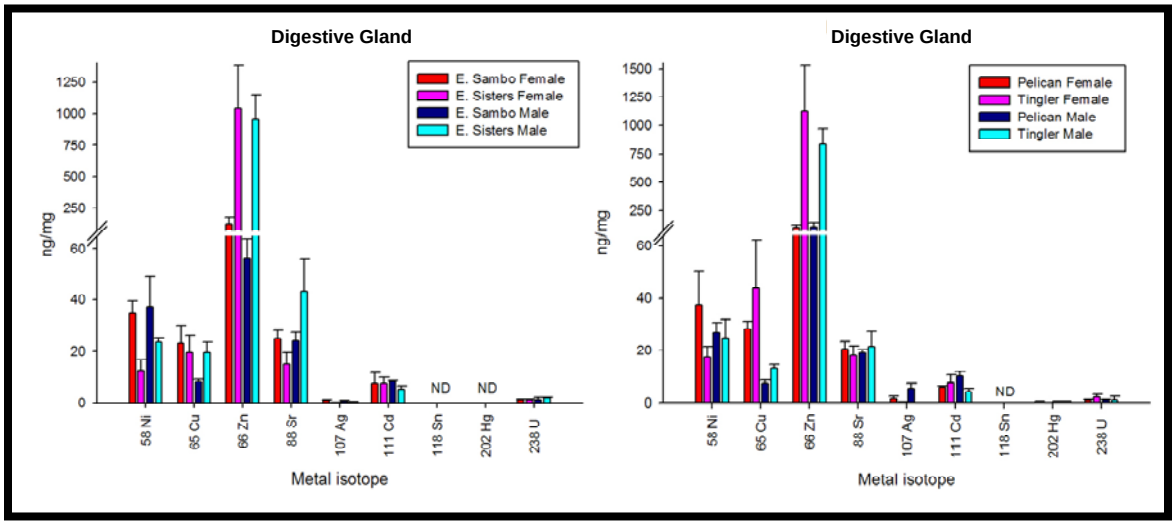

Figure 4.1. Metal concentrations in the digestive gland of queen conch collected on 15-FEB-07 (left) and 7-JUN-07 (rt). E. Sambo and Pelican represent offshore sites. E. Sisters and Tingler are nearshore. Note that y-axis scales are not identical.

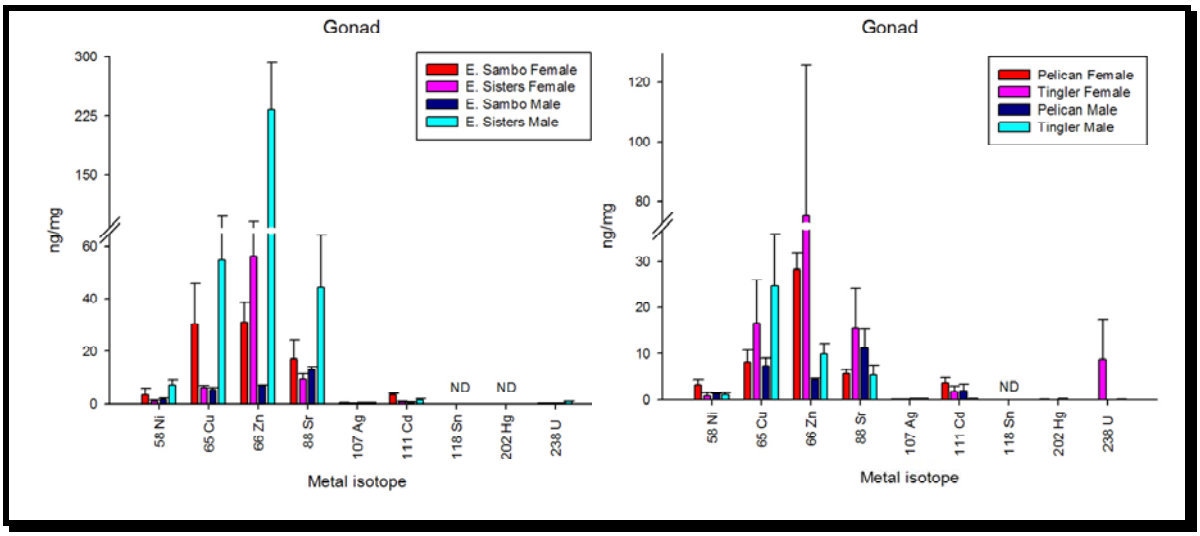

Figure 4.2. Metal concentrations in the gonad of queen conch collected on 15-FEB-07 (left) and 7-JUN-07 (rt). E. Sambo and Pelican represent offshore sites. E. Sisters and Tingler are nearshore. Note that y-axis scales are not identical.

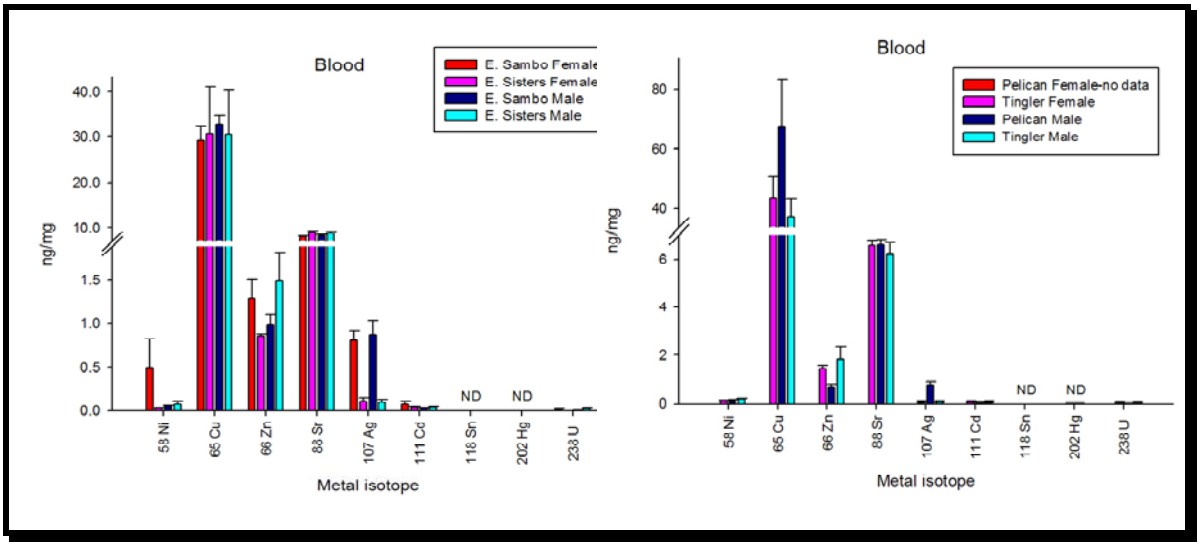

Figure 4.3. Metal concentrations in the hemolymph (blood) of queen conch collected on 15-FEB-07 (left) and 7-JUN-07 (rt). E. Sambo and Pelican represent offshore sites. E. Sisters and Tingler are nearshore. Note that y-axis scales are not identical.

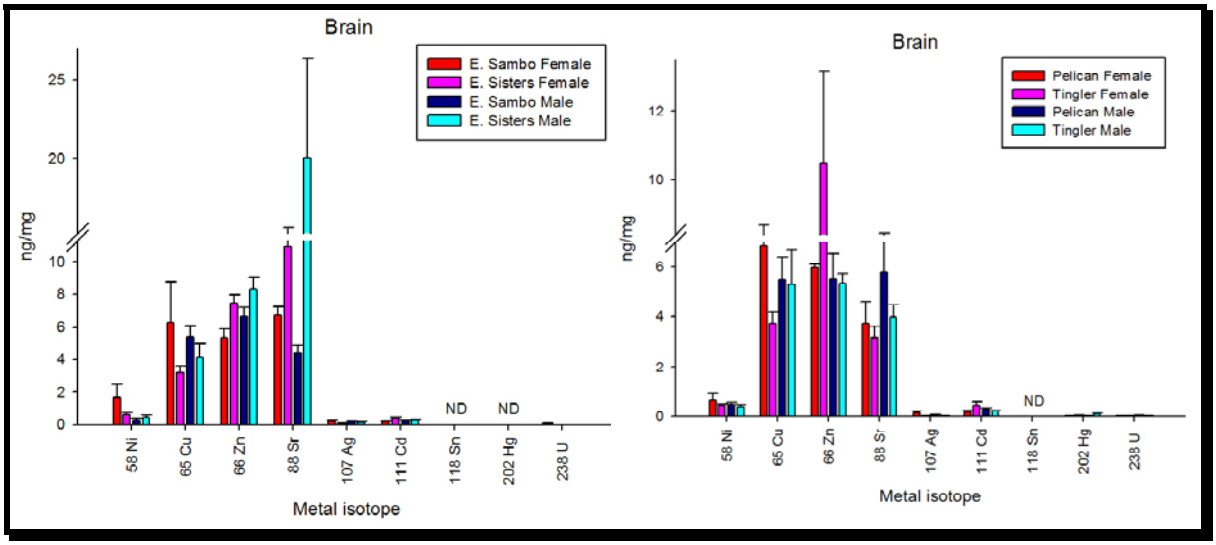

Figure 4.4. Metal concentrations in the neural ganglia (brain) of queen conch collected on 15-FEB-07 (left) and 7-JUN-07 (rt). E. Sambo and Pelican represent offshore sites. E. Sisters and Tingler are nearshore. Note that y-axis scales are not identical.

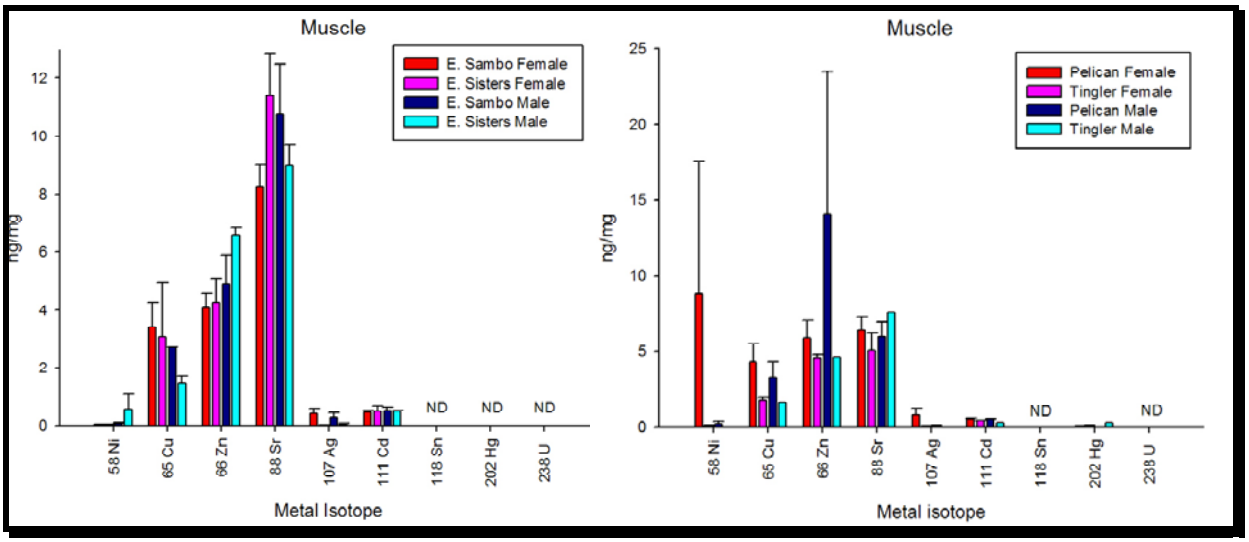

Figure 4.5. Metal concentrations in the muscle of queen conch collected on 15-FEB-07 (left) and 7-JUN-07 (rt). E. Sambo and Pelican represent offshore sites. E. Sisters and Tingler are nearshore. Note that y-axis scales are not identical. Tingler Male Muscle data based on n=1.

Conclusions: Based on the data obtained from this sample set and the metals analyzed, there is little difference between nearshore and offshore sites that would account for the effects observed on reproduction except for zinc and silver. Zinc looks particularly interesting and should be examined further given that sample sizes were relatively small and somewhat unbalanced, which significantly reduced the statistical power of the analyses.

Organic Compounds in Water Column, Sediment, and Queen Conch Tissues

Nancy Szabo, PhD
Center for Environmental and Human Toxicology
University of Florida
Gainesville, FL 32611

Preparation of POCIS and SPMD samplers for chemical analysis

Preparation of the POCIS and SPMD samplers was conducted by Environmental Sampling Technologies (EST), St. Joseph, Missouri. Five ampules of POCIS extract in methanol and five ampules of SPMD dialysate in hexane were sent from EST to the University of Florida Center for Human and Environmental Toxicology on 26 July 2007. The POCIS ampules consisted of one extraction blank (1 POCIS), two field blanks (1 POCIS), and two site samples (composite of 3 POCIS). The five ampules of SPMD consisted of one Day Zero/Dialysis Blank (composite of 3 SPMDs), two field blanks (composite of 3 SPMDs), and two site samples (composite of 3 SPMDs).

Prior to field deployment, the SPMD membranes were spiked with 50ng each of PCBs 4, 14, 29, and 50. The samples were returned to EST from the Florida Fish and Wildlife Conservation Commission on 5 June 2007 in a cooler with ice. The POCIS filters were extracted 11 July and the SPMD membranes were put into dialysis on 10 July. The dialysis process consisted of an exchange every 24 hours for 3 days process to increase the recovery of pyrethroids. Following dialysis, the samples were concentrated and combined using the Kuderna-Danish method, blown down under UHP nitrogen gas (Praxair), filtered through glass fiber filter paper (Fisher, G-8) transferred to methylene chloride (Fisher, Lot 067673) and put into vials for GPC cleanup—the equivalent of one SPMD per vial. The cleanup was done 19-20 July 2007. Sample EST #07-197 Site nearshore, when concentrated, was found to contain about 0.5mL of lipid. This sample was split into eight (8) vials for cleanup so it would not overload the column. Following GPC cleanup the samples were again blown down under UHP nitrogen gas, re-combined, and transferred back into hexane.

The SPMD samples were quantitatively transferred to amber ampules using hexane as the transfer solvent, chilled in a solution of IPA/dry ice and sealed with an oxygen/acetylene torch. The POCIS were extracted using 50mL of methanol, evaporated under UGP nitrogen gas, composited when necessary, and quantitatively transferred to amber ampules using methanol as the transfer solvent. They were sealed in the same manner as the SPMDs.

Analysis of Organic Compounds

Queen conch and sediments from offshore and nearshore sites and SPMD and POCIS water extraction membranes deployed at offshore and nearshore sites were analyzed by

GC-MS for 30 organochlorine pesticides (OCP; aldrin, α-benzene hexachloride (BHC), β-BHC, λ-BHC (also known as lindane), δ-BHC, *cis*-chlordane, *trans*-chlordane, *o,p'*-DDD, *p,p'*-DDD, *o,p'*-DDE, *p,p'*-DDE, *o,p'*-DDT, *p,p'*-DDT, dieldrin, endosulfan I, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, endrin ketone, heptachlor, heptachlor epoxide, hexachlorobenzene, kepone (also known as chlrodecone), methoxychlor, mirex, *cis,*-nonachlor, *trans*-nonachlor, oxychlordane, and toxaphene), 16 polyaromatic hydrocarbons (PAH; acenaphthene, acenaphthylene, anthracene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, benzo[g,h,i]perylene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, and pyrene), and total polychlorinated biphenyls (PCB; sum of mono-substituted to octa-substituted chlorinated biphenyls).

Traces of technical grade chlordane (*cis*- and *trans*-chlordane) and *p,p*-DDE were identified in the conch homogenates (mixed tissues including organs and foot) from both sites, but were not quantifiable. Interestingly, *p,p*-DDT was present at low concentrations in all but one animal with somewhat higher levels found in offshore (7.57 ± 6.335 ppb wet weight; n=6) than in nearshore (2.02 ± 1.470 ppb wet weight; n=6) individuals. Findings in the sediments were in general agreement, with detectable traces of technical grade chlordane, but without detectable *pp*-DDT or *pp*-DDE. In contrast, the PAH naphthalene was present at low levels in each conch, but without the discernable offshore (1.69 ± 0.550 ppb wet weight; n=7) – nearshore differences (1.70 ± 0.621 ppb wet weight; n=6) of *p,p*-DDT. Naphthalene was not detected in any of the sediments. Low levels of phenanthrene were also quantified in most conch and sediments from both offshore (4.34 ± 4.714 ppb wet weight, n=4 conch; 0.39 ± 0.404 ppb wet weight, n=2 sediment) and nearshore (1.69 ± 0.580 ppb wet weight, n=6 conch; 1.30 ± 0.880 ppb wet weight, n=3 sediment) sites. Those OCP and PAH that were not specifically mentioned were not detected in conch or sediment samples. PCBs were not detected in any tissue or sediment sample.

For chlorinated compounds, the SPMD devices showed detectable, but not quantifiable levels of heptachlor epoxide and *cis*-nonachlor in off-shore waters, but only *cis*-nonachlor in near-shore waters. In off-shore waters *cis*-chlordane (0.27 pg/L), chrysene (2.52 pg/L), and benz[a]anthracene (3.21 pg/L) were present at quantifiable levels. In near-shore waters *cis*-chlordane (0.11 pg/L), *p,p'*-DDD (5.02 pg/L), chrysene (3.88 pg/L), benz[a]anthracene (12.68 pg/L), benzo[b]fluoranthene (15.13 pg/L), benzo[k]fluoranthene (6.24 pg/L), benzo[a]pyrene (6.11 pg/L), and indeno[1,2,3-cd]pyrene (7.40 pg/L) were present at quantifiable levels. No other OCP or PAH was found to be present at a concentration above the analytical detection limit and above the level of the field and extraction blanks in waters from either site.

Queen conch and sediments from offshore and nearshore sites and SPMD water extraction membranes deployed at nearshore and offshore sites were analyzed by GC-MS for Naled and its non-specific metabolite DDVP. No tissue sample, sediment, or site water was found to contain detectable levels of Naled or DDVP.

Estrogenic compounds evaluated at nearshore and offshore sites included 17β-estradiol (E2) and ethynylestradiol (EE2). The estrogens were analyzed via LC-MS/MS for all samples. Only one female queen conch from nearshore sites had a quantifiable level of EE2 (0.91 ng/g wet wt), the second female and two of the four males showed

detectable but not quantifiable levels. EE2 was below detection limit in the other two males. In contrast, both off-shore males showed quantifiable levels (0.24 ng/g and 1.61 ng/g wet wt), while two of the five females showed trace levels and the remaining females had levels of EE2 below detectable limits. No offshore conch of either sex gave detectable concentrations of E2. For near-shore animals, one female (0.05 ng/g wet wt) and one male (0.15 ng/g wet wt) had quantifiable concentrations of E2; levels in the others were not detectable. Only one of the three near-shore sediments showed trace levels of both EE2 and E2. One off-shore sediment sample gave a quantifiable concentration of E2 (0.17 ng/g wet wt); another showed a trace amount of EE2; the third was below detectable limits for EE2 and E2. Neither EE2 nor E2 were present at detectable levels in water from either site (sampled using POCIS extraction membranes).

Chapter 5 – Protein Biomarkers

Nancy Denslow and Kevin Kroll
University of Florida
Center for Environmental & Human Toxicology
Dept of Physiological Sciences
Bld 471-Room 52, 471 Mowry Rd
Gainesville, FL 32611
ndenslow@ufl.edu
kkroll@ufl.edu

Introduction

The focus of this project was initially to develop an ELISA for vitellin (Vg), the precursor for egg yolk protein, as a reproductive biomarker (Fig. 5.1). Specifically, we concentrated on the identification and purification of Vg from conch hemolymph and gonads in order to develop antibodies to Vg as a tool to examine stressors in conch reproduction. The first step in this process was the induction of Vg in male conch by injecting male conch with 17β-estradiol (Fig. 5.2). This approach did not work and various other approaches were applied until Vg was ultimately isolated. The approach to Vg isolation as well as the identification of other biomarkers is detailed in the following sections.

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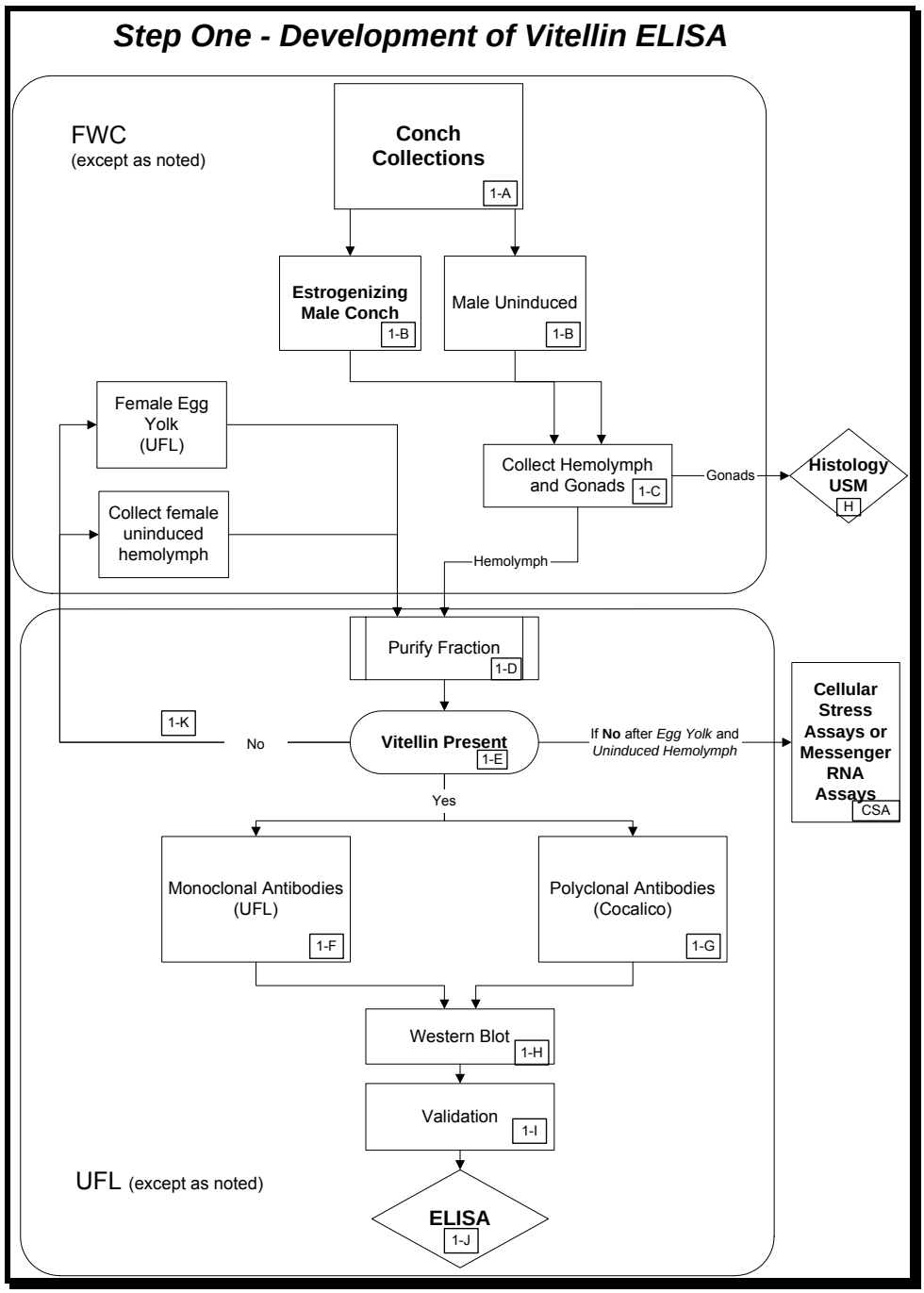

Figure 5.1. Step One in the queen conch endocrine disruption project. The upper box represents the isolation of vitellin, a critical first step in the project as originally designed.

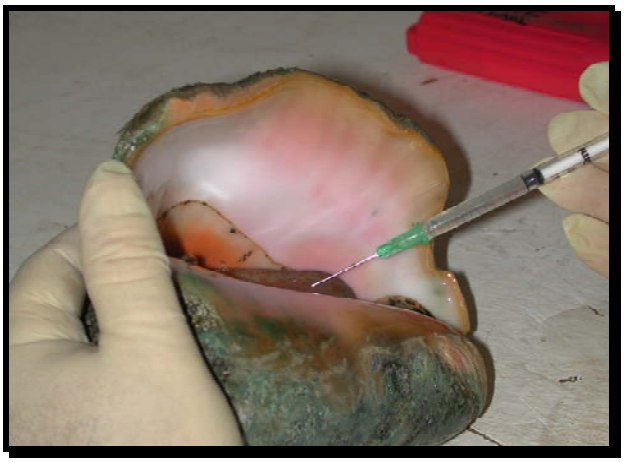

Figure 5.2. Injecting a male conch with 17β-estradiol.

Isolation of Vitellin (Vg)

Analysis of Hemolymph of Estrogenized Male Conch

Hemolymph from wild reproductive males (n=3), females (n=3), and estrogenized male hatchery-reared conch (n=10) was collected on June 8, 2004 by FWC personnel. Wild male and female conch were collected in the process of copulation and their hemolymph was used as the control for comparison. Two different estrogen-induction time points (40 and 178 d post estrogen stimulation with boosters every two weeks) were tested in the event that induction in conch was slow and prolonged. The hemolymph was analyzed by ion exchange chromatography and polyacrylamide gel electrophoresis (PAGE) for the presence of Vg induced by estrogen injection (see Chapter 3 for details on estrogen treatment). A tantalizing protein band was apparent in females and estrogenized male conch and was absent in control males (Fig. 5.3). This band suggested that Vg was present in the hemolymph and propelled the subsequent studies.

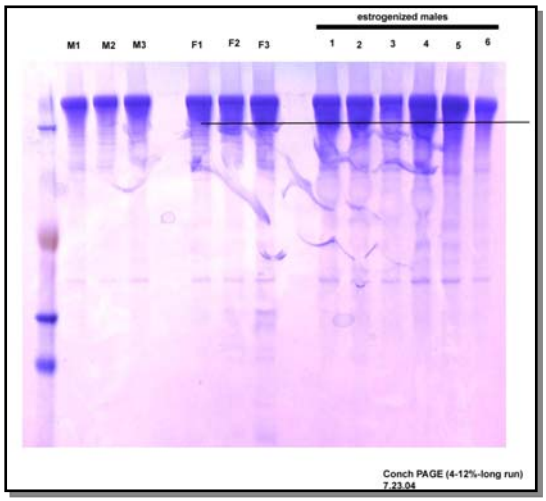

Figure 5.3. Gel run on July 23, 2004 showing the bands of suspected vitellin (denoted by the black line) in estrogenized hatchery, male conch and wild females (F1, F2, and F3). Wild males (M1, M2, and M3) were not expected to exhibit any vitellin and had no protein band in this location.

Hemolymph proteins were separated by anion exchange chromatography using a 20HQ column on the BIOCAD Chromatography system (Perseptive Biosystems TM). In summary, 100 µl of the hemolymph was diluted 1:20 in running buffer (20 mM bis-tris-propane, 75 mM NaCl, pH 9.0)

and then loaded on the column. Non-binding proteins were eluted using five column volumes of running buffer and then a linear salt gradient (75 mM-800 mM NaCl) was applied to elute the proteins that bound to the resin. Chromatographs of the wild males, wild females, and estrogenized males (two sampling time points) were overlapped and compared to check for the presence of a new highly abundant protein in the estrogenized males. However, no induced protein peaks were evident in the chromatograms of hemolymph from males treated with estrogen. In other oviparous vertebrates (fish, birds, reptiles, amphibian) plasma egg yolk precursor protein or "VTG" is induced to very high levels with estrogen treatment in the range of 10-50 mg/ml.

Hemolymph from three different individuals each of wild males, females, and estrogenized male conch was analyzed by PAGE (gradient (3-8%) tris-acetate gel, NuPAGE^{TM}) in the event the induced proteins were expressed at low levels. This gel has higher resolving power than the previous one for high molecular weight proteins. The gel was stained using colloidal blue. After destaining in water, the banding patterns of the males, females, and estrogenized males were compared (Fig. 5.4). There were no additional proteins induced by the treatment with 17β-estradiol.

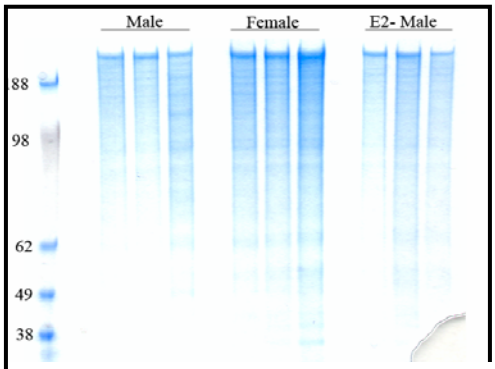

Figure 5.4. PAGE analysis of hemolymph proteins

Since estrogen treatment did not induce VTG synthesis in the hemolymph, the process flow in Step One in the project dictated that we would continue the isolation process by purifying the yolk protein, lipovitellin (LV), from the egg compartment (Step 1K, Fig. 5.1). Concurrent with this approach, we also began examining cellular stress and developing tools for mRNA arrays (CSA, Fig. 5.1).

Isolation of Lipovitellin from the Yolk of Conch Eggs

In January 2005, we began the process of isolating and identifying lipovitellin (LV) from the egg yolk from female conch (Fig. 5.5, Step 1-K). To facilitate this, we collected gonads and hemolymph (Fig. 5.5) from three offshore female conch, three offshore male conch, and three nearshore female conch. Neuroganglia were also collected for evaluation of egg laying hormone and AGPWamide analyses, see below.

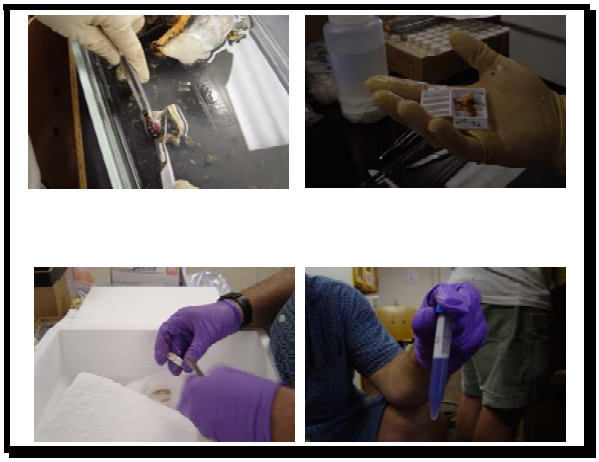

Figure 5.5. Conch tissue collection. Clockwise from upper left: surgical collection of tissues, tissues place in canisters for histological preparation, hemolymph, shipping tissues.

Offshore males and nearshore females were collected in order to compare yolk protein profiles (PAGE, chromatography, Western Analysis). We expected to find a peak (by chromatography) or a protein band (by PAGE) for egg yolk proteins (Lv) in offshore females that would not be present in offshore males and in non-reproductive nearshore females. Samples were shipped to University of Florida and were also preserved for histological examination.

Gel filtration chromatography

An ovary from an offshore mating female conch collected by FWC in June 2004 was used to purify egg yolk proteins by gel filtration chromatography. Frozen conch ovary (1.7 g) was homogenized in a tissue grinder using 10 ml of ice cold gel filtration buffer (25 mM Tris, 300 mM NaCl, 0.05% azide, pH 7.4). This solution was centrifuged at 15,000 x g for 30 min (4 °C) and resulted in three layers. The top layer was fat, the yellow middle layer contained soluble proteins, and the bottom layer consisted of tissue debris. The middle layer was collected and centrifuged again to remove any further debris or fat.

Egg yolk proteins were separated by their size using gel filtration chromatography media Sepharose CL-6B (Pharmacia). This gel filtration media is designed to separate proteins between 2000 Kda and 1 Kda in size. Four ml of this crude yolk mixture was loaded onto a column (1.5 x 90 cm) and fractions were collected every 15 min. The flow rate was maintained at 0.4 ml/min using a peristaltic pump. The purification process was conducted in a cold room at 4 °C. The absorbance (@ 280nm) of each fraction was measured and plotted (Fig. 5.6).

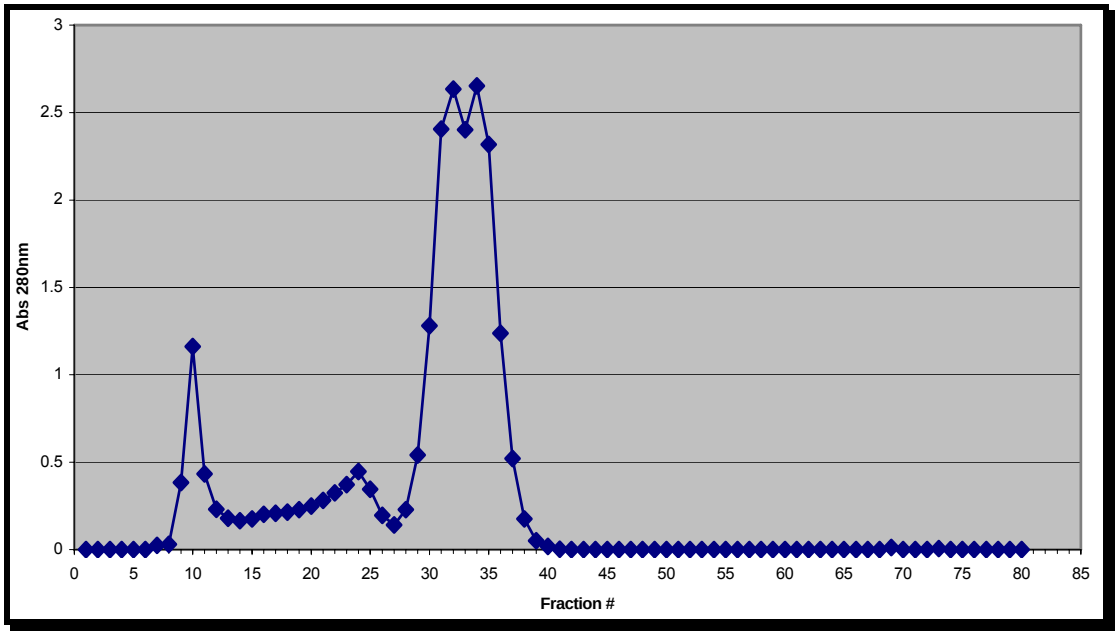

Figure 5.6. Gel filtration chromatograph of Conch Yolk proteins.

The crude egg yolk appears to be composed of four groups of proteins of varying molecular weight. In order to determine the identity of each of these protein peaks, several fractions of each peak were pooled and then analyzed by polyacrylamide gel electrophoresis (PAGE). A PVDF blot of these proteins was analyzed for amino acid sequence by Edman degradation with the hope that the Lv protein would be identified by comparison to the available protein databases at NCBI.

The total protein concentration of each fraction was determined by Bradford assay (Coomassie Plus, PIERCE) using bovine serum albumin as a standard. Each purified peak (Fig. 5.6) was diluted in Laemmli sample buffer and heated at 100 °C for 15min. Ten micrograms of each fraction was loaded onto a 4-20% gradient bis - tris gel (NuPAGE ^{TM}, Invitrogen) and run at 200V for 0.5 hr to separate the proteins. The gel was stained using colloidal blue and a duplicate gel was transferred to PVDF membrane (Invitrolon ^{TM}, Invitrogen). Blotting was conducted for three hrs at 100 V using transfer buffer (10 mM MES, 10% methanol, 0.01% SDS, pH 6.0).

Fig. 5.7 displays the results of the PAGE gel stained by colloidal blue. The last two peaks were not stained by colloidal stain. Similar results are seen in purified fish egg yolk proteins. The last peak(s) are usually very small phospho-proteins or “phosvitins” which are un-stainable by Coomassie-type stains.

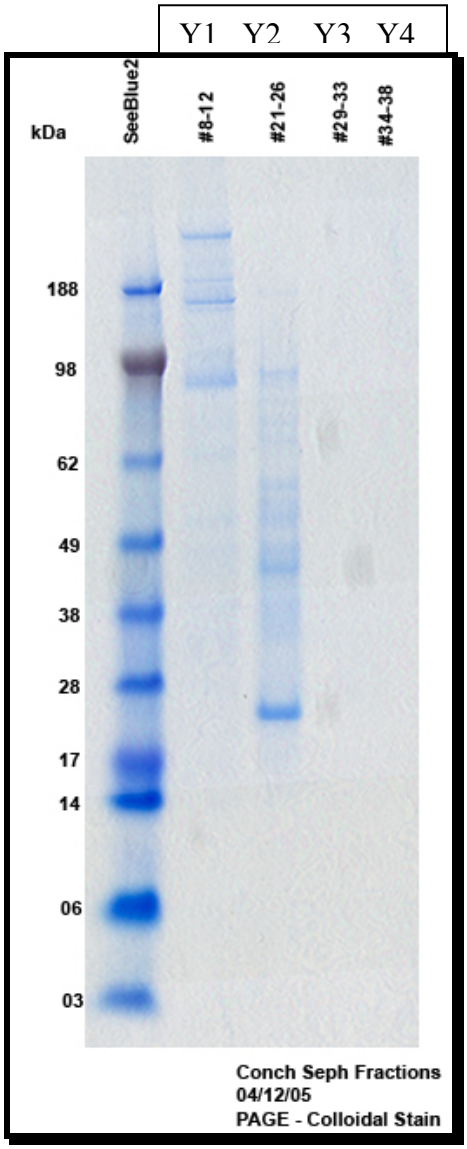

Figure 5.7. PAGE gel of conch egg yolk protein.

Amino Acid Sequencing and Protein Identification

A blot of the purified egg yolk proteins was given to the protein core (University of Florida-Interdisciplinary Center for Biotechnology & Research) for Edman sequencing and comparison to the protein database. This method is used to identify the proteins if the amino acid sequences are similar to other egg yolk proteins in the data base.

The plan was to identify the queen conch LV by amino acid sequencing, in order to start development of specific anti-vitellin polyclonal antibodies, to measure the production of egg yolk proteins nearshore and offshore samples or contaminant-treated conch. Using this antibody we could potentially visually locate egg yolk in histological tissues to verify the source of production by immunohistochemistry.

Analyses of protein bands in fraction Y2 by Edman chemistry were negative, suggesting that all of these proteins were N-terminally blocked. We decided to digest the proteins with the protease Endo-Lys-C in order to get fragments within the sequences and identify them in that manner. The fragments were separated by HPLC and again submitted for Edman sequencing. The major band was identified as glutathione transferase (GST), not lipovitellin or a vitellogenin derivative (Fig. 5.8). (GST is an important enzyme that is used in detoxification.)

Based on these results, phospho-protein staining, and literature on snail yolk (Barre et al, 1991), we turned to sequencing proteins that were present in peak Y1. Four bands of high molecular weight (90, 180, 190, & 200 Kda) were present in gel filtration fraction Y1. Two major bands (90 & 200 Kda) were selected for sequencing (Fig. 5.9).

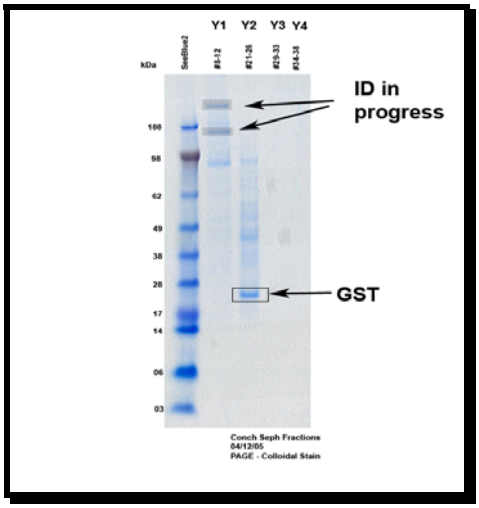

Figure 5.8. Separation of gel filtration peaks Y1-Y4 by polyacrylamide gel electrophoresis (PAGE) on a gradient gel (NuPAGE 4-12%). The box indicates the band that was excised and sequenced to verify identity.

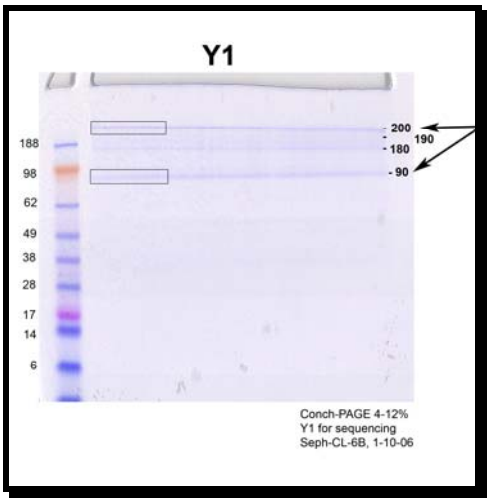

Figure 5.9. PAGE on peak Y1 for Edman sequencing. The two bands indicated by boxes were sequenced.

Unfortunately, both proteins were N-terminally blocked, so we chose to have them identified by mass spec (QSTAR- University of Florida-Protein Core). This technique is state of the art and much cheaper (~10X) than amino acid sequencing. The high molecular weight band (200 KDa) was identified as hemocyanin (also spelled as haemocyanin), an oxygen carrier protein found in most mollusks. The smaller band (90 KDa) was identified as vault protein, a biomarker of drug resistance. The search results for each of these proteins are listed in Table 1.

Table 1. Potential protein identity based on QStar mass spec fragmentation pattern.

I. Y1- 200 KDa Band

1). Hemocyanin type 2 (*Rapana thomasiana*)
2). Haemocyanin (*Aplysia californica*)
3). Haemocyanin, units G & H
4). Hook-associate protein 2 (*Vibrio parahaemolyticus*)

II. Y1- 90 KDa Band

1). Major vault protein (*Ictalurus punctatus*)
2). Major vault protein Isoform 5 (*Strongylcentrottus purpuratus*)
3). Unnamed protein product (*Tetraodon nigroviridis*)
4). Major vault protein (*Mytilus edulis*)
5). Hemocyanin type 2 (*Rapana thomasiana*)
6). Heat shock protein HSP 90 (*Danio rerio*)

While these results were disappointing in that Lv was not identified, we found out that the mass spectrometry method was viable for identifying proteins from queen conch. We continued our search for Lv by other methods.

We turned our attention to fractions Y3 and Y4. These fractions did not stain well by traditional Coomassie blue stains. This is a characteristic of phosvitin, an egg yolk protein derived from Lv that is rich in phosphoserine. We tried two new luminescent stains, Sypro Ruby stain (Invitrogen™), which is a very sensitive general protein stain equal or better than silver and Diamond Pro Q (Invitrogen™), a stain used to visualize phospho-proteins, which are often un-stainable by traditional means (Fig. 5.10). The basis for using the Diamond Pro Q is that Vg is likely to be highly phosphorylated, if it shares characteristics with fish Vtg. This technique may also be used to identify the phosvitins and phosvettes since they are also highly phosphorylated and do not stain by traditional means (*i.e.*, Coomassie).

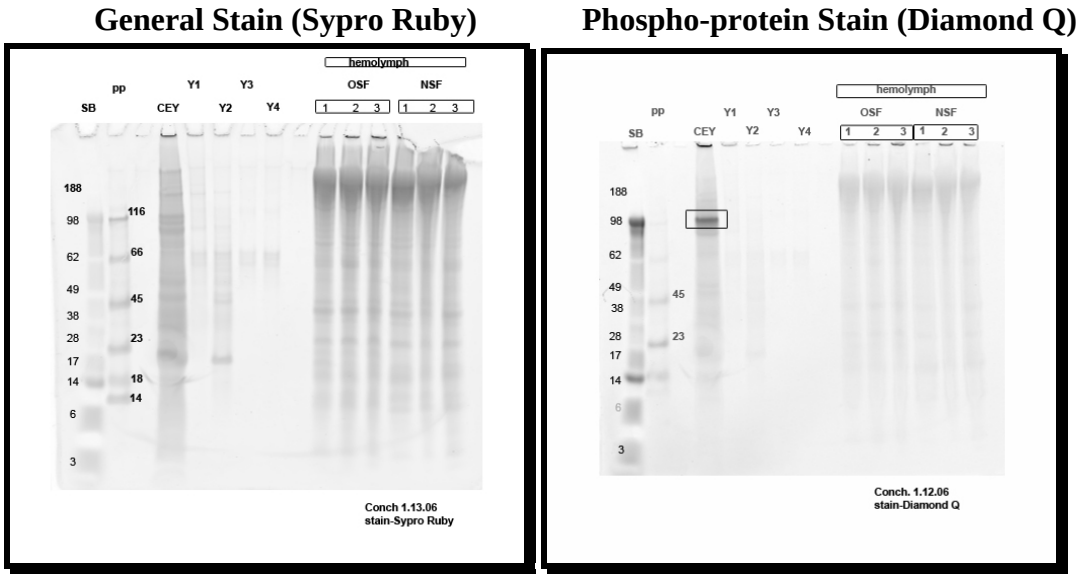

Figure 5.10. PAGE analysis of several protein fractions stained by either Sypro Ruby (a general protein stain) or Diamond Pro Q (a phosphoprotein specific stain). Lane 1, MW ladder (See Blue 2), Lane 2, MW ladder (Peppermint Stick), Lane 3, Crude egg yolk protein (CEY), Lanes 4-7, gel filtration purified fractions (Y1-Y4), Lanes 8-10, hemolymph from off-shore (OSF) female conch, and Lanes 11-12, hemolymph from near-shore (NSF) female conch.

The PAGE analysis showed one major phosphor-protein in conch crude egg yolk (CEY) at about 98 kDa (Fig. 5.10, box in right panel). Unfortunately, the purified egg yolk fractions did not seem to contain a stainable protein possibly because they were too dilute to see the same 98 Kda band by the phospho-protein stain. No differences were evident in hemolymph by the phospho-protein stain between the OSF and NSF samples. This finding supported other work indicating that Vg was not secreted into the hemolymph in conch. According to Barre *et al* (1991) in the garden snail, yolk proteins (4 large egg yolk proteins: 440, 140, 100, and 80 KDa) are produced in the digestive gland and transported to the gonad via hemolymph. Our data suggests that the queen conch egg yolk proteins are directly synthesized in the gonad.

Identification of queen conch Vg with antibodies

Vg and its egg yolk derivative, LV, are the most dominant phosphoproteins in their respective compartments for oviparous vertebrates. The appearance of the 98 KDa band with the phosphoprotein stain suggested that queen conch LV might also be phosphorylated (Fig. 5.10). It was possible that conch LV might share epitopes with Vtg's, so we also tested the CEY fraction by Western analysis using monoclonal antibodies developed against Vtg's from bird (3G6), swordfish (2D10), killifish/sheepshead minnow (5C9/5F8), sturgeon/gar (1H2/1G7), snapping turtle (8G9/8D7,5B12), and a pool of carp/striped bass Mabs (2D3/1C8/3G2) (all displayed in Fig. 5.11.) These monoclonal antibodies have wide cross reactivities with Vtg's from a number of different fish species and they are of high affinity. We hypothesized that they may also bind to the conch Vg. A protein band of the same MW as the one elucidated by the phosphoprotein stain was recognized by the monoclonal antibodies for fish Vtg. The strongest reaction was with antibody 5C9/5F8. This band is indicated by a dashed line.

Based on these data, we were confident that this 98 Kda band was the conch lipovitellin that was present in the egg yolk protein. To again check to see if Vg was present in hemolymph, we analyzed another set of hemolymph fractions by SDS-PAGE followed by Western blot using the 5C9/5F8 monoclonal antibody combination and we also used the phosphoprotein stain. This again proved negative.

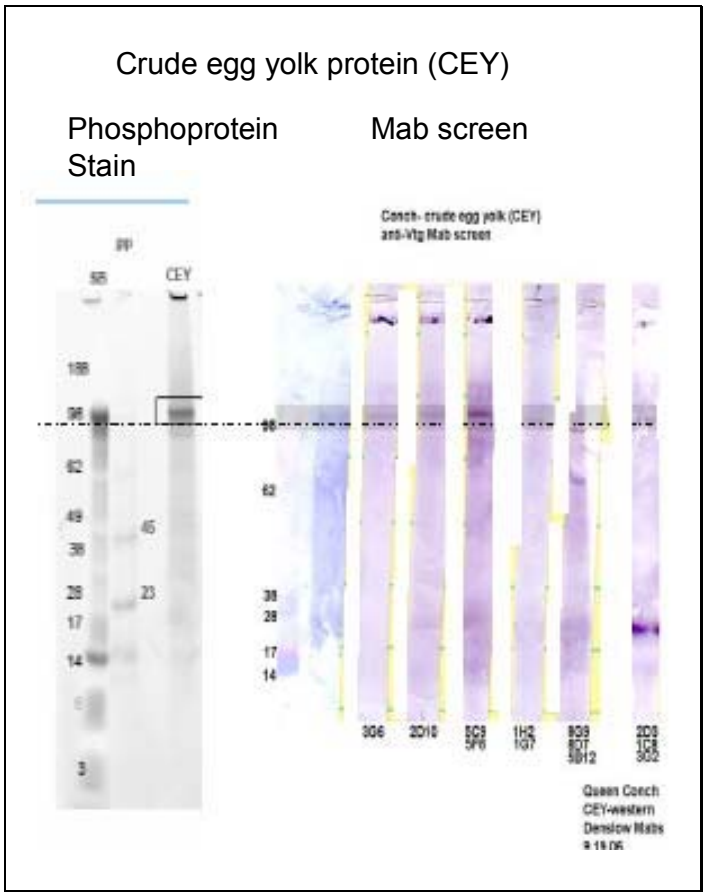

Figure 5.11. Identification of conch vitellin (ie lipovitellin) by phosphor-protein stain and western analysis using anti-fish-vitellogenin monoclonal antibodies. Proteins of crudely purifed conch egg yolk (CEY) was separated by SDS-PAGE and stained by Sypro (general stain), phosopho-proteins (Diamond ProQ), and by Western.

Conclusions from the Vtg isolation

Egg-laying vertebrates synthesize yolk in the liver, transfer the protein thru the blood, and absorb the protein by receptor mediated uptake into developing oocytes in the ovary. Insects and some invertebrates, which do not have a discrete livers, synthesize the yolk in the ovary itself; hence, egg yolk does not enter the blood or hemolymph. Our data suggests that this is the case of conch Vtg.

Female conch hemolymph does not appear to contain the egg-yolk precursor Vtg based on PAGE, phosphoprotein content, and western results. Protein phosphorus, an indirect identifier of

yolk protein was able to identify egg yolk lipovitellin, but did not stain any hemolymph proteins. In addition, western analysis using the anti-fish vitellogenin antibodies (5C9/5F8) that recognized conch egg yolk, did not detect proteins in the hemolymph. Based on these results, the anti-fish Vtg monoclonal antibody 5C9/5F8 will be useful to detect conch Vg directly in the gonad of affected females and it can be used to determine egg yolk protein synthesis and deposition through the reproductive cycle. However, because gonad is virtually non-existent in nearshore females and thus had limited utility in the context of this study, we decided not to produce a new antibody.

Heat Shock Proteins

To determine whether queen conch were impacted by thermal stress, female conch tissues were collected by FWC personnel on 15 and 23 March 2005 from nearshore (n=3) and offshore sites (n=3) for analysis of heat shock proteins (HSPs). Gels and western blots were performed on female conch hemolymph. Three different antibodies against heat shock proteins, HSP-60, HSP-70, and HSP-90, were tested against the conch hemolymph. Initially, the antibodies did not appear to react with conch tissues or with a positive control provided to us by another lab. However, by increasing the protein concentration in the gel well from 10 µg to 40 µg, and improving assay sensitivity using a biotinylated secondary antibody and strepavidin-alkaline phosphatase, it was possible to detect cross-reactivity of the antibodies with conch heat shock proteins (Figs. 5.12, 5.13). We developed a positive heat shock protein control by heating several fathead minnows to 95˚F for 1 week and testing the tissues against the heat shock antibodies. Fathead minnow liver appeared to be the most heat sensitive tissue and was used as a positive control. In addition, purified HSP proteins (Stressgen) were obtained to verify band identities. The banding patterns of heat shock proteins from nearshore versus offshore animals vary considerably, suggesting apparent differences.

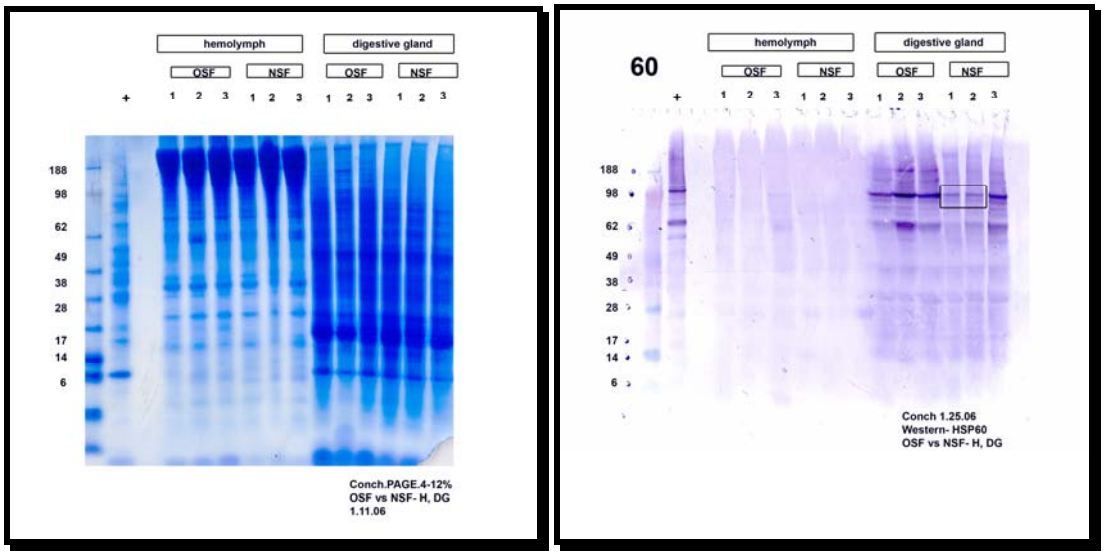

Figure 5.12. PAGE and heat shock protein western analysis of conch hemolymph and digestive gland homogenates from off-shore and near-shore females**.** Note- no gonads are present in near-shore conch. A positive control (heat treated fathead minnow liver) is also displayed (+). Boxes indicate areas of interest or differences between OSF and NSF samples.

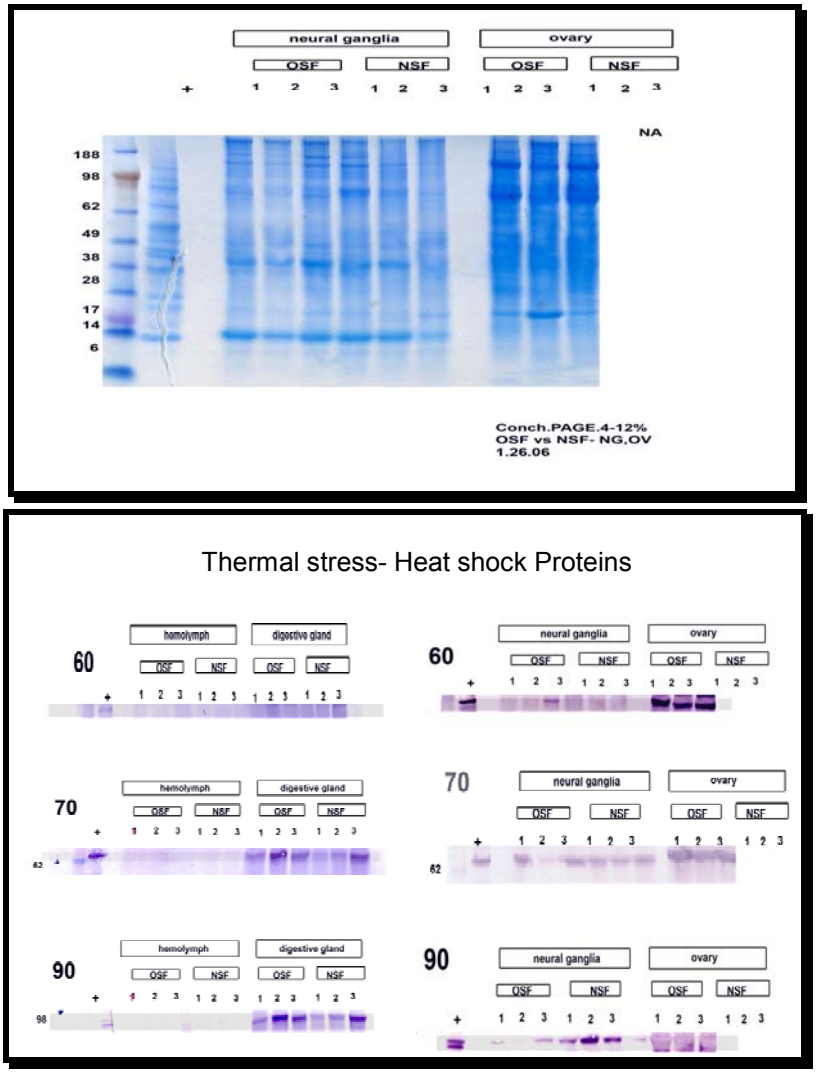

Figure 5.13. PAGE and heat shock protein western analysis of conch neural ganglia and ovary homogenates from off-shore and near-shore females**.** Note- no gonads are present in near-shore conch. A positive control (heat treated fathead minnow liver) is also displayed (+).

Apparently, hemolymph in conch contains no heat shock proteins. The digestive gland, neural ganglia, and ovary reacts with all three heat shock protein antibodies. The only trends that appear to be evident between the offshore and nearshore females: 1) there is a depression in HSP 60, 70, & 90 in the digestive glands of 2 of the 3 nearshore females indicated by boxes, and 2) HSP 60 & 90 appear to be in higher concentration in 2 of the NSF neural ganglia.

Another observation was that the heat shock protein antibodies seemed to recognize several different protein sizes. This may be a result of cross-reactivity to other heat shock proteins, multiple heat shock proteins in the conch, or degradation. The band ID was verified by using fathead minnow heat induced proteins as a standard (Fig. 5.13).

Oxidative and Organic Stress

Oxygen stress and exposure to organic pollutants are other factors that we investigated between offshore and nearshore conch**.** We tested antibodies against an oxidative biomarker-glutathione peroxidase and cytochrome P450 (CYP1A), on conch tissues by western analysis. If these antibodies cross-react with conch tissues, oxygen deprivation and/or exposures to dissolved organic pollutants may be contributing to conch reproductive impairment nearshore.

Conch hemolymph and digestive gland homogenates were analyzed for the presence of an oxygen-stress biomarker, glutathionine peroxidase (GPX, Fig. 5.14) and a biomarker for exposure to polyaromatic hydrocarbons, cytochrome P450 1A1 (Fig. 5.15) by western analysis (Stressgen). Both biomarkers recognized proteins in the conch digestive gland that are the same size as purified GPX and P450 standard (Stressgen). However, the offshore "normal" conch have overall higher levels than the nearshore females. This is the same pattern we found in the other biomarkers (heat, metals) and is contrary to what we would have expected.

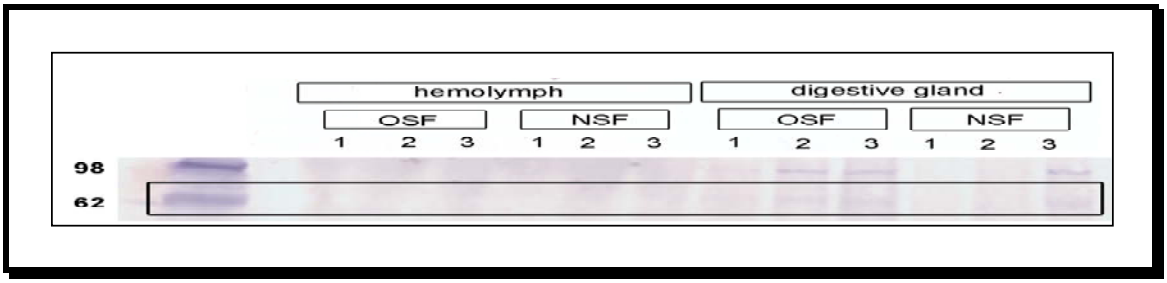

Figure 5.14. Western Analysis of conch hemolymph and digestive gland for the presence of an oxygen-stress biomarker, glutathionine peroxidase (GPX).

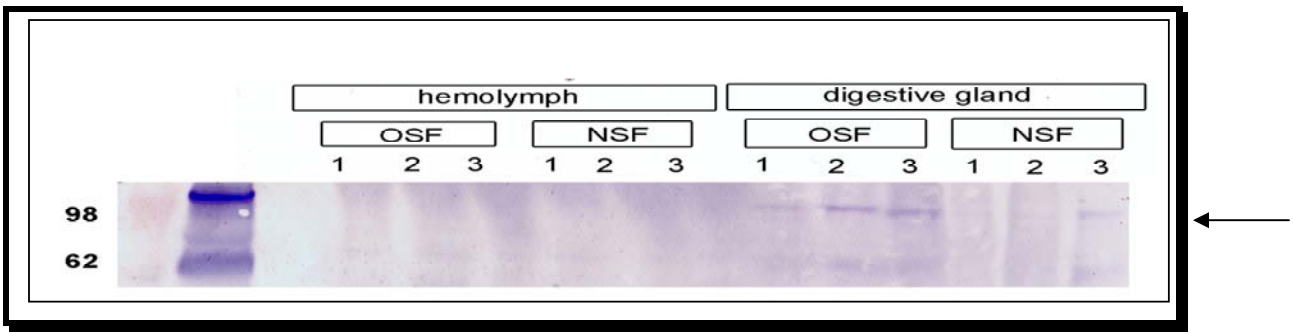

Figure 5.15. Western Analysis of conch hemolymph and digestive gland for the presence of exposure to organic contaminants, cytochrome P450 1A1.

Metal Stress

Conch hemolymph and digestive gland homogenates were analyzed for the presence of the metal-stress biomarker, metallothionine using Western blot from conch collected 9 June 2004 (Fig 5.16). It appears that the offshore conch have more of the metallothionine than the nearshore conch. Metallothionines are sensitive to metals and the response may be a result of the sum of all metals present.

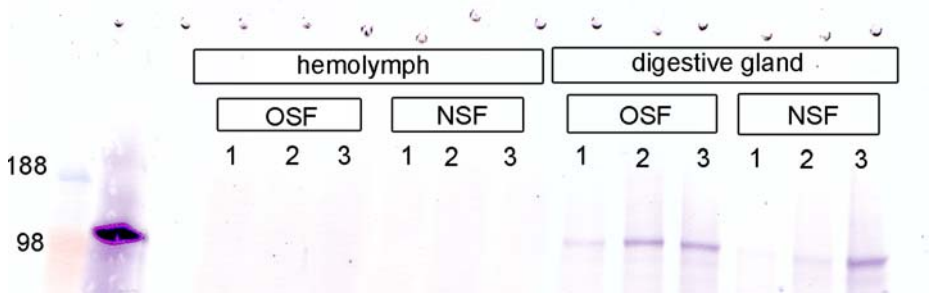

Figure 5.16. Western Blot analysis of conch hemolymph and digestive gland for the presence of exposure to metals using the biomarker metallothionine.

Chapter 6 – Histological Assessment of Queen Conch Tissues

Nancy Brown-Peterson
The University of Southern Mississippi
Institute of Marine Sciences
Gulf Coast Research Laboratory
703 East Beach Drive
Ocean Springs, MS 39564
nancy.brown-peterson@usm.edu

Histological Methods

Tissue from the middle of the gonad and the neuroganglia of each conch were removed and preserved in 10% neutral buffered formalin immediately upon collection and shipped to USM for processing. After 7 to 14 days in fixative, the tissue samples were be rinsed overnight in running tap water, dehydrated in a series of graded ethanols, and loaded into an automatic tissue processor (Shandon Hypercenter XP) for dehydration, clearing, and paraffin infiltration following standard histological techniques. Two 4 µm serial sections from each paraffin embedded tissue sample were mounted on glass slides and stained with hematoxylin and eosin. A detailed histological inspection of each gonadal sample was be made to assess the stage of maturity and the percentage of gametogenic tissue (relative to the entire gonad). Each animal was given a score from 1 to 8 to quantify gonadal maturity following Delgado et al. (2004), derived from a maturity scale developed by Egan (1985). Gonadal maturation classes are listed in Table 6.1. Conch are considered spawning capable histologically if oocytes are in oviducts or if spermatozoa are in sperm ducts. In addition, the percentage of gametogenic tissue was noted using the following index: <25%, 25-50%, 51-75%, and >75%. Neuroganglia sections were inspected for number and morphology of ganglia cells, amount of nerve fibers and the association of the nerve fibers with the ganglia cells.

Table 6.1. Queen conch gonadal maturation classes.

Code	Maturity Class
1	Early Developing
2	Developing
3	Mature
4	Spawning capable
5	Spent/Regressing
6	Atretic
7	Regenerating
8	No gonadal tissue

Histological analysis of tissues collected in 2004

Queen conch were collected from offshore spawning sites in June 2004 (N=6; 3 females, 3 males) during the peak of the reproductive season to asses both gonadal and neural ganglia tissue in reproductive conch. These collections provided a "normal baseline" for subsequent collections, particularly for neural ganglia tissue which had not been previously examined in queen conch. All males and females had abundant (>75%) gonadal tissue in histological sections (Table 6.2). While none of the sections from female conch contained oviducts and thus could not be classified as spawning capable, all females had large, fully grown vitellogenic oocytes in the ovary and were clearly capable of spawning (Fig. 6.1). All gonadal sections of males had sperm ducts containing spermatozoa, and the males were all spawning capable (Table 6.2; Fig. 6.2).

Table 6.2. Histological analysis of queen conch from June 2004. All numbers expressed as percentages.

	Offshore Female (N=3)	Offshore Male (N=3)
<25% gonadal tissue	0	0
25-50% gonadal tissue	0	0
50-75% gonadal tissue	0	0
>75% gonadal tissue	100	100
Early Developing	0	0
Developing	0	0
Mature	100	0
Spawning capable	0	100
Regenerating	0	0
Numerous ganglia cells	100	100
Reduced # ganglia cells	0	0
Ganglia cell morphology normal	100	100
Hypertrophy of ganglia cells	0	0
Excessive nerve fibers	0	0
Ganglia cells without nerve fibers	0	0

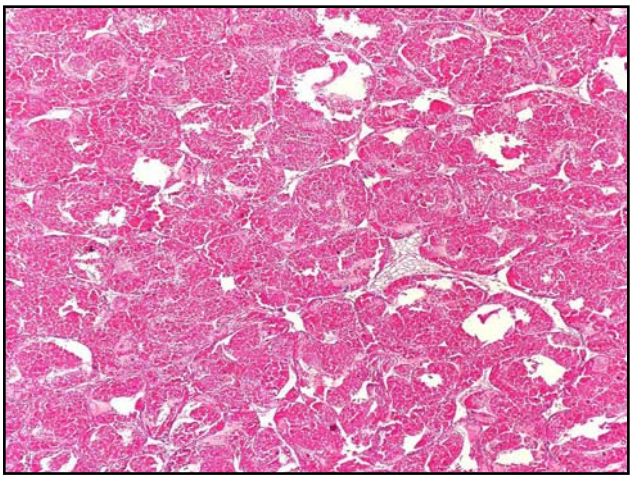

Figure 6-1. Ovarian tissue from an offshore female queen conch during the reproductive season. 40x magnification.

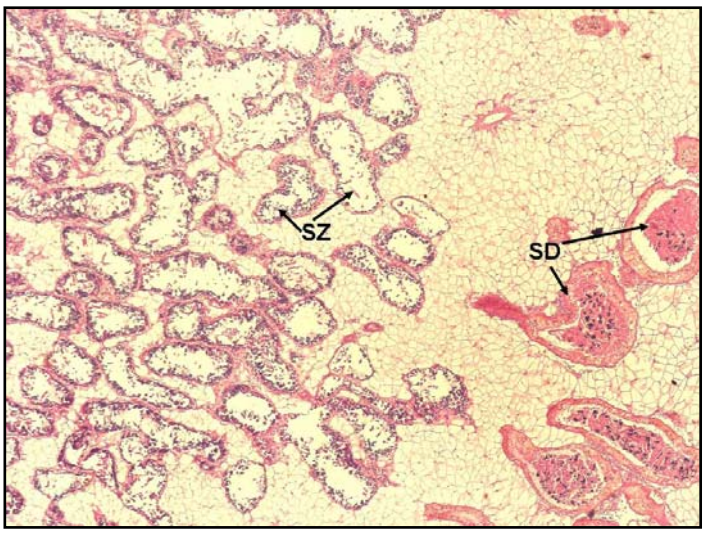

Figure 6-2. Testicular tissue from an offshore male queen conch during the reproductive season season. SD—sperm duct; SZ—spermatozoa. 40x mag

Neural ganglia tissue of offshore conch of both sexes appeared healthy, with large areas of ganglia cells exhibiting normal morphology (Fig. 6.3). Nerve fibers were associated with ganglia cells, but there was no proliferation of nerve fibers in any samples. These histological sections were considered to be a "normal, healthy baseline" example of neural ganglia tissue.

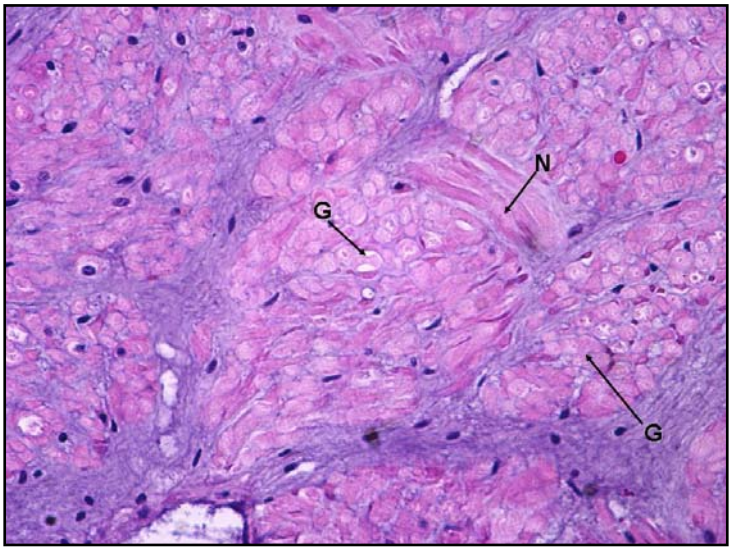

Figure 6.3. Neural ganglia tissue from an offshore female queen conch. G—ganglia cells; N—nerve fibers. 400x magnification.

Histological analysis of tissues collected in 2005

Queen conch from offshore locations were collected in March 2005 (N=5; 3 female, 2 male), at the start of the conch reproductive season to see if there were any differences in gonadal and neural ganglia tissue at the beginning of the season (2005 collections) compared with the peak portion of the season (2004 collections). Additionally, tissues were collected from near shore females (N=3) to evaluate differences in gonadal and neural ganglia tissue between nearshore and offshore female conch.

All offshore males and females had fully developed gonads and appeared capable of spawning (Table 6.3), similar to the June 2004 samples (see Figs. 6.1 and 6.2). Additionally, the neural ganglia tissue of the offshore conch showed healthy ganglia cells and normal morphology (Table 6.3), and appeared identical to the "baseline" ganglia histology from offshore samples in June 2004 (see Fig. 6.3). However, female conch collected from nearshore locations in March had noticeably abnormal neural ganglia histology (Fig. 6.4), with a greatly reduced number of ganglia cells that occurred individually rather than in clumps. Some ganglia cells appeared hypertrophied, and there was proliferation of nerve fibers in the sections, which was often not associated with ganglia cells (Table 6.3). The gonadal tissue of the nearshore females was similarly abnormal (see Fig. 6.5 for example). There was <20% ovarian tissue in the sections, and no evidence of gonadal recrudescence, with only primary oocytes present in greatly reduced numbers (Table 6.3). There was little indication from histological analysis that the ovaries of the nearshore conch would become capable of spawning during the reproductive season.

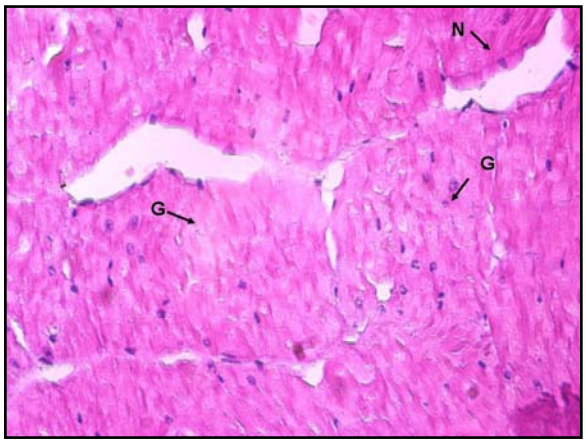

Figure 6.4. Neural ganglia tissue from a nearshore female queen conch in March. G—ganglia cells. N—nerve fiber. 400x magnification.

Table 6.3. Histological analysis of queen conch from March 2005. All numbers expressed as percentages.

	Offshore Female (N=3)	Nearshore Female (N=3)	Offshore Male (N=2)
<25% gonadal tissue	0	100	0
25-50% gonadal tissue	0	0	0
50-75% gonadal tissue	0	0	0
>75% gonadal tissue	100	0	100
Early Developing	0		0
Developing	0	0	0
Mature	100	0	
Spawning capable	0	0	100
Regenerating	0	67	0
No gonadal tissue	0	33	0
Numerous ganglia cells	100	0	100
Reduced # ganglia cells	0	100	0
Ganglia cell morphology normal	100	0	100
Hypertrophy of ganglia cells	0	33	0
Excessive nerve fibers	0	100	0
Ganglia cells without nerve fibers	0	67	0

Histological analysis of tissues collected in 2007

Collections in 2007 were designed to answer three questions raised from collections in the previous years:
(1) Is there a difference in gonadal development in male and female conch prior to the beginning of the reproductive season (February) and during the peak reproductive season (June)?
(2) Are there differences in gonadal tissues of male and female conch collected from offshore and nearshore locations during February and June?
(3) Are there differences in neural ganglia tissue of male and female conch collected from offshore and nearshore locations during February and June?

Gonadal Tissues

Gonadal tissues were histologically analyzed from 16 conch collected in February 2007 (Table 6.4) and 12 conch collected in June 2007 (Table 6.5). During February, all female conch collected from the offshore location (N=4) had large amounts of gonadal tissue (>85% of gonadal area in all cases), and all conch had numerous vitellogenic oocytes in the ovary (see Fig. 6.1). Additionally, all conch had developing oocytes in the cortical alveolar stage in the ovaries. No oocytes were observed in the oviducts in February, suggesting that although the conch had fully developed ovaries, they were not yet in spawning condition. Male conch collected from offshore locations in February (N=4) also exhibited large amounts of testicular tissue (>80%), and all males were undergoing active spermatogenesis and contained spermatozoa in the testis (see Fig. 6.2). Additionally, 75% of the males had spermatozoa in the sperm ducts, indicating they were capable of spawning.

The gonadal condition of conch collected from nearshore locations in February contrasted sharply with their offshore counterparts. Nearshore females (N=4, Table 6.4) all had greatly reduced amounts of ovarian tissue (<20% in all cases) and none had vitellogenic oocytes. Two of the females (50%) were beginning to undergo ovarian recrudescence as indicated by the presence of cortical alveolar oocytes in the ovaries (Fig. 6.5); the other 2 females had only primary oocytes and were considered regressed. It is likely that none of the female conch would have attained spawning condition within the next 1-2 months. Nearshore males collected in February (N=4) had more testicular tissue than the females (25-75%, Table 6.4), but reduced amounts of tissue compared to the offshore counterparts. However, all males were undergoing active spermatogenesis and contained spermatozoa in the testis, although, in small amounts. Two of the males (50%) had small amounts of spermatozoa in sperm ducts and are considered capable of spawning.

Table 6.4. Gonadal condition of queen conch from February 2007. All numbers expressed as percentages.

	Offshore Female (N=4)	Nearshore Female (N=4)	Offshore Male (N=4)	Nearshore Male (N=4)
<25% tissue	0	100	0	0
25-50% tissue	0	0	0	50
50-75% tissue	0	0	0	50
>75% tissue	100	0	100	0
Early Developing	0	50	0	0
Developing	0	0	0	25
Mature	100	0	25	25
Spawning capable	0	0	75	50
Regenerating	0	50	0	0

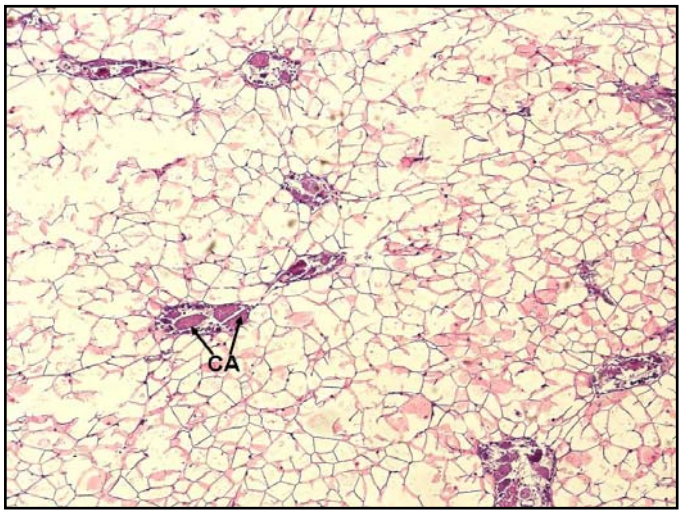

Figure 6.5. Ovarian tissue of female queen conch collected nearshore in February. Note reduced amount of tissue and lack of vitellogenic oocytes. This individual was identified as an imposex due to the presence of a small penis and ovarian gonadal tissue. CA—cortical alveolar oocytes. 100x magnification.

One conch collected from the nearshore location during February and identified macroscopically as a male based on the presence of a small penis was found to be a female upon histological inspection. Ovarian recrudescence was occurring in this conch (Fig. 6.5), and there was no indication of testicular tissue. This conch has been tentatively identified as imposex, based on the occurrence of female gonadal tissue, but having external male sexual characteristics.

All female conch collected during June from offshore locations (N=4, Table 6.5) had well developed gonads with many vitellogenic oocytes and appeared similar to conch collected in February, although one female had a slightly reduced amount of ovarian tissue (60%). Oviducts were only present in the histological sections of two of the females; one of these contained oocytes and is considered to be in spawning condition. All males collected from offshore locations in June (N=2) had large amounts of testicular tissue (>80%), were undergoing active spermatogenesis. All had spermatozoa in the sperm ducts and were considered capable of spawning.

Table 6.5. Gonadal condition of queen conch from June 2007. All numbers expressed as percentages.

	Offshore Female (N=4)	Nearshore Female (N=1)	Offshore Male (N=2)	Nearshore Male (N=5)
<25% tissue	0	0	0	80
25-50% tissue	0	100	0	20
50-75% tissue	25	0	0	0
>75% tissue	75	0	100	0
Early Developing	0	0	0	20
Developing	0	100	0	0
Mature	75	0	0	40
Spawning capable	25	0	100	0
Regenerating	0	0	0	20
No gonadal tissue	0	0	0	20

Only one female was collected from the nearshore location during June. This conch had reduced amounts of gonadal tissue (30%) compared to the offshore June females, but more gonadal tissue than the near shore February females. This conch had some small vitellogenic oocytes in the ovary and is considered to be developing (Table 6.5); it is unclear if this conch would have been able to spawn later in the reproductive season. In contrast, the male conch collected nearshore during June (N=5) were less reproductively active than those collected nearshore in February. The majority (80%) had greatly reduced amounts of gonadal tissue (<25%), and 40% of the males exhibited no spermatogenesis (Fig. 6.6). Of the 60% undergoing active spermatogenesis, none were considered to be capable of spawning due to the small amounts of spermatozoa present in the testis.

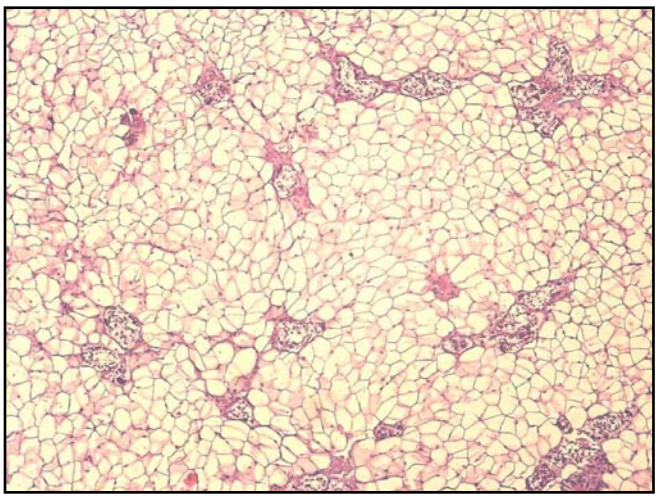

Figure 6-6. Testicular tissue of a male queen conch collected nearshore in June. Note reduced amount of tissue and lack of spermatogenesis. This conch was macroscopically identified as a female due to lack of a penis. 100x magnification.

One conch collected nearshore in June macroscopically identified as a female due to the presence of an oviduct had testicular tissue upon histological inspection, although there was no active spermatogenesis occurring and only a few spermatogonia were present (Fig. 6.6). However, there was no evidence of ovarian tissue in the gonad of this conch. We therefore tentatively identified as this conch as imposex as well.

Neural ganglia tissues

Neural ganglia tissues were histologically analyzed from 16 conch collected in February 2007 (Table 6.6) and 13 conch collected in June 2007 (Table 6.5). The neural ganglia tissue of all males and females captured at offshore locations during February (N=4 each sex) appeared healthy (see Fig. 6.3 for example). There were numerous ganglia cells with normal morphology, and good connections between ganglia cells and associated nerve fibers. In contrast, ganglia tissue of females from near shore locations in February (N=4) showed a reduced number of ganglia cells in the majority of the samples (75%), evidence of hypertrophy of ganglia cells (25% of samples) and large amounts of nerve fibers not closely associated with ganglia cells (75%). These tissues appeared similar to the March 2005 near shore neural ganglia tissue (see Fig. 6.4) Ganglia tissue from near shore males in February (N=4) was more similar to the offshore males, with only 25% of samples exhibiting a reduced number of ganglia cells, hypertrophy of ganglia cells or unassociated nerve fibers (Table 6.6).

Table 6.6. Neural ganglia condition of queen conch from February 2007. All numbers expressed as percentages.

	Offshore Female (N=4)	Nearshore Female (N=4)	Offshore Male (N=4)	Nearshore Male (N=4)
Numerous ganglia cells	100	25	100	75
Reduced # ganglia cells	0	75	0	25
Ganglia cell morphology normal	100	75	100	75
Hypertrophy of ganglia cells	0	25	0	25
Excessive nerve fibers	0	25	0	25
Ganglia cells without nerve fibers	0	75	0	25

Ganglia tissue from males and females captured offshore in June (N=4, both sexes) was similar to that observed in February, with numerous ganglia cells and normal morphology (Table 6.7). Only one female was captured from the nearshore location in June; while this conch had a numerous ganglia cells, many exhibited hypertrophy or appeared to be undergoing apoptosis (debris in cell). The majority of nearshore males sampled in June (N=5) showed a reduced number of ganglia cells and an excessive amount of nerve fiber, often not associated with ganglia cells (see Fig. 6.4). Furthermore, 60% of the male samples exhibited ganglia cell hypertrophy and it appeared many ganglia cells were beginning to undergo apoptosis.

Table 6.7. Neural ganglia condition of queen conch from June 2007. All numbers expressed as percentages.

	Offshore Female (N=5)	Nearshore Female (N=1)	Offshore Male (N=2)	Nearshore Male (N=5)
Numerous ganglia cells	80	100	100	40
Reduced # ganglia cells	20	0	0	60
Ganglia cell morphology normal	100	0	100	0
Hypertrophy of ganglia cells	0	100	0	60
Apoptosis/debris in ganglia cells	0	100	0	40
Excessive nerve fibers	0	0	0	60
Ganglia cells without nerve fibers	0	0	0	40

Conclusions from histological analysis

Collection of queen conch gonadal tissues during 2004, 2005, and 2007 clearly demonstrate that conch collected from offshore locations in the Florida Keys are undergoing active oogenesis and spermatogenesis from February through June, and are capable of spawning. Furthermore, collections during 2005 and 2007 show that conch collected from nearshore areas in the Florida Keys have a greatly reduced amount of gonadal tissue. Females show delayed or no gonadal development from February through June. It is unlikely these conch would have become reproductively capable. These results support previous observations (McCarthy et al. 2002, Delgado et al. 2004) documenting that nearshore female conch are non reproductive. In contrast, males in February showed evidence of spawning capability, although their reproductive contribution would be much less than offshore conch once again supporting previous observations (Delgado et al. 2004). Males collected nearshore in June showed less testicular tissue and spermatogenesis than those collected in February, suggesting spawning would be unlikely even if a ripe female was present. Thus, nearshore females show extreme developmental delay over the course of the reproductive season, while males appear to begin normal testicular development in the spring, but are unable to sustain it through June.

Chapter 7 - Analysis of Queen Conch Neuropeptide Expression

Patricia McClellan-Green, Ph.D.
North Carolina State University
Department of Environmental and Molecular Toxicology
Center for Marine Sciences and Technology
303 College Circle
Morehead City, NC 28557

In mollusks, sexual development and reproduction is largely controlled by the action of peptide neurohormones as opposed to steroid hormones (Köhler *et al*., 2007). Peptide neurohormones that are released by the cerebral, pleural and abdominal ganglia are responsible for many physiological and reproductive functions in gastropods, including sexual differentiation, gonadal development, reproduction and mating. It is apparent that outside factors can influence reproduction and mating, and it has been suggested that activating and inhibiting factors could regulate neurohormone release (Nishikawa *et al*., 2004). This would be analogous to the regulation of pituitary hormones by hypothalamus releasing or inhibiting factors in vertebrates.

The overall goals of this project were to elucidate the role of neurohormonal factors in the reproductive failure of nearshore queen conch and determine what impact changing environmental factors play in their regulation and activity. To accomplish this we must first determine whether known gastropod neuropeptides involved in gastropod mating are expressed in queen conch. All photomicrographs from this section are found in Appendix III.

Quantification of egg laying hormone (ELH) and APGWamide.

Briefly, whole tissue homogenates were prepared in TBS (25 mM Tris-HCl pH 7.4, 150 mM NaCl, plus broad-range protease inhibitors) and stored at -80°C. The wells of a 96 well plate was coated with 100 µg total homogenate protein and allowed to adhere overnight at 4°C. The well were washed 3X with TBS + 0.05% Tween-20 (TBST). The wells were emptied and blocked with 5% non-fat dry milk in TBS for one hour at room temperature (RT). The wells were next washed three times with TBST and once with TBS. The samples were then incubated with a 1:5000 (ELH) or a 1:1000 (APGWamide) dilution of primary antibody in TBST for 2 hours. The wells were washed as previously described and then incubated with a 1:5000 dilution of secondary antibody (donkey anti-rabbit IgG whole or goat anti-mouse IgG) for two hours. The wells were washed as previously described and visualization occurred using 1-STEP™ pNPP (Pierce, Rockford, IL). The plate was read at 405 nm and levels of the expressed peptides are determined using standard curves prepared with purified ELH or APGWamide neuropeptides (Malik and Daymon, 1982, McClellan-Green personal observation). Levels of specific peptides were standardized using total protein concentration (µg ELH or APGWamide/mg total protein).

Immunohistochemistry of ELH and APGWamide

In preparation for immunohistochemistry sections of snails were placed in 10% buffered formalin overnight and then embedded in paraffin. Sections from paraffin blocks were cut (in duplicate) into 7um segments and allowed to dry on warming block overnight by Dr. Nancy Brown-Petersen at USM. Slides were then stained using anti-APGWamide polyclonal antibody or anti-ELH polyclonal antibody. Immunohistochemical (IHC) staining followed basic protocols with some alterations. IHC slides were incubated in xylene substitute (CitriSolve™) 2X for 10 minutes, then 100% EtOH 2X for 2 minutes, followed by 3% hydrogen peroxide for 10 minutes. The slides were then blocked with 5% rabbit serum for 5 hours and rinsed 3X in 1X phosphate buffered saline (PBS). Next the slides were incubated in the primary antibody (polyclonal rabbit anti-APGWamide, 1:100 or polyclonal rabbit anti-ELH, 1:1000) for 4 days then rinsed 4X in 1X PBS. The slides were next incubated overnight in the secondary antibody (Alexa Fluor® 488 goat anti-rabbit IgG (H+L) at a 1:2000 dilution) followed by rinsing 4X in 1X PBS. Antibody recognition was detected via visualization of fluorescence using a FITC filter.

Fluorescent microscopy was carried out using a Zeiss fluorescent microscope at the Duke University Marine Laboratory. Images were captured with a Canon PowerShot A650 IS camera temporarily mounted on the scope. Three slides from each animal were obtained from Nancy Brown-Petersen at The University of Southern Mississippi after histological interpretation. One slide of each set was stained using anti-APGWamide and one slide was stained using anti-ELH. The third replicate is reserved to confirm results from either the APGWamide or ELH staining.

Egg-Laying Hormone

The concentration of egg laying hormone was measured using an ELISA developed for the mud snail and the sea hare, *Aplysia*. ELH was measured in whole tissue homogenates by ELISA in a 96-well plate format. The wells of the plate were coated with 100 μg total homogenate protein. After blocking with 5% non-fat dry milk, the samples were incubated with a 1:5000 dilution of primary antibody and then with a 1:5000 dilution of secondary antibody (donkey anti-rabbit IgG whole or goat anti-mouse IgG). Visualization was through pNPP at 405 nm and levels of the expressed peptides will be determined using standard curves prepared with purified ELH or APGWamide neuropeptides. The assay cross-reacts well with male (M) and female (F) conch digestive gland (L), ovary (O), and testis (T) as displayed in Fig. 7.1.

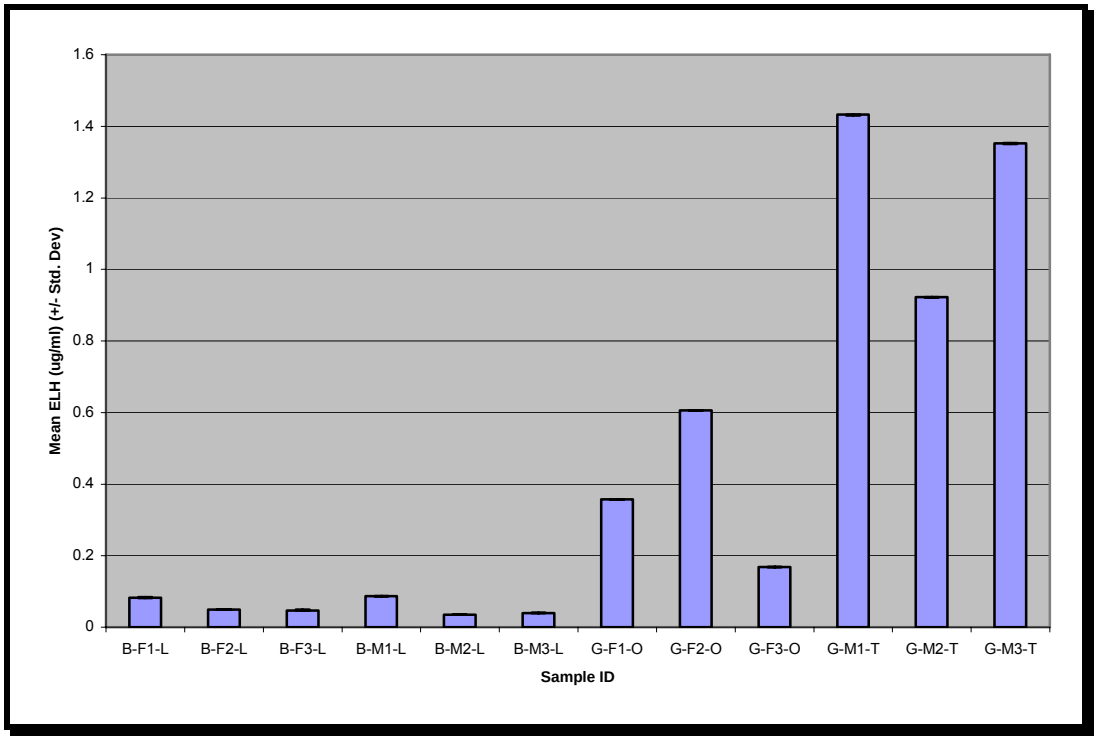

Figure 7.1. Egg laying hormone concentration in queen conch tissues. Three wild, reproductive offshore male and female conch captured on 6/8/04 were used for the validation of this assay.

Conch tissues (hemolymph, digestive gland, gonad, & neural ganglia) from the same offshore and nearshore males and females used in this study were examined for egg-laying hormone (ELH). The data presented in Figs. 7.2, 7.3, and 7.4 show that the nearshore females have much lower ELH levels than the offshore females. Keep in mind that no gonadal tissue was available in near-shore conch. The neural ganglia were available for near and offshore conch and this looks like a promising tissue for analysis.

The relationship between nearshore and offshore conch is less clear in the hemolymph (Fig. 7.5). The nearshore females appear to have higher ELH concentrations than the offshore females.

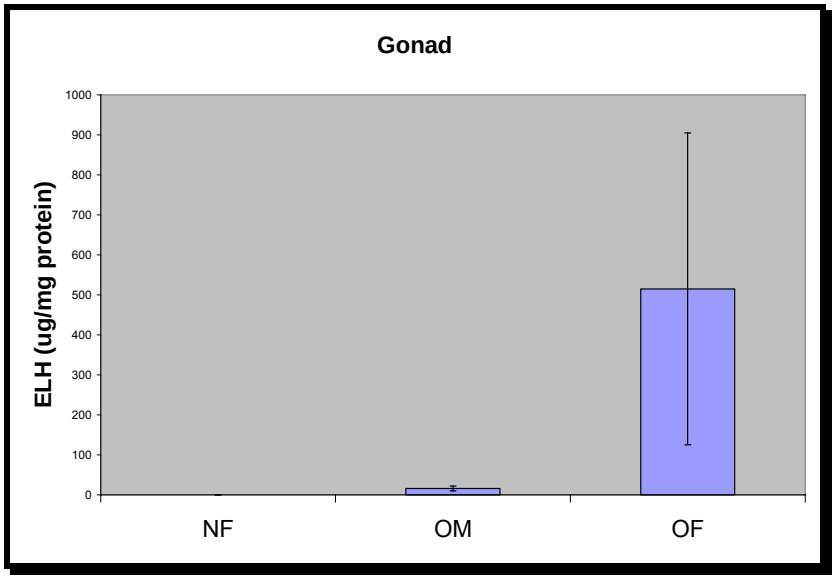

Figure 7.2. Quantification of egg-laying-hormone (ELH) in the gonads of queen conch collected from nearshore and offshore on March 15, 2005. Data are presented as the mean (µg peptide/mg total protein) ± one standard error. NF represents nearshore females; OM represents offshore males; OF represents offshore females.

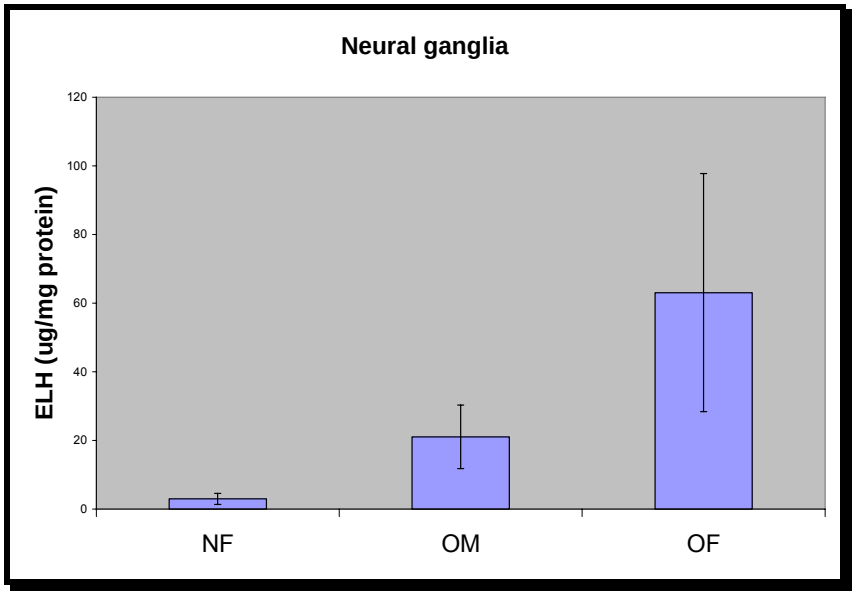

Figure 7.3. Quantification of egg-laying-hormone (ELH) in the neural ganglia of queen conch collected from nearshore and offshore on March 15, 2005. Data are presented as the mean (µg peptide/mg total protein) ± one standard error. NF represents nearshore females; OM represents offshore males; OF represents offshore females.

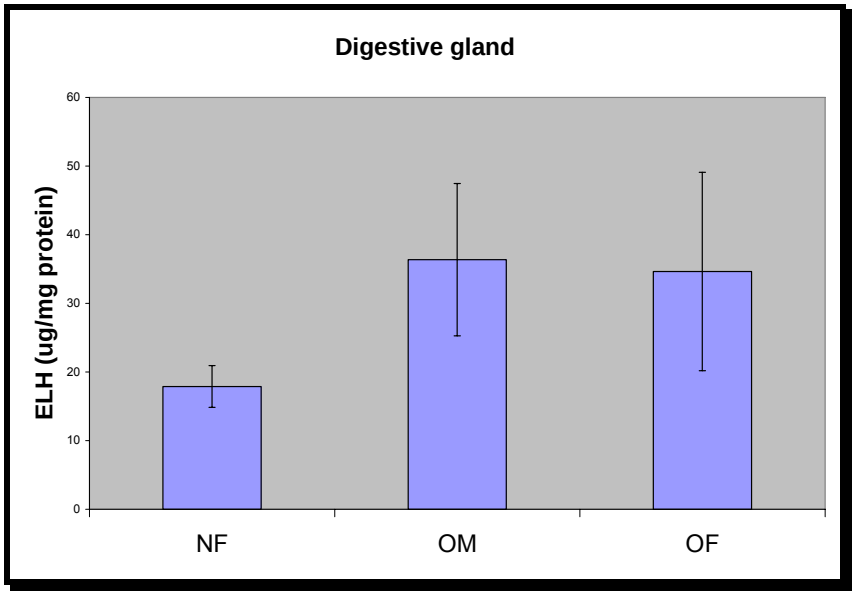

Figure 7.4. Quantification of egg-laying-hormone (ELH) in the digestive gland of queen conch collected from nearshore and offshore on March 15, 2005. Data are presented as the mean (µg peptide/mg total protein) ± one standard error. NF represents nearshore females; OM represents offshore males; OF represents offshore females.

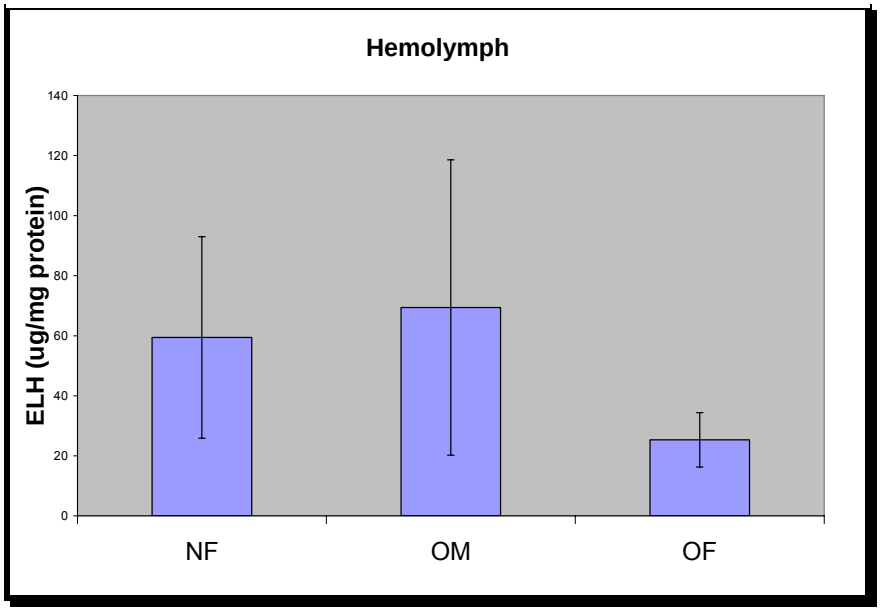

Figure 7.5. Quantification of egg-laying-hormone (ELH) in the hemolymph of queen conch collected from nearshore and offshore on March 15, 2005. Data are presented as the mean (µg peptide/mg total protein) ± one standard error. NF represents nearshore females; OM represents offshore males; OF represents offshore females.

Immunohistochemistry Conclusions

The following conclusions can be reached from the data on APGWamide and ELH expression in queen conch tissues:

1. Both peptides are expressed in the tissues of female and male queen conch.
2. The highest levels of expression for APGWamide and ELH occur in the gonad and neuroganglia.
3. Although no ELISA assays were used for quantification, the offshore female appears to express higher levels of ELH (general observation OF > NM > OM $\underset{\sim}{}$ NF) and the offshore male had higher levels of APGWamide (general observation OM >> OF > NM $\underset{\sim}{}$ NF).
4. The gonad tissue of nearshore males and females was disorganized and appeared "eroded" compared to the gonads from offshore animals. It appeared that the gonads began their seasonal differentiation into male or female structures but that the process was interrupted and differentiation was non-gender specific.

Chapter 8 – Applying Genomics to Queen Conch Ecotoxicology

Nancy Denslow, Iris Knoebl, and Daniel Spade
University of Florida
Center for Environmental & Human Toxicology
Dept of Physiological Sciences
Bld 471-Room 52, 471 Mowry Rd
Gainesville, FL 32611
ndenslow@ufl.edu

Introduction

The genomic component of this study was beyond the original scope of the project. However, we were able to leverage the results of these studies to obtain additional funding from the State of Florida to incorporate genomics into the overall program.

The genomic component is divided into 8 phases: 1) purify RNA, 2) make cDNA from the purified RNA, 3) develop a cDNA normalized library (library development), 4) sequence the normalized library via a massive parallel sequencer from Roche (454 sequencer), 5) assemble the sequences into genes and identify the genes by comparison to the existing gene databases, 6) design oligonucleotides (60'mers) from selected genes to put onto the chip, 7) test the chip, and 8) compare nearshore vs. offshore conch tissues using the arrays. Many of these objectives were accomplished and aspects of the toxicogenomic research remain ongoing.

cDNA Library Preparation (Phases 1-2)

Total RNA was isolated from 12 individual conch tissue samples using the Trizol kit following the manufacturer's protocols. The samples were taken from male and female, nearshore and offshore, and three tissue types (neural ganglia, gonad, viscera) for the 12 samples. RNA concentration and integrity were assessed with the ND-1000 Nanodrop and Agilent 2100 Bioanalyzer instruments. Equal masses of RNA (200 ng / sample) were pooled together from each of the tissues, and used as the template for the cDNA library construction. The library was constructed using the SMART cDNA synthesis kit from Clontech. A total of 650 ug of pooled RNA was used as the template for 1st strand synthesis.

Following 1st strand synthesis, the sample was diluted with TE buffer, and 4 ul was used as template for LD-PCR. PCR cycle number was optimized to produce an amplicon pool that was amplified, but not overcycled. For this library, 15 cycles were determined to be appropriate.

Three 100 ul PCR reactions were performed to generate sufficient cDNA mass to continue. The reactions were purified using a Qiagen PCR purification kit, precipitated with NaOAc, and resuspended to a final concentration of 205 ng/ul. This represented the total cDNA library used for normalization.

Library normalization (Phase 3)

Library amplification was performed using the Trimmer kit from Evrogen, following the manufacturer's protocols; 1000 ng of amplified cDNA library was used in the reaction. Transcripts were heat denatured, then allowed to rehybridize at 68 ^{o}C for 5 hours. The hybridized cDNA was split into 4 pools, and digested with a series of dilutions of the provided DSN (Double Stranded Nuclease) enzyme.

A non-digested control was used to determine the optimal PCR cycle number (Fig. 8.1). For this amplification, the optimal cycle number was determined to be 10.

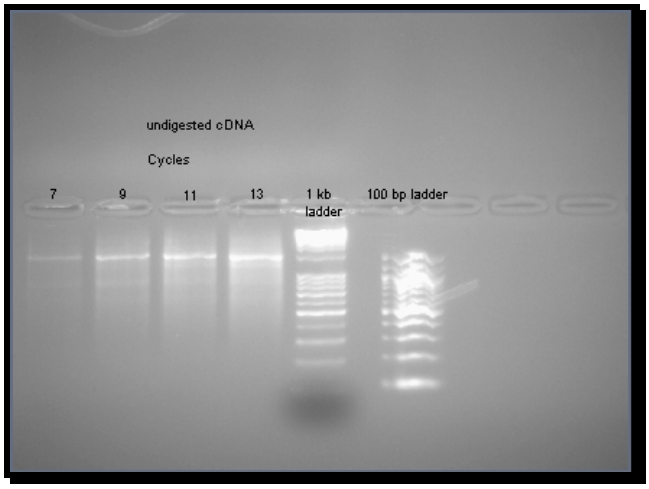

Figure 8.1. Undigested control cDNA test run to determine optimal amplification cycle (10).

The digested cDNA pools were amplified to 10 cycles, and analyzed via agarose gel electrophoresis to determine the appropriate dilution of DSN (Fig. 8.2.)

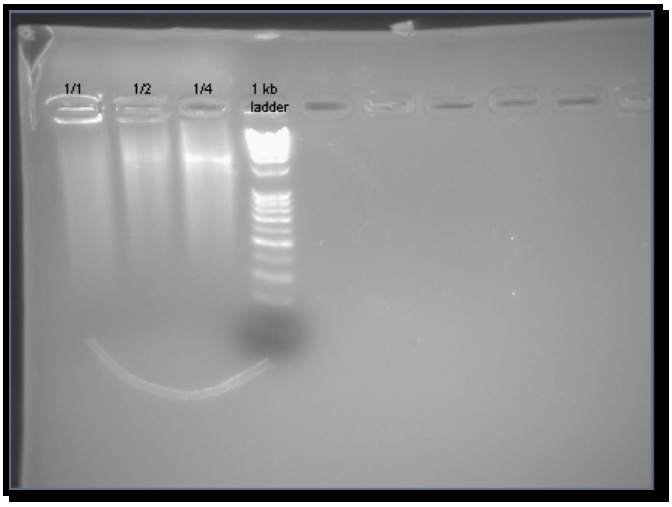

Figure 8.2. Determination of the optimal enzyme dilution which, in this case, was 1:1.

The PCR results indicated that the undiluted (1:1) enzyme provided the best normalization results. This fraction was diluted 1:10 with water, and 2 ul were used as template in three secondary PCR reactions (12 cycles) used to generate sufficient normalized cDNA mass. The secondary amplification reactions were purified, precipitated, and resuspended at a final concentration of 190 ng/ul, for a total of >11.5 ug of normalized cDNA for sequencing on the 454 massively parallel sequencer.

DNA Sequencing on the 454 massively parallel sequencer (Phase 4)

The University of Florida DNA sequencing core operates a Roche 454 FLX massively parallel DNA sequencer that is able to sequence over three hundred thousand DNA fragments in a single four-hour run, reducing the time and cost of obtaining nucleotide sequence. Each DNA fragment generates an average sequence of 200-250 nucleotides which are then assembled into larger overlapping sequences using specialized software. The DNA sequencing core at UFL has experience obtaining sequence from non-model systems, and can obtain sequences from species such as conch. This new sequencer exhibits a quantum leap in sequencing speed and depth, giving not only >20 Mbases of DNA sequence in just a few days, but also providing high quality sequences to give much greater transcriptome coverage at lower cost than traditional Sanger sequencing.

Development of a Queen Conch Library of Expressed Sequence Tags (ESTs) (Phases 5-6)

We have developed a cDNA microarray for queen conch containing approximately 7,740 well-annotated genes to evaluate stress pathways. These sequences were obtained from a normalized cDNA library made from a combination of mRNA from neuroganglia, gonad, digestive gland and muscle. We performed two runs on the instrument and obtained over 81,404 discrete sequences from which we were able to assemble 27,723 contiguous sequences (contigs) and identify 7,740 unique genes BLASTed against the NR and NT libraries with an e-value cutoff of 1x10-4. These 7,740 annotated genes were used to develop an oligonucleotide 8 X 15K microarray by Agilent. Most of the genes that were identified were associated with metabolism; however, there are numerous genes that control various other cellular processes (Fig. 8.3).

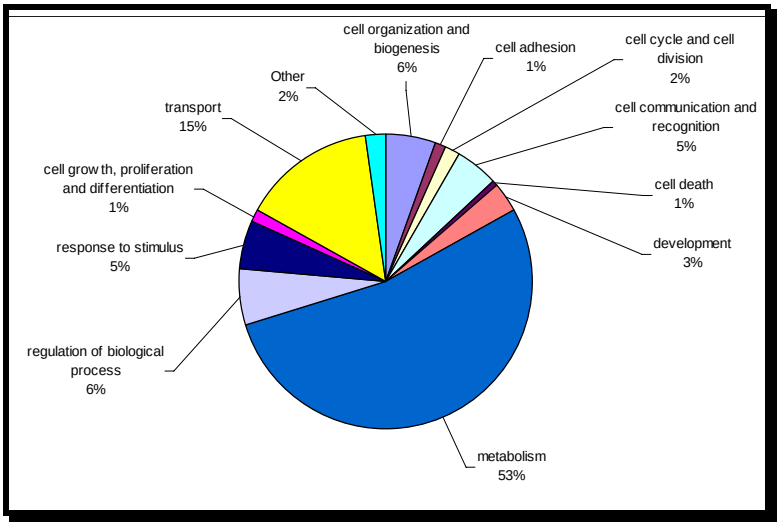

Figure 8.3. Distribution of gene ontology for queen conch genes developed in the sequencing. By far the most genes that were identified were related to metabolism; however, there are genes that regulate a wide variety of processes, many of which may be used to determine how stressors impact physiological processes.

Validation of Microarray and Transcriptomic Comparison of Near shore and Offshore Conch (Phases 7-8)

We used the microarrays with a small sample (n=3) of testis collected from queen conch from offshore and nearshore locations. RNA was extracted from testis samples using the RNA Stat-60 method. We used the one-color Agilent labeling system. Scanning and feature extraction was done using the Agilent scanner. JMP genomics was used to Loess-normalize data and compare expression patterns for the samples (Fig. 8.4). The data for each array were analyzed by one-way ANOVA, using a false discovery rate (FDR) of 0.05 to correct for multiple comparisons. Significantly regulated genes at $p<0.05$ and $p<0.01$ are discussed further. Pathway analysis was conducted in Ariadne Genomics PathwayStudio.

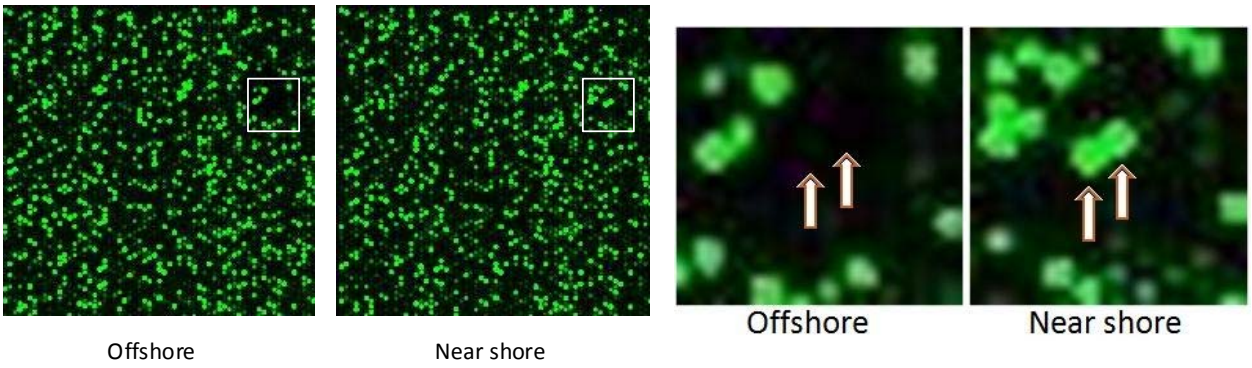

Figure 8.4. Microarray analysis of testis from offshore and nearshore conch labeled with a single color. On the right we have amplified the boxed in area to illustrate changes in gene expression for a few genes between offshore and nearshore conch. The analysis was performed with three different individuals from each location.

A two-way hierarchical clustering based on complete, centered correlation was performed in JMP Genomics for genes that are differentially expressed in the offshore and nearshore samples. Genes used in the cluster were significant at $p < 0.01$. Represented are genes that are up-regulated (red) or down regulated (blue) by the treatments compared to controls (Fig. 8.5). The three samples from offshore segregated together, as did the three samples from nearshore, suggesting that the gene changes represent a robust difference between the groups.

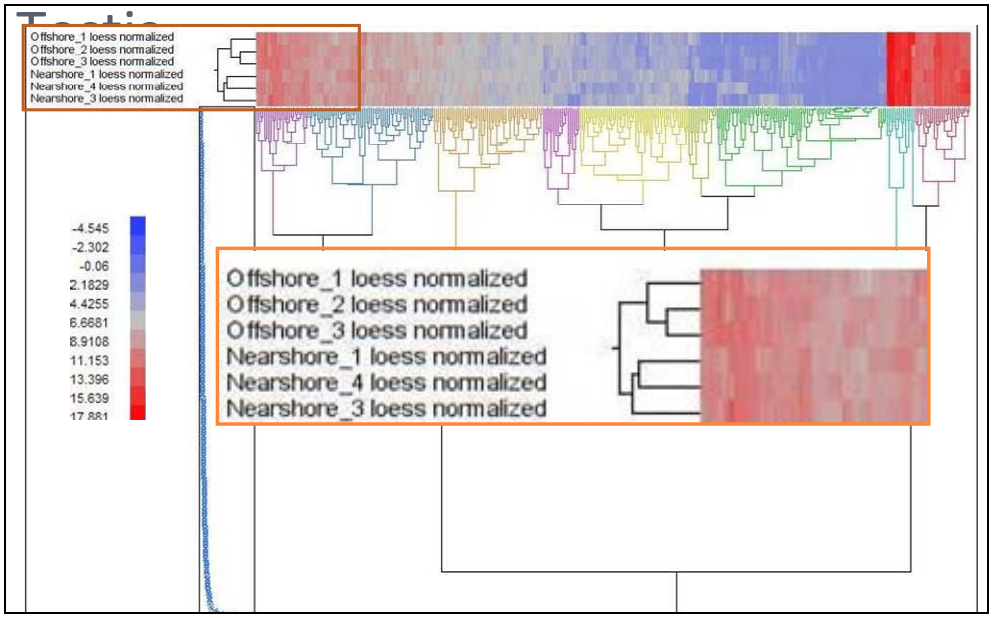

Figure 8.5. Hierarchical cluster analysis showing relationships between gene expression and zone.

At a p-value of 0.05, we found that 1,427 genes were regulated differently between the two samples, with 572 genes up regulated nearshore and 855 genes down regulated nearshore compared to the offshore conch. At a p-value of 0.01, the number of regulated genes drops to 349, with 116 of these up-regulated and 233 down-regulated in the nearshore animals (Fig. 8.6).

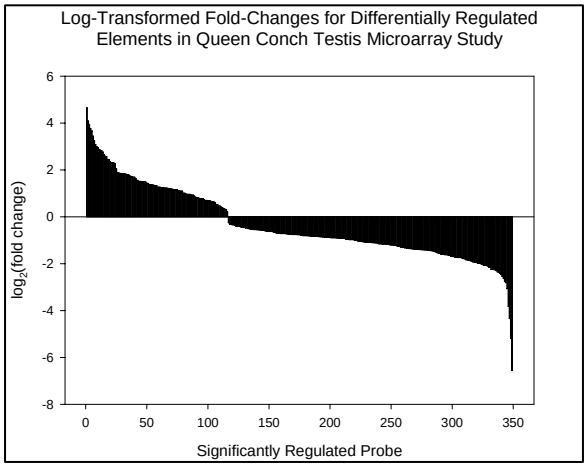

Figure 8.6. Genes regulated at a p-value < 0.01, listed in order from the highest to the lowest fold change.

Tables 8.1 and 8.2 list the genes with human homologues that were most highly up and down-regulated, respectively. Many of the genes are identified as important in stressed animals. Among the genes that were up regulated were many that are first line defense genes for chemical stressors, for example, glutathione-S-transferase and cytochrome P450 26A1. But, even more important were the genes that were down regulated, including the Wnt inhibitory factor1, a known tumor suppressor in humans, as well as glutathione reductase, high mobility group box 3, and oxysterol binding protein. Many of these genes are involved in meiosis, fertilization and reproduction.

Table 8.1. Most highly up-regulated genes in nearshore conch based on fold change. All are significant at $p<0.01$.

ProbeName	Human homologue	Fold Change
UF_Sgi_AF_101461	Glutathione S-transferase A2	15.37
UF_Sgi_CN_113123	Collagen, type V, alpha 1	13.49
UF_Sgi_AF_101173	Vacuolar protein sorting 37 homolog B (S. cerevisiae)	12.66
UF_Sgi_AF_102602	Collagen, type I, alpha 2	10.88
UF_Sgi_AF_103245	Cytochrome P450, family 26, subfamily A, polypeptide 1	7.76
UF_Sgi_AF_107600	Troponin T type 2 (cardiac)	7.18
UF_Sgi_CN_111325	Hedgehog acyltransferase-like	6.31
UF_Sgi_CN_113069	Integrin, beta-like 1 (with EGF-like repeat domains)	5.47
UF_Sgi_CN_111115	Olfactory receptor, family 7, subfamily A, member 17	5.06

Table 8.2. Most highly down-regulated genes in nearshore conch based on fold change. All are significant at p<0.01.

ProbeName	Human homologue	Fold Change
UF_Sgi_AF_103716	WNT inhibitory factor 1	36.56
UF_Sgi_AF_102095	Glutathione reductase	4.73
UF_Sgi_AF_101593	Dolichyl-phosphate mannosyltransferase polypeptide 2, regulatory subunit	4.45
UF_Sgi_AF_104743	Oxysterol binding protein-like 10	4.28
UF_Sgi_CN_110362	Mitogen-activated protein kinase kinase 7	4.25
UF_Sgi_CN_110726	High-mobility group box 3	4.21
UF_Sgi_CN_113575	Protease, serine 27	3.81
UF_Sgi_CN_113745	Olfactory receptor, family 10, subfamily G, member 4	3.71
UF_Sgi_CN_113271	ATPase family, AAA domain containing 2	3.61
UF_Sgi_AF_101068	CSRP2 binding protein	3.58
UF_Sgi_CN_112495	Mannosidase, alpha, class 1B, member 1	3.39

To get a better handle on how the up and down-regulated genes interact with each other and with known biological processes, we used the software program PathwayStudio (Ariadne) to show how the affected genes may interact with each other and contribute to reproduction (Fig. 8.7). To do this analysis, we first had to find the human homologs for the affected genes and then input these into the software program. As can be seen from the figure, many genes that are affected are involved in fertilization, reproduction, spermatogenesis, pachtyne, and meiosis are down regulated (green).

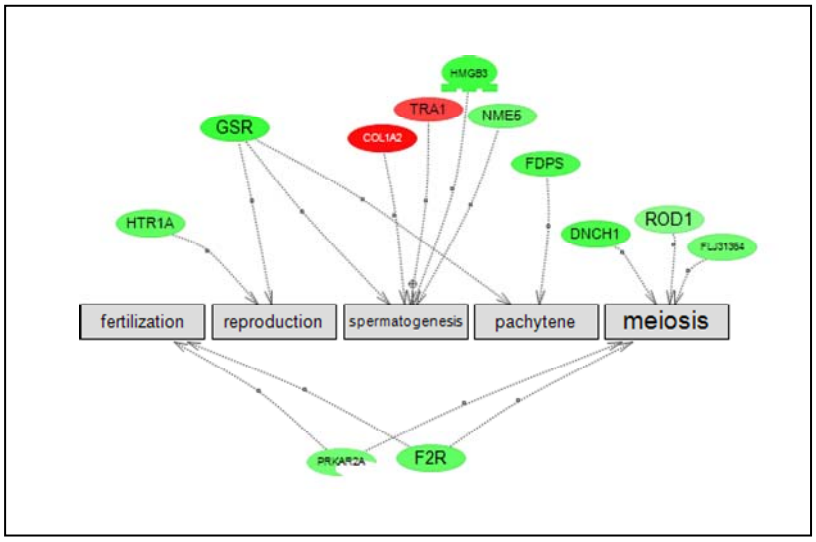

Figure 8.7. PathwayStudio analysis of genes that are changed in nearshore conch compared to offshore conch.

Overall the genomics study indicated that significant pathways affected in the conch testis included mitosis, meiosis, spermatogenesis, cell death and survival. In addition, processes including protein metabolism, RNA metabolism, and cellular energetics were affected. The microarray data were consistent with changes observed by histology and suggests that genes associated with testis development and spermatogenesis are down regulated in the near shore conch. More work is required to determine a causative link between gonad atrophy and a stressor. We need to develop more information to determine which stressor(s) are responsible for the decline in testis quality. Candidates high on the list include chemical toxicity as well as hypoxia and temperature.

Conclusions and Discussion

While significant progress was made during this project, the exact cause(s) for reproductive failure in nearshore conch remain elusive. A variety of organic and inorganic contaminants were evaluated and little correlation with effect was observed. One possible exception is the higher levels of zinc in nearshore conch tissues. Quantification of metals (see Table 4.1) in sediment shows that the levels are low (<1 ng/mg) and slightly higher offshore than nearshore, but the differences may not be significant. However, concentrations in conch digestive gland and gonad show bioaccumulation of this metal and increased levels of Zn in nearshore animals compared to the offshore animals. In the digestive gland, nearshore animals have about 1000 ng/mg Zn compared to about 70ng/mg Zn in the offshore animals. There is information from the literature that Zn can interfere with molluscan reproduction (Laskowski and Hopkin, 1996; Ducrot *et al.*, 2007) as well as survival (Ong and Din, 2001; Gorski and Nugegoda, 2006). Copper also showed a slighter higher concentration in the digestive gland and gonad of nearshore animals, but these levels were not as high as Zn and not as different in offshore and nearshore animals. Concentrations of other metals are similar in nearshore and offshore animals, making them not likely responsible for the reproductive deficiencies.

A suite of organic contaminants, including the pesticide Naled and its more persistent and toxic breakdown product, DDVP, were also evaluated. The organic compounds examined do not seem to be responsible for the reproductive deficiencies observed. A number of the contaminants were actually present at higher concentrations offshore than nearshore, which was the case for DDT and DDE, suggesting that the offshore animals may also be compromised by exposure to these contaminants. This helps explain the increase in biomarkers in offshore animals compared to nearshore conch. For example, Cyp1A was more highly expressed in the digestive gland in offshore females compared to their nearshore counterparts. Cyp1A is known to be responsive to exposure to aromatic hydrocarbons. The concentrations of OCPs in offshore animals were measureable but low and the conch at those locations may have been able to biochemically compensate for the effects of these pesticides, leaving them with intact reproductive gonads. In addition, other biomarkers of stress were tested as described below.

Naled and DDVP were not present in detectable amounts in sediment or tissue samples. Unfortunately, we were unable to test the full suite of possible contaminants, especially other pesticides such as permethrin, due to budget limitations; however, many of those that were tested are known endocrine disruptors. Results from a study conducted concurrent with this project showed that larval settlement is impacted by permethrin (Delgado *et al.*, 2007). To fully evaluate exposure of nearshore and offshore conch to this and other contaminants, additional studies are necessary.

The original tool we hoped to develop to quantify endocrine disruption, vitellin (Vtg), proved to be problematic. After numerous efforts (detailed in Chapter 5), we were finally able to isolate Vtg; however, we concluded that Vtg is not present in the circulatory system (i.e., hemolymph) and Vtg synthesis was not induced by exposure to estradiol. The Vtg that was isolated was found in gonadal tissue in the oocytes. However, this biomarker may have limited utility since conch nearshore have atrophied gonads, and very little tissue thus making comparative studies between nearshore and offshore populations using VTG impractical.

Nevertheless, results of this project have yielded significant progress on methodologies for evaluating biomarkers of chemical stress in conch. We were able to develop, test, and utilize several other biomarkers with broad utility. Glutathione transferases, a suite of heat shock proteins (HSPs), and neuropeptide hormones (egg-laying hormone [ELH], and AGPWAmide), were all identifiable in the conch tissues and we tested each of them in nearshore and offshore conch samples. These biomarkers have highly conserved sequences across species, and antibodies developed for mammals appear to cross react well with the conch homologs. We found differences in expression of these biomarkers between nearshore and offshore conch but were surprised that, for the most part, they were expressed contrary to our formulated hypotheses that the nearshore conch would be more impacted than offshore conch. For example, the HSPs (heat stress), glutathione (oxidative stress response), and cytochrome P450 (polyaromatic hydrocarbons); all had higher concentrations offshore than nearshore (except HSP60). In the case of egg-laying hormone, concentrations were higher in both the gonads and neural ganglia of offshore males than nearshore females; nearshore males were devoid of ELHs suggesting that the reproductive deficiencies are not sex-specific; both sexes nearshore are impacted to some degree. One interpretation of these results may be that nearshore conch exhibit across the board suppression of biomarker proteins in addition to their failure to develop gonadal tissues.

Four biomarkers conformed to the patterns we expected. Hsp60 was higher in the neuroganglia of conch nearshore and evidence now suggests that mitochondrial Hsp 60 is involved in apoptosis (Gupta and Knowlton, 2005). We have already mentioned the concentrations of Zn in the digestive gland and the gonad, which would indicate a problem with reproduction. The other two biomarkers are neuropeptides.

Reproduction in conch, as with many mollusks, appears to have a major component that is neurologically mediated. Suites of neuropeptide hormones have been identified in the marine gastropod *Aplysia* spp. that are involved in the control and regulation of reproduction. Two neuropeptides were examined, including egg laying hormone and APGWamide, a proposed penis morphogenic factor (Oberdorster McClellan-Green, 2000; Oberdorster *et al*., 2005).

Results obtained so far indicate that these neuropeptides are expressed by both male and female conchs. This is similar to the production of androgens (testosterone) and estrogens (estradiol) in both males and females of higher eukaryotes. Our results indicate that ELH is expressed at higher levels in offshore females than nearshore females and that APGWamide is higher in offshore males than nearshore males. More precisely, ELH had the general pattern: (OF > OM > NF $\underline{\sim}$ NM) and APGWamide had a similar pattern (OM >> OF > NM $\underline{\sim}$ NF). Coupled to this was our determination that nearshore conch had neural ganglia tissue abnormalities (Chapter 6). Taken together, this indicates that inshore factors are affecting both sexes of queen conch and there is disruption in the neurological function of conch found nearshore.

In addition to traditional biomarkers, we developed novel tools to analyze gene transcription in queen conch. The use of genomics in ecotoxicology (i.e., toxicogenomics) appears to be an approach that can provide insights into the mechanisms of stress response including altered reproduction. In our case, we developed a library of over 8,000 well annotated genes (Appendix IV on CD) from which we can choose any number to examine how an organism responds to stressful environments. For example, we have identified genes for heat shock proteins, hypoxia inducible factor, vitellin and vitellin receptor, superoxide dismutase,

CYPs, estrogen receptor, among others. The genes that were identified affect many different pathways that regulate energy, metabolism, cell cycle, growth and differentiation, reproduction, cell death, as well as others. These processes are all critical to homeostasis of organisms and we are confident that careful selection of the correct suite of genes for examination will yield insight into what may be causing cessation of reproduction in queen conch that are close to shore.

Interestingly, two genes that were changed on the array in nearshore conch compared to offshore conch are known to be induced by zinc concentrations in mammalian species. These include the DEAD box polypeptide 19a which is known in rat to be responsive to zinc ion and the solute carrier family 39 gene which is a zinc transporter in rat and humans. The DEAD box polypeptide 56 genes was one of the most significantly upregulated genes in the nearshore conch male gonad. In addition, we saw upregulation of a solute carrier family 39 gene and a possible solute carrier 30 gene. Another gene related to zinc that was upregulated was sorbitol dehydrogenase, and we saw upregulation of a copper transporter, as well.

However, genomics alone will not address the problem. In many cases, the issues are manifested in behavioral or morphological deficiencies. For example, the male conch nearshore had significantly smaller penises when compared with their offshore counterparts (Chapter 3). This has implications for fertilization success should these conch mate. Why these structures are smaller is not currently known (although further examination of AGPWamide may provide some clues), but by integrating histology and molecular mechanisms in experimental animals, we may be able to better elucidate the problem. Future examinations of reproductive behavior and responses may help elucidate the connection among tissue morphology, protein concentrations, and gene expression.

Interestingly, one nearshore conch with external male morphology (i.e., possessing a penis) had gonadal tissue similar to female conch and was characterized histologically as imposex (Chapter 6). The most often described cause of imposex induction in marine snails is exposure to various organotin compounds (Matthiessen and Gibbs, 1998) used in bottom paints (e.g., tributyl tin, triphenlytin; Horiguchi *et al.,* 2002; Voulvoulis *et al.*, 2002). However, we found no difference in total tin concentrations in either sediment or conch tissues, thus, the imposex we observed does not appear to be a result of tin exposure.

One aspect beyond the scope of this project was the examination of parasites and their effects on reproduction. Depressed reproduction in queen conch populations in Colombia and elsewhere may be a result of the presence of a parasite found in the digestive gland (Baqueiro *et al*., 2007). In response to this observation, we analyzed samples of digestive gland from nearshore and offshore conch. The parasites were ubiquitous and very common in samples from both reproductive and non-reproductive populations. Because there does not appear to be a relationship between parasite loading and reproductive competence, we conclude that while parasites may contribute to the overall problem, they are not by themselves responsible for the observed reproductive deficiencies.

While the data from this study did not reveal overt differences in chemical contaminant levels between nearshore and offshore sites, detectable concentrations of estradiol and ethynylestradiol were present in both nearshore and offshore waters of the Florida Keys. Despite the fact that 17β-estradiol did not induce Vtg synthesis in the hemolymph of conch (unlike in fish and other species), the presence of estrogenics may still induce changes in other biomarkers

that were not examined and chronic exposure may ultimately result in reproductive impairment. This still needs to be investigated.

Because a component of reproduction in marine gastropods appears to be neurologically controlled by the gonads, estrogen or estrogen-like compounds in the environment may have no effect on their reproduction. However, the estrogenic compounds that we found should be considered a warning flag for the overall health of the Florida Keys ecosystem. Many species other than marine gastropods, including fish and other invertebrates (e.g., scallops) do respond directly to exogenous sources of estrogen and phenolic compounds by producing Vtg, and are likely to be sensitive to these contaminants (Castro *et al*., 2007; Bannister *et al*., 2007; Iguchi *et al.*, 2007; Köhler *et al*., 2007). This has implications for the healthy function of the Florida Keys marine ecosystem.

We are now including in our focus environmental perturbations as adding to the problem of reproductive deficiencies. Reproduction nearshore occurred as recently as the mid-1980's, approximately the same time that the causeways for the new Florida Keys bridge system were installed. This undoubtedly had the effect of reducing flow and likely reducing dissolved oxygen, changing the oxidation reduction potential of the seawater, and disturbing the sediment pH balance. The resulting altered flow through the numerous passes has contributed to stagnation nearshore, resulting in consideration of tidal flow restoration projects (Braynard and Hunt, 2005). In some locations where conch are found nearshore, temperatures have been observed as high as 35°C, well above the more common offshore temperature maximums of 31ºC. Dissolved oxygen (DO) under these conditions is expected to dip. Chronic and cyclic hypoxia have been shown to decrease the female reproductive potential of grass shrimp, *Palaemonetes pugio* (Brouwer *et al*., 2007; Brown-Peterson *et al*., 2008) and various fish species (Landry *et al*., 2003; Thomas *et al*, 2006; Wu *et al*., 2003}, suggesting hypoxia may be implicated in the observed reproductive dysfunction of queen conch. Additionally, carp showed a significant decline in sperm production and steroid hormone levels under low DO conditions (Wu *et al*., 2003).

The observed reproductive effects may also be due to increased temperature. Studies by the Florida Fish and Wildlife Commission have shown that at least two queen conch populations (i.e., Conch Reef and French Reef) that historically reproduced in shallow-water, back-reef environments offshore have shifted their distribution into deeper waters where, presumably, temperatures are cooler and oxygen is increased. Reproductive neurohormones are altered in Aplysia, another gastropod, which are exposed to higher temperature (Wayne, 2001). In our analyses of heat shock proteins ('chaperones' that ensure that the cell's proteins are in the right shape and in the right place), we noted that positive bands in the western blot probed with the human Hsp60 antibody were higher in the neuroganglia of nearshore conch. Hsp expression is induced by heat, ischemia, hypoxia, ATP depletion, heavy metals, and free radicals.

Furthermore, there are increasing nutrient loads nearshore in close association with on-land development, and these nutrients decline from nearshore to offshore (Szmant and Forrester, 1996). Many nutrients are likely discharged from inadequate sewage treatment and disposal (Paul *et al*., 2000). Nearshore eutrophication may cause a number of processes to occur, including increasing the biomass of the algae in the order Ceramiales. Members of this order have very high concentrations of the carotenoid pigments and β-carotene is a precursor to Vitamin A and other retinoic acids; 9 cis-retinoic acid was recently reported to be an endocrine

disruptor in marine gastropods (Nishikawa *et al.*, 2004). Within this order, *Ceramium* spp. and *Spyridea* spp. are both preferred foods for conch (Creswell, 1984; Davison, 1990) and are found almost exclusively in the nearshore zone. Increased consumption of Ceramiales due to eutrophication may have a significant impact on queen conch reproduction.

Future experiments should include translocation of conch from offshore to nearshore in order to elucidate the temporal changes that occur with respect to gene expression and concentrations of biomarkers. Pristine sites outside Florida should be included as baseline controls. Additionally, behavioral components should be incorporated into these studies to couple the physiology to organismal function. As the cause(s) of reproductive failure is narrowed, directed exposure studies (bioassays) will provide direct cause and effect relationships.

Finally, the deficiencies we detailed here are not necessarily immutable. Previous studies have demonstrated that the deficiencies are mediated relatively rapidly when conch are translocated offshore into breeding aggregations (Delgado *et al*., 2004). The physiology of organismal recovery as well as the temporal changes in gene expression may hold a key to understanding both the environmental factors as well as the physiological processes impairing reproduction in conch and, ultimately, impacting population health. The results of these studies will provide resource managers with information on potential mitigation techniques to improve the nearshore habitat which may result in reproduction of nearshore queen conch.

Literature Cited

Anderson, R. L. 1982. Toxicity of fenvalerate and permethrin to several non-target aquatic invertebrates. Environmental Entomology. 11: 1251-1257.

Arcand-Hoy, L. D., A.C. Nimrod, and W. H. Benson. 1998. Endocrine-modulating substances in the environment: estrogenic effects of pharmaceutical products. Int. J. Toxicol. 17: 139-158.

Atkeson, T. D., J. J. Delfino, T.W. La Point, S.C. McCutcheon, and J.H. Rodgers. 1998. Linking ecotoxicity and risk management to sustainable restoration of south Florida ecosystems. Advisory Panel Report to Workshop Steering Committee and Task Force. 23 pp.

Bannister, R., N. Beresford, D. May, E. J. Routledge, S. Jobling, M. Rand-Weaver. 2007. Novel estrogen receptor-related transcripts in *Marisa cornuarietis*; a freshwater snail with reported sensitivity to estrogenic chemicals. Environ Sci Technol. 41: 2643-2650.

Baqueiro Cárdenas, E., L. Frenkiel, A.Zetina Zarate, and D. Aldana Aranda. 2007. Coccidian (Apicomplexa) parasite infecting *Strombus gigas* linné, 1758 digestive gland. J. Shellfish Research. 26: 319-321.

Barre, P., M. Bride, R. Beliard, B. Petracca. 1991. Localization of yolk proteins and their possible precursors using polyclonal and monoclonal antibodies, in *Helix aspersa*. Cell. Mol. Biol. 37: 639-50.

Berg, C. J., Jr. and R. A. Glazer. 1995. Stock assessment of a large marine gastropod *(Strombus gigas*) using randomized and stratified towed-diver censusing. ICES mar. Sci. Symp. 199: 247-258.

Braynard, M. and J. H. Hunt. 2005. Florida Keys tidal restoration monitoring project. Final report. South Florida Water Management District Contract No. ML040281, FWC File Code F2398-03-06-F**,** pp. 101.

Bird, S. L., D. M. Esterly, and S. G. Perry. 1996. Off-target deposition of pesticides from agricultural aerial spray applications. Journal of Environmental Quality. 25: 1095-1104.

Bright, T., W. Jaap, and C. Cashman. 1981. Ecology and management of coral reefs and organic banks. *In:* Proc Environ Res Needs in the Gulf of Mexico. Miami, FL, U.S. Department of Commerce, National Oceanic and Atmospheric Administration, Environmental Research Laboratory: 53-160.

Brouwer, M. et al. Molecular and while animal responses of grass shrimp, *Palaemonetes pugio*, exposed to chronic hypoxia. J. Exp. Mar. Bio Ecol. 43:812.

Brown-Peterson, N., C. S. Manning, V. Patel, N. D. Denslow, and M. Brouwer. 2008. Effects of cyclic hypoxia on gene expression and reproduction in a grass shrimp, *Palaemonetes pugio*. Biological Bulletin 214: 6-16.

Castro L. F., C. Melo, R. Guillot, I. Mendes, S. Queirós, D. Lima, M. A. Reis-Henriques, and M.M. Santos. 2007. The estrogen receptor of the gastropod Nucella lapillus: modulation following exposure to an estrogenic effluent? Aquat Toxicol. 30:465-468.

Celius, T. and B.T. Walther. 1998. Differential sensitivity of zonagenesis and vitellogenesis in Atlantic salmon (*Salmo salar* L.) to DDT pesticides. J. Exper. Zool. 281: 346-353.

Creswell, R. L. 1984. Ingestion, assimilation, and growth of juveniles of the queen conch *Strombus gigas* Linne fed experimental diets. J. Shellfish Research. 4: 23-30.

Cross, J. N. and J. E. Hose. 1988. Evidence for impaired reproduction in white croaker (*Genyonemus lineatus*) from contaminated areas off southern California. Mar. Environ. Res. 24: 185-188.

Daniel, W. W. 1977. *Introductory Statistics with Applications*., Boston, MA: Houghton Mifflin Company.

Davison, D. A., Feeding preferences, growth responses, and food-conversion ratios of juvenile conchs (*Strombus gigas* Linné) reared under experimental conditions. 1990, University of West Florida: Pensacola, FL. p. 1-79.

Delgado, G. A., C. T. Bartels, R. A. Glazer, N. J. Brown-Peterson and K. J. McCarthy. 2004. Translocation as a strategy to rehabilitate the queen conch (*Strombus gigas*) population in the Florida Keys. Fishery Bulletin 102: 278-288.

Delgado, G. A., R. A. Glazer, and D. Wetzel. 2007. The Effects of Water Quality on Embryogenesis and Larval Development of Queen Conch: Implications for Recruitment to and Coastal Development of the Florida Keys. Final Report to the South Florida Water Management District. SFWMD #OT050676. 35p.

Denslow, N. D., M. C. Chow, K. J. Kroll, and L. Green. 1999. Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. Ecotoxicology 8: 385-398.

Ducrot, V., A. R. R. Péry, R. Mons, H. Quéau, S. Charles, and J. Garric. 2007. Dynamic energy budget as a basis to model population-level effects of zinc-spiked sediments in the gastropod *Valvata piscinalis*. Environmental Toxicology and Chemistry. 26: 1774-1783.

Egan, B.D. 1985. Aspects of the reproductive biology of *Strombus gigas.* M.S. Thesis, University of British Columbia, Vancouver, Canada. 147 p.

Fowles, J.R., A. Fairbrother, L. Baecher-Steppan, and N.I. Kerkvliet. 1994. Immunologic and endocrine effects of the flame retardant pentabromodiphenyl ether (DE-71) in C57BL/6J mice. Toxicol. 86: 49-61.

Gerpe M. S., J. E. A. Moreno, V. J. Moreno, and M. L. Patat. 2000. Cadmium, zinc, and copper accumulation in the squid *Illex argentinus* from the southwest Atlantic Ocean. Mar. Biol. 136: 1039-1044.

Glazer, R. and I. Quintero. 1998. Observations on the sensitivity of queen conch to water quality: Implications for coastal development. Proceedings of the Gulf and Caribbean Fisheries Institute. 50: 78-93.

Glazer, R. A. and C. J. Berg Jr. 1994. Queen conch research in Florida: an overview. *In:* Queen conch biology, fisheries, and mariculture. R. S. Appeldoorn and B. Rodriguez, Eds. Caracas, Venezuela, Fundación Cientifica Los Roques: 79-95.

Glazer, R. A., G. A. Delgado, D. Hawtof, and P. Kubilis. In review. Depensation shifts to compensation as a depleted population recovers. Conservation Biology.

Gorski, J. and D. Nugegoda. 2006. Sublethal toxicity of trace methals to larvae of the blacklip abalone, *Haliotis rubra*. Environmental Toxicology and Chemistry. 23: 1360-1367.

Gronen, S., N. Denslow, S. Manning, S Barnes, D. Barnes, and M. Brouwer. 1999. Serum vitellogenin levels and reproductive impairment of male Japanese medaka (*Oryzias latipes*) exposed to 4-tert-Octylphenol. Env. Health. Persp. 107: 385-390.

Gupta, S. and A. A. Knowlton. 2005. HSP60, Bax, apoptosis and the heart. Journal of Cellular and Molecular Medicine. 9: 51-58.

Hartley, D. and H. Kidd. 1983. The Agrochemicals Handbook. Nottingham, England, Royal Society of Chemistry.

Heatwole, D. W. 1987. Water Quality Assessment of Five Selected Pollutant Sources in Marathon, Florida Keys. Florida Keys Monitoring Study: 1984-1985. South Florida District. Marathon Branch Office, Marathon, Florida.

Hill, I. R. 1985. Effects on non-target organisms in terrestrial and aquatic environments. *In:* The Pyrethroid Insecticides. J. P. Leahy, Ed. London, Taylor and Francis.

Horiguchi T, Kojima M, Kaya M, Matsuo T, Shiraishi H, Morita M, Adachi Y. 2002. Tributyltin and triphenyltin induce spermatogenesis in ovary of female abalone, *Haliotis gigantean*. Mar Environ Res. 54: 679-84.

Iguchi T., H. Watanabe, Y. Katsu. 2007. Toxicogenomics and ecotoxicogenomics for studying endocrine disruption and basic biology. Gen Comp Endocrinol. 153: 25-29.

Jaap, W. C. 1984. The Ecology of the South Florida Coral Reefs: A community profile. U.S. Fish and Wildlife Service, Office of Biological Services, Washington, D.C. FWS/OBS-82-/08.

Köhler H. R, W. Kloas, M. Schirling, I. Lutz, A. L. Reye, J. S. Langen, R. Triebskorn, R. Nagel, G. Schönfelder. 2007. Sex steroid receptor evolution and signalling in aquatic invertebrates. Ecotoxicology. 16: 131-143.

Kruczynski, W. 1999. Water Quality Concerns in the Florida Keys: Sources, effects, and solutions. Florida Keys National Marine Sanctuary, Water Quality Protection Program**,** pp. 75.

Lapointe, B. E. and M. W. Clark. 1990. Spatial and Temporal Variability in Trophic State of Surface Waters in Monroe County During 1989-1990. Final report to Florida Keys Land and Sea Trust, Marathon, FL**,** pp. 25.

Lapointe, B. E., D. A. Tomasko and W. R. Matzie. 1994. Eutrophication and trophic state classification of seagrass communities in the Florida Keys. Bulletin of Marine Science. 54: 696-717.

Landry, C.C., S. Manning, and A.O. Cheek. 2003. Hypoxia affects reproduction in gulf killifish (*Fundulus grandis*). Integrative and Comparative Biology 43: 812.

Laskowski, R. and S. P. Hopkin. 1996. Effect of Zn, Cu, Pb, and Cd on fitness in snails (*Helix aspersa*). Ecotoxicology and Environmental Safety. 34: 59-69.

Malik, N. J. and M. E. Daymon. 1982. Improved double immunoenzyme labelling using alkaline phosphatase and horseradish peroxidase. J. Clinical Pathology. 35: 1092-1094.

Matthiessen, P. and P. E. Gibbs. 1998. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. Environmental Toxicology and Chemistry 17: 37-43.

Meerts, I.A.T.M., J.J. van Zanden, E.A.C. Luijks, I. van Leeuwen-Bol, G. Marsh, E. Jakobsson, E. Bergman, and Å. Brouwer. 2000. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro. Toxicol. Sci. 56: 95-104.

McCarthy, K.J., C.T. Bartels, M.C. Darcy, G.A. Delgado and R.A. Glazer. 2002. Preliminary observation of reproductive failure in nearshore queen conch (*Strombus gigas*) in the Florida Keys. Proceedings of the Gulf and Caribbean Fisheries Institute 53: 674-680.

Nishikawa, J., S. Mamiya, T. Kanayama, T. Nishikawa, F. Shiraishi, and T. Horiguchi. 2004. Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. Environmenatal Science Technology 38: 6271-6276.

Oberdorster, E. and P. McClellan-Green. 2000. The neuropeptide APGWamide induces imposex in the mud snail, *Ilyanassa obsoleta.* Peptides 21: 1323-1330.

Oberdorster, E., J. Romano, and P. McClellan-Green. 2005. The neuropeptide APGWamide as a Penis Morphogenic Factor (PMF) in gastropod mollusks. Integrative and Comparative Biology 45: 28-42.

Ong E.S. and Z. B. Din. 2001. Cadmium, copper, and zinc toxicity to the clam, *Donax faba* C, and the blood cockle, *Anadara granosa* L. Bull. Environ. Contam. Toxicol. 66: 86-93.

Paul, J.H., et al. 2000. Rapid movement of wastewater from on-site disposal systems into surface waters in the Lower Florida Keys. Estuaries 23: 662-668.

Pierce, R. H. 1998. Effects of mosquito control measures on non-targeted organisms in the Florida Keys National Marine Sanctuary. Final report submitted to the

Environmental Protection Agency. Mote Marine Laboratory. Technical Report 609**,** pp. 40.

Pierce, R., M. Henry, D. Kelly, P. Sherblom, W. Kozlowsky, G. Wichterman, and T. W. Miller. 1996. Temephos distribution and persistence in a southwest Florida salt marsh. J. American Mosq. Control Assoc. 12: 637-646.

Safe, S. H., W. G. Foster, J. C. Lamb, R. R. Newbold, and G. Van Der Kraak. 2000. Estrogenicity and endocrine disruption. Council for Agricultural Science and Technology. 16 p.

Snedaker, S. C., P. W. Glynn, D. G. Rumbold and E. F. Corcoran. 1995. Distribution of n-alkanes in marine samples from Southeast Florida. Marine Pollution Bulletin. 30: 83-89.

Spies, R.B. and D.W. Rice. 1988. Effects of organic contaminants on reproduction of the starry flounder *Platichthys stellatus* in San Francisco Bay. Marine Biology 98: 191-200.

Stevely, J. M. and R. E. Warner. 1978. The Biology and Utilization of the Queen Conch, *Strombus gigas* L., in the Florida Keys and Throughout its Geographic Range. Marine Resource Inventory. Monroe County Cooperative Extension Service**,** pp. 48.

Szmant, A. M. and A. Forrester. 1996. Water column and sediment nitrogen and phosphorus distribution patterns in the Florida Keys, USA. Coral Reefs 15: 21-41.

Thomas, P., M. S. Rahman, J. A. Kummer, and S. Lawson. 2006. Reproductive endocrine dysfunction in Atlantic croaker exposed to hypoxia. Marine Environmental Research 62: S249-S252.

Voulvoulis N, Scrimshaw MD, Lester JN. 2002. Comparative environmental assessment of biocides used in antifouling paints. Chemosphere. 47: 789-795.

Wayne, N. 2001. Regulation of Seasonal Reproduction in Mollusks. Journal of Biological Rhythms 16: 391-402.

Wu, R.S., et al. 2003. Aquatic hypoxia is a disrupter and impairs fish reproduction. Environmental Science and Technology 37: 1137-1141.

Zheng, W. and E. Van Vleet. 1988. Petroleum hydrocarbon contamination of the Dry Tortugas. Marine Pollution Bulletin. 19: 134-136.