NSU Florida
NOVA SOUTHEASTERN UNIVERSITY

Nova Southeastern University
NSUWorks

HCNSO Student Theses and Dissertations

HCNSO Student Work

12-6-2016

Effects of 17 β-estradiol and Progesterone on Acropora cervicornis and Porites astreoides Growth and Reproduction

Joshua L. Stocker
Nova Southeastern University, js2887@nova.edu

Follow this and additional works at: https://nsuworks.nova.edu/occ_stuetd

Part of the Marine Biology Commons, and the Oceanography and Atmospheric Sciences and Meteorology Commons

Share Feedback About This Item

HALMOS COLLEGE OF NATURAL SCIENCES AND
OCEANOGRAPHY

EFFECTS OF 17 β-ESTRADIOL AND PROGESTERONE ON
ACROPORA CERVICORNIS AND *PORITES ASTREOIDES*
GROWTH AND REPRODUCTION

By

Joshua Stocker

Submitted to the Faculty of
Halmos College of Natural Sciences and Oceanography
in partial fulfillment of the requirements for
the degree of Master of Science with a specialty in:

Marine Environmental Science
and
Coastal Zone Management

Nova Southeastern University

December 6, 2016

Thesis of

Joshua Stocker

Submitted in Partial Fulfillment of the Requirements for the Degree of

Masters of Science:

Marine Environmental Science and Coastal Zone Management

Joshua Stocker
Nova Southeastern University
Halmos College of Natural Sciences and Oceanography

December 2016

Approved:

Thesis Committee

Major Professor: _____
Nicole D. Fogarty, Ph.D.

Committee Member: _____
Esther C. Peters, Ph.D.

Committee Member: _____
David Gilliam, Ph.D.

Committee Member: _____
Donald McCorquodale, Ph.D.

Acknowledgements

First, I would like to thank my major advisor Dr. Nicole Fogarty for allowing me to volunteer in her lab and perform coral ecotoxicology research. Previous to graduate school, I had no prior research experience with marine organisms and am grateful for all of the experiences I have received working in this lab. In addition, I was encouraged and received great insight from my thesis proposal all the way through to my final drafts and defense preparations. This process has been challenging, incredibly rewarding, and I could not have asked for better support and guidance throughout this experience.

I am grateful for all of the support I received from my thesis committee members for their help throughout this process. Dr. Esther Peters' vast research experience, expertise in coral histology, and meticulous editing has been invaluable throughout the entire process. Dr. David Gilliam provided excellent information for obtaining corals and excellent feedback. Dr. Donald McCorquodale was also extremely helpful throughout by providing excellent feedback and his ecotoxicology course helped inspire me to do this research.

I also could not have completed this research project without the enormous help I received from my "2nd Lab." Dr. Abigail Renegar allowed me to use the SEACORE system and on-shore coral nursery to conduct my research while providing excellent experimental feedback and guiding me through the entire histological process. Also, I am grateful for the opportunity to be a Research Assistant in her lab, which allowed me to work on countless research projects while gaining invaluable knowledge. Also, I must thank Nick Turner, whose experience in coral aquaculture and feedback has been invaluable for my experiment and statistical analysis.

I also am grateful for the support I have received from my family throughout this endeavor, without their help and encouragement throughout my entire education I would not be here today. I am also grateful for the experimental assistance and excellent feedback I received when going over presentations from all of my lab members. In addition, I must thank my wonderful girlfriend Lauren for all of her help, support, and for simply putting up with me throughout this entire process.

Table of Contents

List of Figures

List of Tables

Abstract

Reef-building coral populations throughout the world are being threatened by numerous stressors and continue to decline. As potent endocrine-disrupting compounds, exogenous sex steroid contamination has been a largely overlooked stressor to corals. Previous research indicates these compounds are prevalent in marine environments, fluctuate annually along with reproductive cycles, can bioaccumulate, and have had variable effects on growth and reproduction in several cnidarian species. This project had three primary objectives: (1) establish environmental estradiol and progesterone concentrations in Broward County and lower Florida Keys reef environments, (2) conduct 17 β-estradiol and progesterone larval assays on *P. astreoides* larvae to determine the effects of these compounds on settlement and viability, and (3) conduct 17 β-estradiol and progesterone dosing experiments on adult *Acropora cervicornis* and *Porites astreoides* fragments to determine the effects on growth, zooxanthellae, reproduction, and overall tissue health. Estradiol was detected in surface and at-depth water samples from Broward County and lower Keys reef sites at effect level concentrations for marine organisms. Broward County larvae treated with low progesterone (5 ng/L) had decreased survival, while lower Keys larvae in low estradiol treatments (1 ng/L) had increased on-disc settlement. No other treatment effects were observed, however, lower Keys larvae had greater overall survival in comparison to Broward County larvae. There were no significant differences between estradiol and progesterone treatments in the adult-dosing experiment for growth, zooxanthellae density, reproduction, and overall tissue health. This is the first study to detect estradiol at Broward County reefs sites and our results, while inconclusive, indicate these compounds may have the potential to affect coral reef ecosystems.

Keywords: *Acropora cervicornis*, *Porites astreoides*, endocrine disrupting compounds, sex steroids, estradiol, progesterone.

Chapter 1: Introduction

1.1 Problem Statement

Coral reefs are an invaluable ecological and economical resource worldwide. Despite occurring in oligotrophic waters, tropical reefs are highly productive, sustain great biological diversity, and support other marine ecosystems. Reef-building corals provide a vital structural and functional role in the ecosystem by providing habitat to marine organisms, coastal buffering, energy flow, island formation, and biogeochemical cycling (Goreau and Hilbertz 2005). Economically, coral reefs provide societies with building materials, medicines, tourism, fisheries, and sustenance that are critical to less developed countries (Moberg and Folke 1999, Hoegh-Guldberg et al. 2007). Cesar et al. (2003) estimated that coral reefs provide a net benefit of approximately $30 billion (bn), coming from fisheries ($5.7 bn), coastal protection ($9.0 bn), tourism and recreation ($9.6 bn), and biodiversity ($5.5 bn). Although coral reefs have intrinsic ecological and economic value, in recent decades, numerous anthropogenic stressors have led to significant declines in reef-building coral populations throughout the world (Cesar et al. 2003).

On a global scale, increasing water temperatures from climate change have had profound consequences (Hoegh-Guldberg et al. 2007). Atmospheric carbon dioxide concentrations (~400 ppm) are approximately 100 ppm above values recorded in the past 740,000 years and will continue to rise under current practices (Petit et al. 1999, Hooidonk et al. 2013). Thermal stress often leads to coral bleaching from the loss of their symbionts, zooxanthellae, with prolonged exposure increasing rates of mortality (Wilkinson 2008). One severe bleaching event in 1998 destroyed 16% of corals globally (Wilkinson 2008). Along with climatic changes, localized stressors have had significant impacts on coral populations regionally. Destructive fishing practices (blast and cyanide fishing, exploitation of fisheries-(Edinger et al. 1998, Worm and Lenihan 2014, Zaneveld et al. 2016), land based pollution (industrial waste, domestic waste, agricultural runoff, wastewater effluent-(Futch et al. 2011, Zaneveld et al. 2016), sedimentation [dredging, runoff, and effluent-(Lapointe et al. 2004)], tourism, urbanization, invasive species, and shipping are among the most prevalent localized anthropogenic stressors (Cesar et al. 2003, Wilkinson 2008, Jackson et al. 2014). The synergy of these stressors leaves corals

vulnerable to bleaching and disease outbreaks. Zaneveld et al. (2016) reported that overfishing and nutrient pollution along with elevated water temperatures can intensify bleaching events and disease outbreaks, disrupt coral microbiomes, and increase pathogens, macroalgal cover, and ultimately, coral mortality. In 2008, it was estimated that approximately 19% of coral reef area has been destroyed, with a further 35% at risk of being eliminated over the next couple decades (Wilkinson 2008). All of these stressors have led to wide-spread coral degradation and inhibited recovery. One region that has been hit particularly hard is the greater Caribbean (Jackson et al. 2004).

Disease outbreaks associated with coral bleaching have had dramatic impacts on coral populations in the greater Caribbean. Coral cover has decreased from 34.8% to 16.3% from 88 sites since 1970 (Jackson et al. 2014). Meanwhile from 1984 to 1998, macroalgal cover increased from 7% to 23.6%, indicating a phase shift from coral to macroalgal communities (Jackson et al. 2014). Throughout the Caribbean and western Atlantic, *Acropora cervicornis* populations have decreased 80–98% with losses up to 97% in the Florida Keys from thermal stress events and disease outbreaks (Aronson et al. 2008). Recently, in 2014–2015 following a thermal stress bleaching event, surveys throughout south Florida recorded white-plague disease mortality in 81% of susceptible coral species (Precht et al. 2016). Among white-plague disease susceptible species at monitoring sites, 81% of diseased corals were previously bleached with an overall prevalence of 51% (Precht et al. 2016). Considering previous impacts from thermal stress, disease outbreaks, and future climate change predictions, it is alarming to think of future reefs in the greater Caribbean and south Florida along with the environmental and economic ramifications. Without widespread global changes, there will be continued dramatic declines in reef-building coral populations that will have profound socio-economic consequences and great losses in marine biodiversity.

Natural and artificial reefs in southeastern Florida support more than 71,000 jobs and generate approximately $6.3 bn annually between Palm Beach, Broward, Miami-Dade, and Monroe counties through fishing, diving, and boating industries (Fauth et al. 2011). These reefs lie adjacent to three of the most populated counties in the United States that have undergone significant urbanization and coastal development leading to wastewater pollution, sedimentation, and eutrophication of coastal waters (Fauth et al.

2011). Florida reefs have experienced gradual long term declines while being subjected to periodic and chronic stressors. Previous research done by the Southeast Florida Coral Reef Initiative reported that sites located near open ocean outfalls contain more bare-bottom and uncolonized substrate than distant locations (Fauth et al. 2011). Substantial funding and research has been invested in monitoring, restoring, and studying numerous stressors affecting these reefs (Young et al. 2012); however, the potential contributions of hormonal endocrine disrupting pollutants (sex steroids) to coral degradation has been largely overlooked regionally and worldwide.

1.2 Sources of Contamination

Sex steroids contaminate coastal environments through sewage treatment plant effluent, septic tanks, cesspits, coastal runoff, industrial discharge or runoff, and agricultural or livestock waste runoff (Langston et al. 2005, Wright-Walters and Volz 2009, Corcoran et al. 2010). However, the most common source for sex steroid contamination is through treated and untreated human sewage. Sex steroids are commonly found in sewage treatment plant influent and effluent at small concentrations in the ng/L to pg/L range (Belfroid et al. 1999, Wright-Walters and Volz 2009). The geology of South Florida and the Florida Keys provides for easy groundwater flow of pollutants into the ocean from tidal pumping since they lie on a thick layer of porous limestone that lacks soil (Darden 2000); hence, untreated wastewater may contaminate the coastal environment during high precipitation and flooding events. Despite their pervasive nature and resistance to bacterial degradation, not much is known about the residence times, concentrations, distribution, adsorption capabilities, and potential impacts in marine environments (Atkinson et al. 2003).

The primary route for these compounds to enter marine environments in South Florida and the Florida Keys is from wastewater treatment plants, septic systems, and cesspits. In south Florida, approximately 57% of the population is connected to a sewage treatment plant, while the remaining 43% utilize in-ground waste disposal through septic systems or injection wells (Fauth et al. 2011). South Florida has six open ocean outfalls throughout Palm Beach, Broward, and Miami-Dade counties. Cumulatively, they release about 400 million gallons of effluent per day that receives secondary treatment with basic-level disinfection estimated to remove 70–80% of sex steroids from the wastewater

(Auriol et al. 2006, Koopman et al. 2006). Depending on treatment processes, location, temperature, rainfall, and demand, natural and synthetic steroids removal can range from 38–83% (Ternes et al. 1999, Desbrow et al. 2002, Singh et al. 2010). No advanced water treatment techniques are used to remove all nutrients and sex steroids since they are not practical for full-scale sewage treatment and are very expensive. The Florida Current dilutes the effluent northward unless abnormal weather patterns redirect currents east, exposing the reef tract to outfall discharge (Fauth et al. 2011). These events are estimated as a 10% probability (Koopman et al. 2006). Sewage nitrogen has been detected in macroalgae, sponges, and gorgonians through $\delta15N$ isotope analysis, linking effluent to nearby reefs (Koopman et al. 2006). Like Broward County, the Florida Keys face similar issues but do not utilize open ocean outfalls.

Throughout the Florida Keys, there were approximately 30,000 on-site sewage disposal systems composed of septic tanks, cesspits, and injection wells (Lapointe et al. 2004). All three types of sewage disposal in the Keys are susceptible to leaching into coastal waters from groundwater discharge and tidal pumping through the porous limestone substrate (Lapointe et al. 2004, Singh et al. 2010). Studies in the Keys have linked human sewage to coastal environments using stable isotope analysis and found elevated nitrogen concentrations beyond natural fixation, implicating local sewage sources and agricultural runoff (Lapointe et al. 2004). In 2000, the Monroe County Sanitary Wastewater Master Plan provided the foundation for the development of central sewage and treatment systems throughout the Keys. Once developed, all residents are required to tie into their cities' sewer systems to eliminate the use of on-site sewage systems. In the 2013 Status of Wastewater Implementation, approximately 72% of Keys residents are connected to their service area's sewage system. As with centralized sewage treatment provided in south Florida, sex steroid contamination is still a concern since these processes do not completely eliminate these pollutants.

Sex steroids can be transformed in the marine environment and throughout sewage treatment processes. Despite being primarily excreted as inactive waste byproducts, unconjugated sex steroids (i.e. estradiol or progesterone) are commonly found in sewage influent, effluent, and in aquatic environments (Desbrow et al. 1998). Typically, estradiol is removed during the sewage treatment process better than estrone

and ethinylestradiol, but estradiol glucuronides can be deconjugated into estradiol throughout water treatment, elevating concentrations in sewage effluent under certain conditions (Ternes et al. 1999). Legler et al. (2002) found that the bacterium *Escherichia coli* can efficiently deconjugate inactive metabolites into their unconjugated state via enzymatic hydrolysis throughout sewage treatment. Various bacteria (aerobic and anaerobic) that are found in aquatic environments are also capable of hydrolyzing sex steroids along with *E. coli*. Therefore, it is plausible that conjugated estrogens in the environment and in sewage treatment plants can be restored back to unconjugated biologically active compounds during the wastewater treatment processes prior to being released back into the environment (Desbrow et al. 1998).

Throughout the world, few data have been collected on sex steroid concentrations in aquatic environments, even less so in coastal marine environments of south Florida, the Florida Keys, and the Caribbean region. Open ocean water samples collected near Hawaii, Marianas Islands, French Polynesia, and the Florida Keys by Atkinson et al. (2003) had very low estrone concentrations averaging 0.052 ng/L, with many of the samples being non-detectable (ND). Two studies sampled throughout the Florida Keys for estrogenic compounds in 1998 and 1999 (Atkinson et al. 2003), and several years later in 2004 and 2006 (Singh et al. 2010). Estrone has been detected in water samples from the following locations in the Florida Keys: Key Largo and South Big Pine Key offshore patch reefs (0.26 ng/L), Looe Key offshore reef (ND–0.88 ng/L), Key Largo Shore (0.85 ng/L), Key Largo Harbor (0.66–5.2 ng/L), Big Pine Key canal (0.66 ng/L), Key West Channel (0.81 ng/L), and Key West Harbor (1.58 ng/L) near sewage effluent (Atkinson et al. 2003, Sing et al. 2010). Estradiol was detected in Key Largo Harbor (ND–1.8 ng/L) but not at the offshore reef near Looe Key (Singh et al. 2010). Singh et al. (2010) also detected (0.9–2.9 ng/L) of estrone but not estradiol (ND) in the Miami River, which is considered one of the most polluted areas in South Florida and drains into Biscayne Bay. The precursor for steroid synthesis, cholesterol, was detected in nearly every surface water sample in the Miami River, Key Largo Harbor, and Looe Key at concentrations ranging from the ND–2,896 ng/L. No studies measuring progesterone concentrations in the water along with estrogenic compounds in coral tissue have been completed for this region. This limited research indicates that sex steroids exist in

quantifiable concentrations along coastal marine habitats and highlights the need for more research to determine annual concentrations and potential effects they may have on important coral species.

1.3 Sex Steroids

Endocrine-disrupting compounds (EDCs) have the ability to affect the synthesis, metabolism, secretion, transport and processes of natural hormones responsible for physiological homeostasis, development, reproduction, and behavior (Ketata et al. 2008). EDCs can act in several ways: they can interfere with endogenous hormone production, bind or block steroid receptors, mimic hormones, or dislodge hormones from carrier proteins (Rotchell and Ostrander 2003). Sex steroids, phytoestrogens, hydrocarbons, and a wide variety of industrial pollutants comprise the major classes of endocrine-disrupting compounds (Auriol et al. 2006). Natural and synthetic sex steroids are typically the most potent forms of endocrine disruptors to marine and terrestrial organisms, but are often found at lower concentrations in the environment (Auriol et al. 2006).

The three major classes of natural sex steroids in vertebrates are progestins, androgens, and estrogens. They are produced endogenously and are primarily responsible for development, reproduction, and maintaining homeostasis (Baker 2002, Le Curieux-Belfond et al. 2005, Blomquist et al. 2006). In vertebrates, they act through steroid specific nuclear receptors: progestins (PR), androgens (AR), and estrogens (ERα and ERβ) (Thornton et al. 2003). Typically, sex steroids act as a ligand, binding to and activating specific nuclear receptor proteins (e.g. ERα) to stimulate or suppress DNA transcription of specific genes (Thornton 2003, Tarrant 2005).

Progesterone is the only naturally occurring progestin among many synthetic progestins and is a precursor for steroidogenesis of androgens and estrogens (Porte et al. 2006, King and Brucker 2010). Progesterone binds to progesterone receptors and is essential throughout pregnancy, fetal development, and menstrual cycles (Brinton et al. 2008, Corcoran et al. 2010). Clinically, progesterone is most often administered in various contraceptive and menopausal hormone replacement therapies, often in concordance with estrogens (Corcoran et al. 2010, King and Brucker 2010). Progesterone also appears to have neuroprotective and neuroregenerative effects in certain brain regions by increasing the viability and function of neurons and glial cells in the central

and peripheral nervous systems (Brinton et al. 2008). Androgens are mainly composed of testosterone and andrenostenodione in vertebrates. Androgens support growth and development in males and are a precursor to estrogens (Porte et al. 2006).

Natural estrogens are mainly composed of estrone, 17β-estradiol (estradiol), and estriol. Physiologically, natural estrogens have many important physiological functions; most notably they stimulate tissue growth, cellular division, and development of female reproductive organs and secondary sex characteristics (Atkinson and Atkinson 1991, Ruggiero and Likis 2002). Estrone helps maintain female reproduction, secondary sex characteristics, and becomes the primary estrogen post-menopause (Ruggiero and Likis 2002). Clinically estrone is most often used as a hormone replacement therapy for menopause and to prevent osteoporosis (NCBI 2016). Estradiol is the most potent natural estrogen and detected at the greatest concentration in pre-menopausal women (Ruggiero and Likis 2002, NCBI 2016). Estradiol is mostly essential for maintaining fertility and sex characteristics, but also has roles in anabolism, metabolism, and coagulation (NCBI 2016). Estradiol is most commonly used as a hormone replacement therapy for menopause, but is also used in treatments for cancer (breast and prostate) and infertility (King and Brucker 2010, Ruggiero and Likis 2002). Estriol is a metabolite of estradiol and mainly produced by the placenta throughout pregnancy (NCBI 2016). Estriol is also used as a treatment for menopause and given to pregnant women with multiple sclerosis (NCBI 2016). Among natural estrogens, estradiol has greater estrogenic potency than estrone or estriol since it has greater affinity towards estrogen receptors; however, synthetically derived 17α-ethinylestradiol (ethinylestradiol) is more potent and stable than natural estrogens (Atkinson et al. 2003, Langston et al. 2005). Ethinylestradiol is a component in contraceptive medications along with progestins and does not get fully metabolized after ingestion (Andersen et al. 2003, Andrew et al. 2010).

In vertebrates, estrogens are synthesized from androgen and progesterone precursors, they stimulate cellular proliferation and hydration through specific nuclear receptors. Cytochrome P450 is an enzyme that aids in steroidogenesis, and transforms androgens into estrogens (Gassman and Kennedy 1992, Snyder 2000, Armoza-Zvuloni et al. 2012). Cytochrome P450 enzymes are capable of oxidizing endogenous sex steroids

for steroid metabolism, as well as initiating detoxification processes in the presence of exogenous pollutants through oxidation (Gassman and Kennedy 1992).

Sex steroids are also metabolized through vertebrate P450 enzymatic pathways, and through either sugar or sulfate conjugation enzymes in the liver before excretion (Langston et al. 2005). The metabolized estrogens become hydroxlyated metabolites in the liver, and are excreted through urine as glucuronide conjugates (i.e., 17β-estradiol glucuronide) (Legler et al. 2002). The conjugation of hydroxlyated metabolites renders these estrogenic compounds inactive and occurs through either glucuronidation, sulfonation, or O-methylation (Zhu and Conney 1998, Legler et al. 2002). They are excreted through urine after being conjugated to glucuronides or sulfonates that are more water soluble compared to the unconjugated bio-active parent compound (Belfroid et al. 1999, Atkinson et al. 2003). Sex steroids are also excreted in humans through fecal matter at significantly lower concentrations. Unlike estrogens excreted through urine, fecal matter contains more unconjugated or biologically active forms. *Escherichia coli* bacteria found in the digestive system deconjugate steroid metabolites through ß-glucuronidase enzyme synthesis (Desbrow et al. 1998). Synthetic estrogens (ethinylestradiol) are also excreted in the unconjugated active form in greater concentrations than natural estrogens or sex steroids (Legler et al. 2002). When used clinically, more than 60% of ethinylestradiol is excreted through urine, whereas 3% remains in the blood plasma (Carr and Griffin 1998). As persistent endocrine-disrupting compounds, sex steroids have the potential to bioaccumulate in organisms living in aquatic environments and affect a wide variety of organisms at low concentrations.

Medications containing synthetic and natural steroidal hormones from heavily prescribed contraceptive and hormone replacement therapies substantially increase the prevalence and concentration of these compounds in sewage influent, septic tanks, and cesspits (Corcoran et al. 2010). The National Center for Health Statistics reported that approximately 28% of childbearing age women (15–44 years) use a contraceptive pill (or similar hormonal contraceptive) as a form of birth control (Jones et al. 2012). Surveys conducted from 1988–1998 estimated that nearly half of all post-menopausal women have used hormone replacement therapy (Brett and Chong 2001). In addition, 14% of post-menopausal women have used hormone replacement therapies for at least ten years

(Brett and Chong 2001). A Medical Expenditure Panel Survey for 2014 estimated that a total number of 48,462,525 people had hormone or hormone-modifier-related expenditures. Therefore, based on the most recent U.S. census bureau data, nearly 15% of the population has purchased hormone or hormone-modifier medications in the past year. Along the Florida reef tract, the combined population of Palm Beach, Broward, Miami-Dade, and Monroe Counties exceeds 6 million people. If extrapolated using the numbers above, that is approximately 900,000 people on hormone or hormone-modifier medications in southeast Florida and the Florida Keys. Considering this estimate does not include the millions of tourists who visit this region each year and sex steroids are naturally excreted through urine and feces. Thus, there is much potential for these endocrine-disrupting compounds to contaminate the marine environment.

1.4 Sex Steroids in Marine Organisms

Substantial evidence exists that natural and synthetic estrogens have endocrine-disruptive effects in fish and other vertebrates. Incidences of feminization in male fish worldwide have been associated with increased vitellogenin production from natural and synthetic estrogens in treated sewage effluent (Desbrow et al. 1998, Routledge et al. 1998, Lai et al. 2000). Multiple studies have shown that natural and synthetic estrogens at low concentrations (0.1–10 ng/L) can induce vitellogenin in male fish species; this is considered to be an effect-level range for sensitive species (Purdom et al. 1994, Rotchell and Ostrander 2003, Langston et al. 2005, Auriol et al. 2006). In teleost fish, progesterone is produced by the gonads, induces the final stages of oocyte maturation, and likely has a role in spawning (Rotchell and Ostrander 2003). In teleosts, progesterone is an intermediate steroidogenic mediator between pregnenolone and 17α-hydroxyprogesterone, acting as an essential intermediate among three pathways that produce hormones controlling oocyte and sperm maturation, spermatogenesis, and oocyte growth (Nagahama 1994).

In contrast to vertebrates, it is unclear how the endocrine system regulates reproduction and development in invertebrates. The early divergence between invertebrate and vertebrate phyla created differences in endocrine system processes and steroid metabolic pathways (Janer et al. 2005). Due to the physiological differences between vertebrates and scleractinian corals, research depicting the potential effects sex

steroids may have on marine invertebrates has greater relevance. There are large gaps in information and data due to the many different invertebrate groups, methodologies, hormones, and quantification techniques (Janer et al. 2005). Of all invertebrate groups, endocrine-disrupting compounds have been studied the most among molluscs. Research suggests that two general types of endogenous hormones exist in invertebrates, vertebrate-like sex steroids (i.e., estradiol, progesterone, and testosterone) and invertebrate-specific hormones such as ecdysteroids, mostly found in crustaceans (Lafont 2000). Vertebrate-like sex steroids have been identified in most invertebrate groups, including important cnidarian species.

Among cnidarians, the majority of sex steroid studies have looked into daily and monthly fluctuations corresponding with gametogenesis and spawning. Previous research has detected variable concentrations of estrone, estradiol, estradiol glucuronide, progesterone, and testosterone among anthozoan and scleractinian corals. In *Sinularia polydactyla*, Slattery et al. (1999) observed significantly greater progesterone concentrations in females than males, ranging from 824–3,855 ng/g and 604–1,740 ng/g, respectively. Estradiol concentrations were very similar among females and males, ranging from 5–16 ng/g and 5–19 ng/g, respectively (Slattery et al. 1999). In the scleractinian coral *Euphyllia ancora*, Twan et al. (2003) detected variable estradiol, estradiol glucuronide, testosterone, and testosterone glucuronide concentrations during a two-year period. Both estrogens were detected at significantly greater concentrations than either form of testosterone, and estradiol was detected at significantly greater concentrations than estradiol glucuronide (Twan et al. 2003).

In coral tissue, sex steroids have been detected at variable concentrations, often coinciding with reproductive events. In *Euphyllia ancora*, Twan et al. (2003) observed peak annual concentrations of estradiol, estradiol glucuronide, and testosterone glucuronide concentrations in coral tissue prior to spawning. Estradiol concentrations were significantly greater than estradiol glucuronide, while testosterone concentrations were comparatively lower than other months. Estradiol concentrations increased 8-fold and estradiol glucuronide concentrations increased 100-fold from one month prior to the spawning period (Twan et al. 2003). In *Renilla koellikeri*, Pernet and Anctil (2002) detected an increase in estradiol prior to and during spawning (in June), with significantly

greater concentrations in female corals. Estradiol concentrations immediately dropped to baseline following the spawning period where it remained until the following March. In *Montipora verrucosa*, Tarrant et al. (1999) detected increasing estrone and decreasing estradiol concentrations in the coral tissue days before spawning compared to concentrations from the previous month. Similarly, in *Pocillopora damicornis*, estrone and progesterone concentrations were approximately 6 times greater than estradiol over two monthly spawning periods; cholesterol (a precursor for sex steroid synthesis), progesterone, and testosterone were also detected (Rougee et al. 2015). In contrast to other studies, there were no significant differences in concentrations measured before, during, or after spawning when comparing each sex steroid to itself (Rougee et al. 2015).

Several sex steroids have also been detected in the water column near coral colonies during mass spawning events. Seven hours prior to *Sinularia polydactyla* spawning, Slattery et al. (1999) was unable to detect estradiol in the water column near any colonies. While spawning, water samples collected near female colonies had significantly greater concentrations than male colonies at 15.4 ng/L and 0.201 ng/L, respectively (Slattery et al. 1999). Similarly, Twan et al. (2003) was unable to detect estradiol, estradiol glucuronide, testosterone, and testosterone glucuronide in water samples collected near coral colonies one day before and after spawning for three consecutive years. During mass spawning, estradiol glucuronide samples were approximately 3.5 times greater than estradiol, and both were significantly greater than testosterone glucuronide. Testosterone was not detected in any water sample throughout the three-year experiment (Twan et al. 2003). In a study by Atkinson and Atkinson (1991), water samples were collected before, during, and after a mass coral spawning event to determine estradiol fluctuations. As expected, estradiol concentrations peaked during the spawning event at 4.2 ng/L from 1.0 ng/L pre-spawn, before decreasing back down to 1.5 ng/L post-spawn. All of these values were significantly greater than the open ocean control sample, which had an estradiol concentration of 0.55 ng/L (Atkinson and Atkinson 1991). These studies provide evidence that vertebrate-like sex steroids are prevalent in, and released by, scleractinian coral species during spawning events.

Evidence that corals are releasing sex steroids during spawning events and the fluctuations in steroid concentrations detected are important for several reasons. The

increases of estrogens (estrone and estradiol) before and during spawning indicates it may play a role in the late stages of gametogenesis (oogenesis) and/or spawning events (Pernet and Anctil 2002, Twan et al. 2003). Tarrant et al. (1999) detected increases of estrone following decreases of estradiol during stages of gametogenesis and prior to spawning; this indicates that corals may be capable of converting estradiol into estrone. The detection of free and conjugated estradiol and testosterone glucuronide detected in the water near corals during spawning events suggests they may have a functional role in gamete release and spawning (Twan et al. 2003). Estradiol glucuronide may play a role in spawning synchrony and act as a pheromone since it was detected at greater concentrations (3.5-fold) and has greater water solubility than estradiol (Twan et al. 2003, Tarrant 2005). Conjugated sex steroids have been shown to act as pheromones in several aquatic vertebrate species (Hurk and Lambert 1983, Sorensen et al. 1995). These studies provide evidence that several coral species can release and metabolize sex steroids that appear to have an important role in reproduction, gametogenesis, and spawning.

1.5 Steroid Metabolism

Vertebrate-like sex steroids likely have functional roles in marine invertebrates, but the complete physiological role of these compounds for any single species is fragmentary (Lafont and Mathieu 2007). The physiological processes of these compounds are relatively unknown in cnidarians and important metabolic enzymes are necessary for steroidogenesis and metabolism. It is also unknown whether sex steroids are activated in invertebrates. No vertebrate-like androgen-, estrogen-, or progestin-specific nuclear receptors have been found in invertebrates (Thornton et al. 2003). However, invertebrates have various nuclear receptors for other compounds that sex steroids may be able to bind to non-genomically. For example, nuclear receptor genes have been detected in cnidarians that researchers have been unable to classify to a specific nuclear receptor subfamily (Tarrant 2007). However, an estrogen receptor- (ER-) like ortholog was found in the mollusc, *Aplysia californica (*Thornton et al. 2003). The identification of this receptor provides evidence that other species may have steroid-like receptors, especially for estrogens (Janer and Porte 2007). Among marine invertebrates, the majority of scientific studies evaluating the synthesis, metabolic pathways, physiological roles, and impacts of these compounds (endogenous or exogenous) have

been completed using mollusc and echinoderm species (Lafont 2000, Janer and Porte 2007).

Recent research indicates that invertebrates may be able to synthesize these compounds endogenously from cholesterol. Molluscs and echinoderms appear to have most of the necessary enzymes to synthesize estrogens and progesterone from cholesterol (Lehoux and Sandor 1970, Sandor 1981, Lafont and Mathieu 2007, Andrew et al. 2008). The precursors and enzymes used for steroid metabolism and steroidogenesis are necessary to synthesize a variety of sex steroids (Janer and Porte 2007). Porte et al. (2006) mapped the steroidogenic pathways for molluscan and echinoderm species based on past research and vertebrate models beginning with cholesterol. Cytochrome P450s convert cholesterol into pregnenolone, then hydroxysteroid dehydrogenase converts pregnenolone into progesterone. Various cytochrome P450s can transform progesterone into 17α-hydroxyprogesterone, cortisol (in molluscs), or androstenedione. Androstenedione, an androgen and estrogen precursor, can be converted into estrone, estradiol, and testosterone via cytochrome P450 aromatase and 17β-hydroxsteroid dehydrogenases (17β-HSDs) (Porte et al. 2006, Andrew et al. 2008). While the entire steroidogenic pathway has not been proven for cnidarians, previous research has detected some of the necessary metabolic enzymes and precursors for endogenous production.

Gassman and Kennedy (1992) were the first to detect cytochrome P450s and other metabolic enzymes in a scleractinian coral (*Favia fragum*) or cnidarian species. It was hypothesized that cytochrome P450 and similar metabolizing enzymes may play a role in the reproductive cycle of *Favia fragum*, however they did not find variability in annual concentrations (Gassman and Kennedy 1992). Aromatase (part of the cytochrome P450 family) activity was also observed for the first time in coral tissue by Twan et al. (2003). Increasing aromatase concentrations were detected and peaked during the spawning period. Hypothetically, these enzymes may be able to biosynthesize estradiol from testosterone endogenously since there was a decrease in testosterone coinciding with increases in estradiol preceding the spawning period (Twan et al. 2003). Twan et al. (2003) is the first to suggest that corals may be capable of endogenously synthesizing sex steroids since sex steroids remained non-detectable in the water column while sex

steroids were detected in coral tissue along with crucial metabolizing enzymes throughout the year.

Several other important steroidogenic enzymes have been detected in various cnidarian species. In two Antarctic soft coral species, Slattery et al. (1997) reported physiologically relevant concentrations of steroidogenic enzymes: 5α-reductase, 3β-hydroxysteroid dehydrogenase, and 17βhydroxysteroid dehydrogenase. Tarrant et al. (2003) showed the presence of 17β-HSD and 5α-reductase enzymes in three different coral species. Research by Rougee et al. (2014) observed the CYP2E1, β-glucuronidase, and arylsulfatase C (ASC) metabolizing enzymes in the cnidarian (*Pocillopora damicornis*) for the first time. β-glucuronidase and ASC are phase II regenerating enzymes that catalyze conjugated glucuronic acid and sulfate molecules, respectively. Cytochrome P450 reductase, cytochrome P450 2E1, UDP glucuronosyltransferase (UGT), and glutathione-S-transferase were also detected. UDP glucuronosyltransferase is a phase II enzyme able to catalyze steroid hormones with annual variations appearing to coincide with the brooding coral *P. damicornis* reproductive cycle. All of these identified enzymes have potential to affect steroidogenesis in corals.

Reproductive enzymes have also been detected in corals coinciding with spawning events. Gonadotropin-releasing hormone (GnRH) is a hypothalamic neuropeptide that regulates reproduction in vertebrates; an immunoreactive GnRH-like (irGnRH) compound was detected in *E. ancora* for the first time by Twan et al. (2006). Annual variations in irGnRH also fluctuated with the spawning period similarly to aromatase activity, estradiol, and estradiol glucuronide. The greatest irGnRH concentrations occurred during the spawning period and were comparatively 10 times greater than outside of the spawning season. Based on these reports detecting increases in estradiol, estradiol glucuronide, aromatase activity, and irGnRH coinciding with *E. ancora's* spawning season, it seems likely that these compounds play an important role in steroidogenesis, gametogenesis, and mass spawning events (Twan et al. 2003, Twan et al. 2006).

Genomic work is crucial to fully understanding the physiology of sex steroids in cnidarian species. Putnam et al. (2007) completed the genome for the sea anemone *Nematostella vectensis*. This is the first genome completed for any cnidarian species

providing an entirely new route of research examining sex steroid metabolism in cnidarians (Tarrant et al. 2009). Analysis of this genome indicates that *N. vectensis* has short chain dehydrogenase enzymes with homologs of genes capable of metabolizing steroids in other organisms (Tarrant et al. 2009). Two of these orthologous genes (17β-HSD4 and 17β-HSD8) are associated with oxidative enzymes that can convert estradiol into estrone, but most likely do not have as large a role in steroid metabolism as other 17β-HSD's (Tarrant et al. 2009). However, *N. vectensis* did not contain homologs for many other enzymes necessary for steroidogenesis, and homogenates have yet to prove their ability to metabolize steroid substrates as several coral species have through 17β-HSD enzymes (Tarrant et al. 2009).

Past research has not proven that corals can endogenously produce sex steroids; however, this research indicates through the presence of cholesterol, steroidogenic enzymes, estrogenic, progestogenic, androgenic, and other metabolizing enzymes that corals have the potential to do so. Much information about the various roles, pathways, and effects that exogenous sex steroids may have on the physiological processes of scleractinian corals are still unknown. The effects of exogenous sex steroids are further complicated by the lack of data on marine concentrations of various sex steroids and synergistic effects.

1.6 Effects of Sex Steroids

The majority of ecotoxicological studies have been done on molluscs because they are sessile organisms, filter feeders, and bio-accumulate inorganic and organic compounds (Andrew et al. 2010). Exogenous sex steroids studied have produced a wide variety of results ranging from changes in reproductive development and physiology to no adverse effects. Le Curieux-Belfond et al. (2005) observed that the Pacific oyster *Crassostrea gigas* accumulated E2 and E2 metabolites at a rate 31 times greater than saltwater controls in its tissue. Additionally, this oyster was able to metabolize nearly all of the estradiol into estrone through 17β-hydroxysteroid dehydrogenase enzymes that were present in treatment tissue samples. Previous research indicates that exogenous estrogens can initiate vitellogenesis, increase oocyte development, and feminize males (Langston et al. 2005). Vitellogenins are precursor proteins and essential for developing egg yolk (vitellin) during reproductive development and may be regulated by estradiol

(Andrew et al. 2008). Vitellogenins can be induced in juvenile male oysters by estrogens and reduce male development, cause intersex gametes, and ultimately, sex reversal (Andrew et al. 2010). In an ethinylestradiol treatment (50 ng/L), there was a three-fold increase in vitellogenin production in the female oyster *Saccostrea glomerata* compared to the control, whereas males also had a significant increase in vitellogenin production but to a lesser extent (Andrew et a. 2008). The authors also found linear dose-responses between ethinylestradiol treatments and vitellogenin production through four days. Andrew et al. (2010) concluded that females are receptive to estrogenic compounds with increased vitellogenin production and oocyte growth; however, males had endocrine disruption through vitellogenin production inhibiting male development and increasing sex reversal.

Several studies have reported sex steroids having direct effects on reproduction in molluscs and echinoderms. Wang and Croll (2004) injected estradiol, progesterone, and testosterone into the sea scallop *Placopecten magellanicus,* observing that each sex steroid induced masculinization by accelerating gonad differentiation, thereby increasing the male-to-female ratio. Female scallops had morphological changes from the estradiol and testosterone treatments that induced oocyte growth and deteriorated oocytes, respectively. However, they found no significant differences between body, shell, and gonad weights (Wang and Croll 2004). Studies treating echinoderms with in vitro estradiol treatments have generally found an increase or stimulation of ovarian or oocyte growth (Schoenmakers et al. 1981, Barker and Xu 1993). A more recent study has found that estradiol treatments can disturb sea urchin embryological development (Roepke et al. 2005). Despite the variation in response to exogenous estrogens and other sex steroids, evidence suggests that they can affect vitellogenin synthesis, reproductive development, and sex determination in some invertebrates. However, there are no clear dose-response relationships between sex steroids and vitellogenin production, reproductive development, and sex determination for invertebrates (Ketata et al. 2008).

Due to the presence of various sex steroids and related enzymes in corals and other organisms, it has been hypothesized that exogenous sex steroid pollution can affect coral physiology, reproduction, and ultimately reef ecosystems (Tarrant et al. 2004). Due to the importance of successful coral reproduction and recruitment in long-term reef

resilience, it is evident that further research is necessary to determine dose-response relationships (the response of an organism when exposed to varying levels of a stressor) with common sex steroids such as estradiol and progesterone. The distribution and accumulation of sex steroids in corals are unknown; these lipophilic compounds may bind to the lipid-rich epidermis and be distributed through the polyp by cellular diffusion into the gonads (Armoza-Zvuloni et al. 2012). Furthermore, gastrovascular canals are hydrophilic in nature, but carrier proteins could facilitate the passage of these compounds to reproductive cells (Gateño et al. 1998, Baker 2002).

Corals can immediately obtain lipophilic or hydrophobic chemicals (i.e., sex steroids) from the water column at a rate exceeding metabolism and release (Gassman and Kennedy 1992, Peters et al. 1997, Tarrant et al. 2001). Tarrant et al. (2001) provided evidence that corals can bioaccumulate dissolved estrone very quickly in an experimental system with environmentally relevant estrone concentrations by analyzing tank water samples. At a concentration of 2 ng/L, the estimated uptake rate for live corals was 0.17 ng $m^{-2} s^{-1}$, whereas the calculated release rate was 0.033 ng $m^{-2} s^{-1}$. After 2.5 hours of dosing, corals released approximately 20% of the radiolabeled estrone back into the water column. The estrone released was fully active and unaltered by the coral. The presence of conjugated estrogens or estradiol in the tissue or tank was not analyzed in this study. Estrone concentrations in the corresponding tanks were significantly lower for live corals in comparison to fixed corals and frozen control fragments, indicating adsorption to the lipid-rich epidermis of coral tissue is not the primary pathway and diffusion across cell membranes is needed (Tarrant et al. 2001). This research indicated that corals can potentially bioaccumulate estrone at concentrations as low as 0.3 ng/L (Tarrant et al. 2001, Atkinson et al. 2003). Bioaccumulation of the more estrogenically potent forms (estradiol and ethinylestradiol) has not been studied. This research suggests that estrogens and other sex steroids have the potential to bioaccumulate in coral tissues since uptake rates appear to be greater than metabolic processes. Twan et al. (2006) observed that dosing corals with GnRH increased aromatase activity and sex steroid concentrations, resulting in a 20% increase in oocyte growth.

In the freshwater cnidarian *Hydra vulgaris*, males and females exposed to high ethinylestradiol treatments (500 µg/L) had significantly lower numbers of testes and

oocytes produced, respectively (Pascoe et al. 2002). However, these concentrations were far greater than environmentally relevant concentrations, and produced no reproductive effects in this study. Tarrant et al. (2004) observed that *Montipora capitata* colonies treated with estradiol for 3 weeks prior to spawning had a 29% decrease in the release of egg-sperm bundles at greater than environmentally relevant concentrations. Additionally, in two replicated seasonal experiments, *Porites compressa* fragments treated with estrone for 2–8 weeks had a 13% and 24% decrease in skeletal growth and had greater tissue thickness compared to the controls at environmentally relevant concentrations (2 ng/L). Tarrant et al. (2004) hypothesized that exogenous sex steroids may inhibit skeletal growth by controlling cellular proliferation.

A study along the Israeli-Mediterranean coast looked into the effects of exogenous steroid hormone pollution on reproductive development in scleractinian corals at sites contaminated with acute and chronic sources of pollution. Colonies exposed to acute municipal pollution for one year had incomplete or disrupted gametogenesis, characterized by smaller oocyte diameter and a lower proportion of fully developed testes compared to the reference and chronically contaminated sites (Armoza-Zvuloni et al. 2012). Sites with chronic exposure to these contaminants (several decades) appeared to better tolerate steroidal hormone pollution. Analysis of water samples indicated significantly higher estradiol concentrations at the contaminated sites compared to reference sites, yet there were no significant differences for testosterone. Chronically contaminated sites did not have reduced gametogenesis, indicating some exogenous sex steroid inputs may not reduce reproductive capabilities, even though there were significantly greater concentrations of estradiol, progesterone, and testosterone in coral tissues collected from contaminated sites (Armoza-Zvuloni et al. 2012). It is possible that acute stressors may initiate adaptation processes favoring hardy genotypes that can withstand chronic pollution (Armoza-Zvuloni et al. 2012). This study is the first to find high concentrations of estradiol, progesterone, and testosterone in scleractinian coral colonies from sewage-contaminated sites, suggesting bioaccumulation is at least relevant at contaminated sites. Also, *O. patagonica* is a non-indigenous coral much more tolerant to stressors and abnormal environmental conditions compared to indigenous species,

perhaps decreasing relevance to threatened Caribbean species (Armoza-Zvuloni et al. 2012).

Despite many studies, the information and data depicting the various roles, pathways, and synergistic effects exogenous sex steroids and other contaminants have on the physiological processes of scleractinian coral remains unclear. The effects that sex steroids and other exogenous pollutants can have on coral reproduction and health can vary greatly depending on species, period of exposure, and concentration (Armoza-Zvuloni et al. 2012). Few studies have examined the effects of exogenous estrogens and testosterone on coral growth and reproduction, but there is some evidence that impacts vary among many factors, such as concentration, form of steroidal hormone, time of year, and coral species (Twan et al. 2003, Tarrant et al. 2004, Armoza-Zvuloni et al. 2012). More research is needed to determine whether or not sex steroids are affecting or have the potential to affect future reef coral populations. Some concerns regarding the quantification and analysis of sex steroids and related enzymes should be addressed.

Scientists have raised questions about sex steroid identification and quantification methodologies (Markov et al. 2009). Most studies examining invertebrate sex steroid concentrations have used radioimmunoassay (RIA) with vertebrate antibodies. These antibodies can cross react with similar sex steroids or endogenous non-vertebrate steroids. Kime and Larsen (1987) and Lowartz et al. (2003) showed that vertebrate steroids quantified using RIA in a sea lamprey were actually a more water soluble 15α-hydroxylated steroid using high-performance liquid chromatography (HPLC), which did not detect any of the classical vertebrate sex steroids. Markov et al. (2009) found a lack of homologous vertebrate genes for steroid-generating enzymes in cnidarians and other invertebrates. This questions whether vertebrate-like steroids are more likely to act as hormones than natural endogenous steroids in invertebrates. Markov et al. (2009) suggested the use of GC-MS to identify all endogenous steroids and precursors to model metabolic pathways to validate physiological effects.

Vertebrate sex steroids have been determined in cnidarians, however it is unclear whether they are biologically active or misidentified cnidarian homologs (non-vertebrate steroids), which can occur through radioimmunoassay analysis (Tarrant et al. 2005, Markov et al. 2009). Although a steroid-specific nuclear receptor (e.g., estrogen receptor)

has yet to be found in cnidarians, sex steroids may be able to function through a homologous invertebrate-specific steroid receptor or other receptors activating non-genomic effects (Tarrant et al. 2005).

1.7 Study Species

Acropora cervicornis is an extant branching coral on reefs of the greater Caribbean region, and is found as far north as Palm Beach County. Its unusual branching morphology provides fisheries and invertebrate habitat that is unlikely to be replicated by other species in this region (Young et al. 2012). Colonies prefer shallow reefs with low to moderate wave action at various depths ranging from approximately 5–25 meters (Aronson and Precht 2001). It is a fast growing coral species with growth rates ranging from 3–11.5 cm/year, with rates in Florida reported at 10–11.5 cm/year (Shinn 1976, Boulon et al. 2005). This species can reproduce asexually through fragmentation or sexually as a hermaphroditic broadcast spawner. Broadcast spawning colonies simultaneously release their gamete bundles into the water column for fertilization. In south Florida, *A. cervicornis* spawns a few days before and after the full moon in the months of August and September. The short spawning period coincides with a long reproductive cycle. Oocytes develop slowly taking up to 10 months, while spermaries are often not seen in the tissue until a month before spawning in the months of July and August (Szmant 1986, Soong 1991). Once released, gamete bundles break down at the ocean surface allowing sperm to externally fertilize eggs. Successfully fertilized eggs will develop into planula larvae, which settle for suitable substrate for metamorphosis.

In the past three decades, *A. cervicornis* populations have been reduced by more than 80% (Aronson et al. 2008) due to sensitivity to bleaching and white-band disease. Boulon et al. (2005) have reported population reductions of 97% in Belize, Dry Tortugas, Florida Keys, Jamaica, and St Croix. Throughout South Florida, the Florida Keys, and the Caribbean, there are more than 60 restoration projects from 14 different countries using the threatened *Acropora* species (Young et al. 2012). *A. cervicornis* was used in 48% of these research projects, while an additional 40% utilized it and *Acropora palmata* (Young et al. 2012). Each location has different stressors and the potential to contain physiologically significant sex steroid concentrations (Young et al. 2012). Determining potential effects that these sex steroids may have on the growth and health of *A.*

cervicornis will be beneficial to the many conservation and restoration efforts being conducted.

In contrast, *Porites astreoides* is a common hermaphroditic brooding coral found in the greater Caribbean, southeast Florida, and the Florida Keys with relatively high recruitment and settlement rates (Cooper 2009). After internal fertilization, this species releases planulae into the water column with spawning occurring around the new moon primarily in the months of April, May, and June. The planulae can swim around for days before finding a suitable substrate to settle and metamorphose on. As a relatively resilient and abundant reef species in this region, it has been used as an indicator species for model systems simulating reef resiliency under variable climate change scenarios (Cooper 2009). Thus, it is a suitable species to examine the influence sex steroids may have on coral reproductive success.

1.8 Objectives

This project examined the effects of 17 β-estradiol and progesterone contamination on *Acropora cervicornis* and *Porites astreoides* growth, overall health, and reproduction. *Porites astreoides* larvae were collected in the spring of 2014 to examine any impacts these hormones might have on larval settlement and viability. Dose-response experiments were conducted on adult *P. astreoides* and *A. cervicornis* fragments to examine any effect these compounds may have on coral health, reproduction, and growth. Each species was collected from Broward County and lower Keys reefs to evaluate potential effects from two distinct regions. The dosing experiments were conducted at the Halmos College of Natural Science and Oceanography's Central Experimental Pollution Facility Seacor System. In addition, water samples were collected from Broward County (2013 and 2014) and lower Florida Key's reefs (2014) to determine baseline estradiol and progesterone concentrations. This established environmentally relevant concentrations critical to the larval and dosing experiments.

Chapter 2

Does exposure to 17 β-estradiol and progesterone influence growth and reproduction of two scleractinian corals?

2.1   Introduction

Coral reefs are an invaluable ecological and economical resource worldwide. They fill a vital structural and functional role in the ecosystem by providing habitat to marine organisms, coastal buffering, energy flow, island formation, and biogeochemical cycling (Goreau and Hilbertz 2005). Economically, coral reefs provide societies with building materials, medicines, tourism, fisheries, and sustenance that are critical to less developed countries (Moberg and Folke 1999, Hoegh-Guldberg et al. 2007). Increasing water temperatures and ocean acidification from climatic changes have had profound consequences, leading to coral bleaching events and disease outbreaks worldwide (Hoegh-Guldberg et al. 2007). In addition, reefs are affected by many localized stressors, such as destructive fishing practices, land based pollution, sedimentation, urbanization, invasive species, and shipping (Cesar et al. 2003, Jackson et al. 2004, Wilkinson 2008). Globally, approximately 19% of coral reef area has been destroyed with 35% more at risk of being eliminated over the next couple decades (Wilkinson 2008). The greater Caribbean has been hit hard with coral cover decreasing from 34.8% to 16.3% since 1970 (Jackson et al. 2014). The important shallow reef-building coral, *Acropora cervicornis*, has decreased more than 80%, with losses up to 97% in the Florida Keys (Boulon et al. 2005, Aronson et al. 2008).

Substantial funding and research has been invested in monitoring and restoring coral reefs, and studying numerous stressors (e.g., disease, bleaching, and ocean acidification) affecting these reefs; however, the potential contributions of sex steroids to coral reef degradation have been largely overlooked. Sex steroids are endocrine-disrupting compounds (EDCs) that can affect synthesis, metabolism, secretion, and transport of natural hormones responsible for physiological homeostasis, development, reproduction, and behavior (Baker 2002, Le Curieux-Belfond et al. 2005, Blomquist et al. 2006, Ketata et al. 2008).

The three major classes of natural sex steroids in humans and vertebrates are androgens, estrogens, and progestins. Natural estrogens are mainly composed of estrone,

23

17β-estradiol (estradiol), and estriol. Progesterone is the only naturally occurring progestin and is a precursor for steroidogenesis of androgens and estrogens (Porte et al. 2006, King and Brucker 2010). In vertebrates, sex steroids are produced endogenously and excreted through urine and feces (Daughton 1999). Medications containing synthetic and natural steroidal hormones, such as contraceptive and hormone replacement therapies, substantially increase the prevalence and concentration of these compounds in sewage influent (Corcoran et al. 2010).

The primary route for these compounds to enter coastal environments in southeast Florida and the Florida Keys is from wastewater treatment plants, septic systems, and cesspits. Southeast Florida has six open ocean outfalls throughout the highly urbanized Palm Beach, Broward, and Miami-Dade counties. Cumulatively, they release approximately 400 million gallons of effluent per day that receives secondary treatment and basic-level disinfection, estimated to remove 38–83% of natural and synthetic sex steroids from the wastewater (Ternes et al. 1999, Desbrow et al. 1998, Auriol et al. 2006, Koopman et al. 2006). Throughout the Florida Keys, there are central sewage facilities that utilize injection wells, as well as approximately 30,000 on-site sewage disposal systems (septic tanks or cesspits) that are all susceptible to leaching (Lapointe et al. 2004). The geology of southeast Florida and the Florida Keys provides for easy groundwater flow of pollutants into the ocean from tidal pumping and high precipitation since it is composed of a thick layer of porous limestone that lacks soil (Darden 2000). The large human population in this region along with current wastewater practices highlight the potential for contamination along the Florida Reef Tract (FRT).

The FRT stretches 150 miles from Martin to Monroe County through two distinct regions (southeast Florida and the Florida Keys) that are exposed to many stressors including sewage contamination (Fauth et al. 2011). There are few data on sex steroid concentrations in the marine environment and in marine organisms along the FRT and greater Caribbean. At offshore reefs throughout the Keys, estrone concentrations have ranged from not detectable (ND)–0.88 ng/L, while estradiol concentrations were not detectable (Atkinson et al. 2003, Singh et al. 2010). Samples from near-shore environments (canals, shore, channels, and harbors) had greater estrone and estradiol concentrations ranging from 0.66–5.2 ng/L and ND–1.8 ng/L, respectively (Atkinson et

al. 2003, Singh et al. 2010). To my knowledge, progesterone has not been quantified near any reef or coastal environment. Since there are large gaps in the data for many invertebrates along with differences among methodologies and quantification techniques making it complicated to compare results, it remains unknown if sex steroids found at the above concentrations will affect scleractinian corals (Janer et al. 2005).

In contrast to vertebrates, it is still unclear how the endocrine system regulates reproduction and development in invertebrates. Vertebrate-like sex steroids have been identified in most invertebrate groups, including important cnidarian species. The physiological processes of these compounds are relatively unknown in cnidarians and important metabolic enzymes are necessary for steroidogenesis and metabolism. Recent research indicates that cnidarians may be able to endogenously synthesize sex steroids. Cholesterol (the precursor for steroidogenesis in vertebrates), intermediate steroidogenic enzymes, progesterone, estrogens, androgens, and other intermediate metabolizing enzymes have been detected in various species (Gassman and Kennedy 1992, Twan et al. 2003, Slattery et al. 1997, Tarrant et al. 1999, Twan et al. 2003, Armoza-Zvuloni et al. 2012). Multiple studies have reported annual fluctuations of estrone and estradiol in coral tissue, with increases often coinciding with gametogenesis and mass spawning events (Tarrant et. 1999, Pernet and Anctil 2002, Twan et al. 2003). Twan et al. (2003) detected elevated estradiol and estradiol glucuronide concentrations prior to and during spawning events in *Euphyllia ancora* tissue. Throughout the year, estradiol was significantly higher in the tissue compared to its inactive conjugated form, except during spawning, in which estradiol glucuronide was greater in tissue and the water column compared to estradiol. This suggests estradiol and conjugated estradiol may have a functional role in gamete release and spawning events (Twan et al. 2003). A follow-up study detected cholesterol (precursor to steroid synthesis), estrone, estradiol, progesterone, and testosterone in *Pocillopora damicornis* tissue (Twan et al. 2006). The detection of free and conjugated estradiol and testosterone in the water near corals during spawning events suggests they may have a functional role in gamete release and spawning synchrony (Twan et al. 2003).

The presence of cholesterol, steroidogenic enzymes, estrogenic, progestogenic, and androgenic hormones highlights the potential for corals to metabolize and synthesize these compounds; however, there is also evidence that exogenous sex steroids can affect

and may bioaccumulate in corals. Estradiol, progesterone, and testosterone have also been detected at increased levels near sewage-contaminated sites that are close to reef environments (Armoza-Zvuloni et al. 2012). Tarrant et al. (2001) showed that three coral species can accumulate dissolved estrone in an experimental system with environmentally relevant estrone concentrations. After 2.5 hours of dosing, corals released approximately 20% of the radiolabeled estrone back into the water column, indicating uptake rates can be greater than metabolic processes. Environmentally, acute exposure (1 year) to sex steroids was reported to disrupt gametogenesis, decrease oocyte diameter and proportion of fully developed testes in the coral *Oculina patagonica* (Armoza-Zvuloni et al. 2012). Meanwhile, chronic exposure (several decades) had no observable effects on coral health or reproduction (Armoza-Zvuloni et al. 2012). The limited information on the effects of sex steroids along with the presence of various sex steroids and necessary enzymes in corals indicates exogenous sex steroid pollution may potentially affect coral physiology, reproduction, and ultimately reef ecosystems (Tarrant et al. 2004).

Much is unknown about the pathways and concentrations of sex steroids in coastal environments and the synergistic effects on the physiological processes of scleractinian coral. This project had three primary objectives: (1) establish environmental estradiol and progesterone concentrations in Broward County (southeast Florida) and lower Florida Keys reef environments, (2) conduct estradiol and progesterone larval assays on *P. astreoides* larvae to determine the effects of these compounds on settlement and viability, and (3) conduct estradiol and progesterone dosing experiments on adult *Acropora cervicornis* and *Porites astreoides* fragments to determine the effects on growth, zooxanthellae, reproduction, and overall tissue health. The baseline estradiol and progesterone concentrations were used to establish environmentally relevant concentrations for each experiment. *Acropora cervicornis* is a threatened broadcast spawning species sensitive to bleaching and disease, and is used in many restoration projects throughout the region (Aronson et al. 2008, Young et al. 2012). In contrast, *P. astreoides* is an abundant and relatively resilient hermaphroditic brooding coral with high settlement and recruitment rates.

2.2   *Materials and Methods*

2.2.1   *Water sample analysis*

To determine baseline estradiol and progesterone concentrations in Broward County and lower Key's reef environments, surface and coral-depth water samples were collected in spring 2013 and 2014. In Broward County, samples were collected at the HCNSO offshore coral nursery, Southeast Florida Coral Reef Evaluation and Monitoring Project sites (SECREMP), and the city of Hollywood open ocean outfall (2014 only). In the lower Keys, samples were collected from Mote's Tropical Research Laboratory's offshore coral nursery and Birthday Reef near Summerland Key. When possible, three separate samples were collected at the surface and at coral-depth for each site in 1-L Nalgene bottles. These samples were kept in a cooler on ice until arrival in the lab where they were immediately stored in a -20°C freezer.

The sex steroid extraction procedure began by pouring the thawed water samples through a prepped C-18 column attached to a vacuum flask. After each sample, a rinse of RO water (100 mL's) were run through the column. The columns were stored in the -20°C freezer prior to being eluted. The sex steroids were eluted from the C-18 columns by three separate 100-mL changes of solvents in a clean vacuum flask. The first solvent was a 1:1 solution of reverse osmosis (RO) water and methanol, followed by 100% methanol, and lastly 100% ethyl acetate. Solvents for each sample were combined in flasks with a glass stopper or foil lid and stored in the -20°C freezer. A rotary evaporator reduced sample volume until there were 2–3 mL of the sample remaining in the solvent trap, this volume was pipetted into pre-cleaned scintillation vials. The scintillation vials were placed under an air manifold in a fume hood to evaporate off the remaining volume of solvent and stored in the -20°C freezer.

These vials were analyzed using estradiol- and progesterone-specific enzyme-linked immunosorbent assay (ELISA) kits from Cayman Chemical. The estradiol kit had an assay range of 0.0066–4.0 ng/mL, with 100% specificity for estradiol; in this kit, estradiol antibodies have cross reactivity with estradiol-3-sulfate (14.5%), estradiol-3-glucuronide (14%), estrone (12%), and estradiol-17-glucuronide (10%). The progesterone kit had an assay range of 0.0078–1.0 ng/mL with 100% specificity for progesterone; the progesterone antibodies have cross reactivity with pregnenolone (14%), estradiol (7.2%),

and 5β-pregnan-3α-ol-20-one (6.7%). EIA buffer (1 mL) was added to each vial (diluted later if necessary) and vortexed for 10 seconds. Each sample was loaded in triplicate following the estradiol- and progesterone-specific protocol provided in each assay kit. The 96-well plates were analyzed using a Thermo Scientific Multiskan Microplate Spectrophotometer with a 412 nm wavelength. The plate was developed 60–90 minutes until the absorbance readings of each B_0 (maximum binding) well were between 0.3 and 1.0 absorbance after subtracting the average absorbance from the blank wells. The values from each plate were analyzed using the estradiol- and progesterone-specific Excel workbooks provided by Cayman Chemical online.

2.2.2 Larval dose-response experiment

For the larval settlement and viability experiments, *P. astreoides* colonies were collected from Broward County reefs in April and May 2014. All colonies were kept in onshore nursery tanks and placed in plastic pitchers each night before the new moon each month. In the pitchers, each colony was supplied with running water that flowed down the handle into plastic tri-pour cups that had their bottoms replaced with mesh to allow water circulation. Larvae from each colony were collected in the tri-pour cups and counted the next morning with glass pipettes before being pooled together. Lower Keys *P. astreoides* colonies were collected from a reef near Summerland Key and brought back to Mote's Tropical Marine Laboratory (MTML). Released larvae were transported to the Guy Harvey Oceanographic Center (GHOC) three days after collection for the larval experiments.

For all larval experiments, 20 larvae were dosed in plastic petri dishes containing pre-conditioned aragonite settlement discs using filtered (0.2 µm) seawater. These discs were conditioned at the offshore coral nursery for at least four weeks to develop a biofilm layer and crustose coralline algae to induce settlement. The 2014 Broward County (n=13 replicates) and lower Keys (n=5 replicates) assays were exposed to the control, 1 ng/L estradiol, 10 ng/L estradiol, 5 ng/L progesterone, and 30 ng/L progesterone treatments. Throughout the 10-day experiments, 50% water changes were completed every third day. Stock steroid solutions for each treatment were used to maintain proper sex steroid concentrations during these water changes. The total number of larvae surviving 1, 4, 7 and 10 days were quantified and scored depending on the following settlement

categories: swimming larvae, freely metamorphosed, settled on petri dish, or settled on top, side, and bottom of disc. Larvae that had not metamorphosed, settled on the disc or dish, and appeared to be healthy were scored as swimming even if they were not actively swimming.

2.2.3   Adult dose-response experiment

In April 2014, 12 large *P. astreoides* colonies were collected from Broward County (Fort Lauderdale) and lower Keys (Summerland Key) reefs. In addition, 90 *Acropora cervicornis* fragments (~7 cm long) from 18 different genotypes were collected from the HCNSO and Mote's Tropical Marine Laboratory (MTML) offshore coral nurseries. Corals collected from the lower Keys were transported to Broward County after being wrapped loosely in seawater soaked bubble wrap. At the on-shore nursery, two 1-cm fragments were clipped from the base of each *A. cervicornis* fragment for pre-exposure zooxanthellae density and sex steroid analysis. Each remaining *A. cervicornis* fragment was affixed to a 2" x 2" travertine tile using CorAffix gel. *Porites astreoides* colonies were cut into 6 equally sized fragments using a wet saw, resulting in five experimental fragments and one pre-experimental control fragment. These fragments were given a 2-week recovery period prior to the experiment.

Corals were placed in 40-gallon experimental tanks for the 3-week dosing experiment. There were two separate trials, each having a control, low estradiol (1 ng/L), high estradiol (5 ng/L), low progesterone (5 ng/L), and high progesterone (30 ng/L) treatment tank. A stock solution was made for each treatment by diluting estradiol and progesterone into 1 mL of ethanol, these were serially diluted to create a working solution to dose the experimental and dosing tanks. Every three days, the 40-gallon experimental tanks received a 50% seawater change to maintain water quality and replenish sex steroids from the 20-gallon dosing tanks using peristaltic pumps. Stock steroid solutions for each treatment were injected into the dosing tanks to maintain proper sex steroid concentrations. Prior to and following the 3-week dosing period, all fragments were photographed with a ruler placed vertically next to each fragment. The *A. cervicornis* fragments were measured for skeletal growth using ImageJ and buoyant wet weight methods. Additionally, tissue samples from each fragment were fixed in a solution

of a buffered zinc formalin fixative [1-part Z-Fix Concentrate (Anatech, Ltd.) diluted with 4 parts of seawater] for histological and zooxanthellae density analyses.

2.2.4 *Skeletal growth measurements*

Skeletal growth rates of *A. cervicornis* fragments were measured using the buoyant wet-weight method described by Davies (1989), prior to and immediately after the 3-week dosing experiment using an analytical balance. To calculate skeletal mass, the following formula was used: $M_{air} = M_{water} / [1 - (D_{water} / D_{object})]$ (Jokiel et al. 1978, Ferrier-Pages et al. 2000). Temperature and salinity were recorded throughout weighing to determine seawater density, which was consistently 1.023 mg/L. Growth rates for buoyant wet weight and linear extension were normalized to fragment size since growth is dependent on fragment weight (Ferrier-Pages et al. 2000). The normalized growth (G) was reported as percent change in mass per time period by using the formula: $G = (M_{t+1} - M_t) / [M_t * (T_{t+1} - T_t)]$ (Ferrier-Pages et al. 2000). Linear extension rates were measured through ImageJ analysis and also reported as normalized growth using the same formula as above. Pictures were taken of each *A. cervicornis* fragment before and after the 3-week dosing experiment outside of the acclimation and experimental tanks. A ruler was placed parallel to each fragment to create an accurate scale bar for measurement in ImageJ. Each photograph was carefully taken at approximately the same distance from the coral fragment to reduce error.

2.2.5 *Zooxanthellae density*

Zooxanthellae are the algal symbionts found in coral. Their densities were measured in pre- and post-treatment fragments for *A. cervicornis* and post-treatment for *P. astreoides* as an indicator of coral health. All coral fragments were decalcified with 5% HCL/EDTA and stored in 70% ethanol. A corer (13 mm diameter) was used to standardize the amount of tissue per sample resulting in a surface area of 13.27 cm^2. Coral samples were homogenized using a 15-mL VWR® PTFE tissue grinder and transferred to scintillation vials with 10 mL (*P. astreoides*) and 4 mL (*A. cervicornis*) of 70% ethanol, respectively. From the scintillation vial, 1 mL was centrifuged at 6500 RPM for 4 minutes. The supernatant was discarded and pellet was diluted with 1-mL of RO water. Ethanol was not used to dilute since it would evaporate quickly, interfering with the zooxanthellae counts. The zooxanthellae were loaded into a hematocytometer

with a glass pipette to be counted. Cells were counted in the outside four squares and replicated five times. The formula used to determine the cell density (cells/cm^2) is: (Number of cells per square)*(dilution factor)*(10^4).

2.2.6   Histological analysis

Prior to and immediately preceding the dosing experiment, small fragments were collected from each *A. cervicornis* and *P. astreoides* colony. Each fragment was fixed in a 50-mL plastic centrifuge tube using the Z-Fix solution. Coral samples were decalcified using a 5% HCL/EDTA solution that was replaced daily until all skeleton was removed. Endolithic organisms and algae were also removed when present. Once decalcified, tissue samples were trimmed and cut to obtain cross and longitudinal sections. Coral tissue samples were processed through a graded series of increasing ethanol concentrations, 100% xylene, and embedded in blocks using Paraplast Plus®. Each block was sectioned at 5 µm at three depths 50 µm apart for a total of three slides per coral sample. Each slide was stained using Harris's hematoxylin and eosin staining protocol. Slides were analyzed on an Olympus BX 43 light microscope at 10x and 40x magnification. The number of oocytes and spermaries were counted for each sample.

2.2.7   Statistical analysis

All larval settlement, skeletal growth, and zooxanthellae density data were analyzed using R Studio statistical software. Data were tested for normality, equal variance, and transformed to run a one- or two-way ANOVA. If the data were non-parametric, a Kruskal-Wallis ANOVA (one-way and two-way) test was used. Larval survival over the 10-day experiment was analyzed using a Kaplan Survival Curve in JMP software.

2.3   Results

2.3.1   Water sample analysis

Estradiol and progesterone was detected in every surface and coral-depth water sample at each location (Table 1). Since the progesterone assay has a narrower range of detection, progesterone was detected in every sample but below the detection limit of 7.8 ng/L. The standard curve is necessary for accurate quantification, therefore, these data should be interpreted cautiously and are estimates. The water samples from the

Hollywood city open ocean outfall were greater than the highest calibration standard, thus outside the linear range and not quantifiable. However, this indicates that these samples had estradiol concentrations greater than 4 ng/L.

Table 1. Concentration (ng/L) of estradiol and progesterone at the surface and depth for Broward County and lower Keys reefs (5–8m), and Hollywood open ocean outfall samples (from 20 m).

	Estradiol		Progesterone	
Location	Surface	At depth	Surface	At depth
Broward County (2013)	0.08–0.58	0.10–0.22	2.37–5.44*	1.97–3.84*
Broward County (2014)	0.18–0.29	0.04–0.19	2.21–2.79*	1.63–2.47*
Hollywood Outfall	>4.0[†]	0.28–0.87	3.19–3.94*	1.97–3.39*
Lower Keys	0.46–1.04	0.55–3.38	2.39–5.7*	2.97–5.24*

*Represents samples <DL, these results are outside linear range of calibration standards for the progesterone assay.
[†]Data greater than linear range of calibration standards for the estradiol assay.

When comparing the two regions, the lower Keys had significantly greater estradiol concentrations at the surface (Kruskal-Wallis, χ^2=11.781, p=0.0006, Fig. 1A) and depth (Kruskal-Wallis, χ^2=14.039, p=0.0009, Fig. 1B). There were no significant differences for the Hollywood outfall depth samples when compared to Broward County and lower Keys reefs (p>0.05). For progesterone, there were significantly greater concentrations detected for depth water samples collected from the lower Keys than Broward County reefs (ANOVA, $F(2,15)$ = 6.244, p = 0.009, Fig. 1D). There were no significant differences in progesterone between any sites for the surface water samples or at depth between Broward County to Hollywood outfall, and Hollywood outfall to the lower Keys (p>0.05, Fig. 1C). When comparing surface to coral-depth samples, only surface samples for Broward County reefs had significantly greater concentrations of progesterone than at depth samples (Kruskal-Wallis, χ^2 = 4.532, p = 0.033). There were no significant differences in estradiol detected between surface and coral-depth samples for any location (p>0.05). However, these data should be interpreted cautiously since all values were below the detection limit, therefore it is likely there is some error in these estimates.
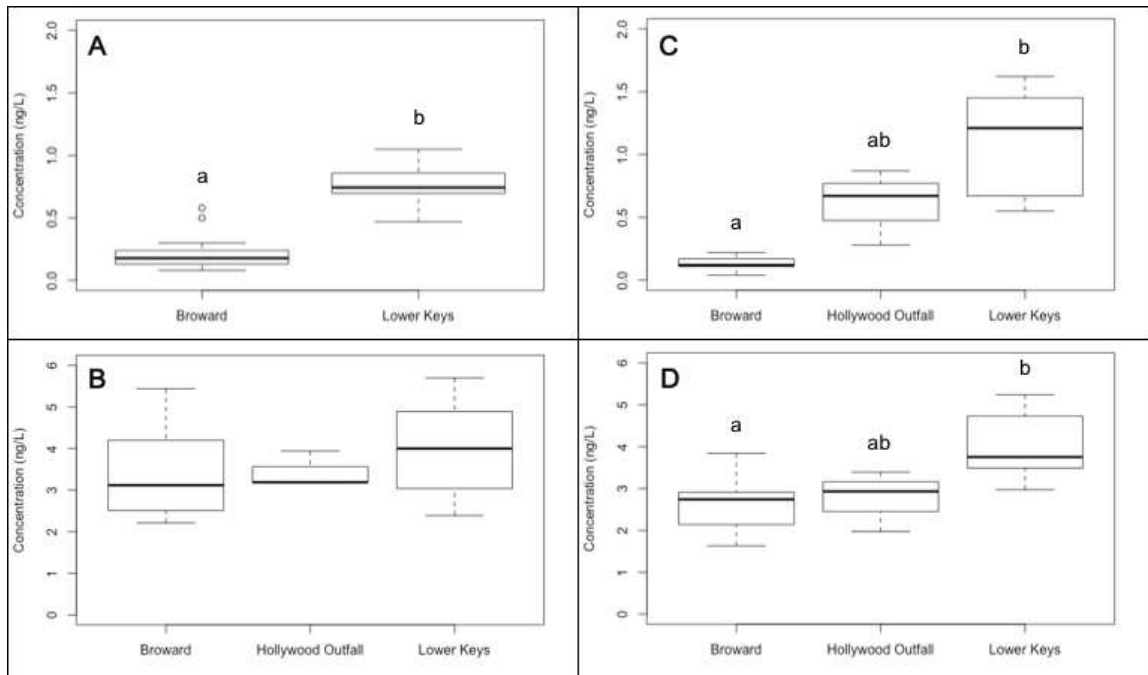
Figure 1. Concentration (ng/L) of estradiol and progesterone for each location. (A) Estradiol at surface for Broward County (n=17) and lower Keys (n=6). (B) Progesterone at surface for Broward County (n=17), Hollywood outfall (n=3), and lower Keys (n=6). (C) Estradiol at depth for Broward County (n=10), Hollywood outfall (n=3), and lower Keys (n=6). (D) Progesterone at depth for Broward County (n=10), Hollywood outfall (n=3), and lower Keys (n=6).

2.3.2 Larval settlement and viability

For the 2014 Broward County and lower Keys *P. astreoides* larval experiments, the proportions of larvae swimming, settled on disc, settled on petri dish, metamorphosed, and alive were scored after 1,4,7, and 10 days. The figures (2 and 3) only show the proportion of average settlement after days 1 and 4 since nearly all settlement occurred by day 4. Settlement was expected to be greater on the pre-conditioned settlement discs; however, the lower Key's larvae settled randomly on the disc and the petri dish. For this reason, the amount of larvae settled on disc and petri dish (total settled) was also used. A two-way ANOVA or Kruskal-Wallis test, depending on whether data were normally distributed with equal variance, was used to determine if there were significant differences between treatments and groups over the 10-day experiment. Estradiol and progesterone treatments were analyzed independently along with each scoring group. Due to datasets being non-normally distributed, great variance

33

within treatments, and the number of independent variables (scoring group, days, location, and treatment), it was difficult to detect precise treatment effects.
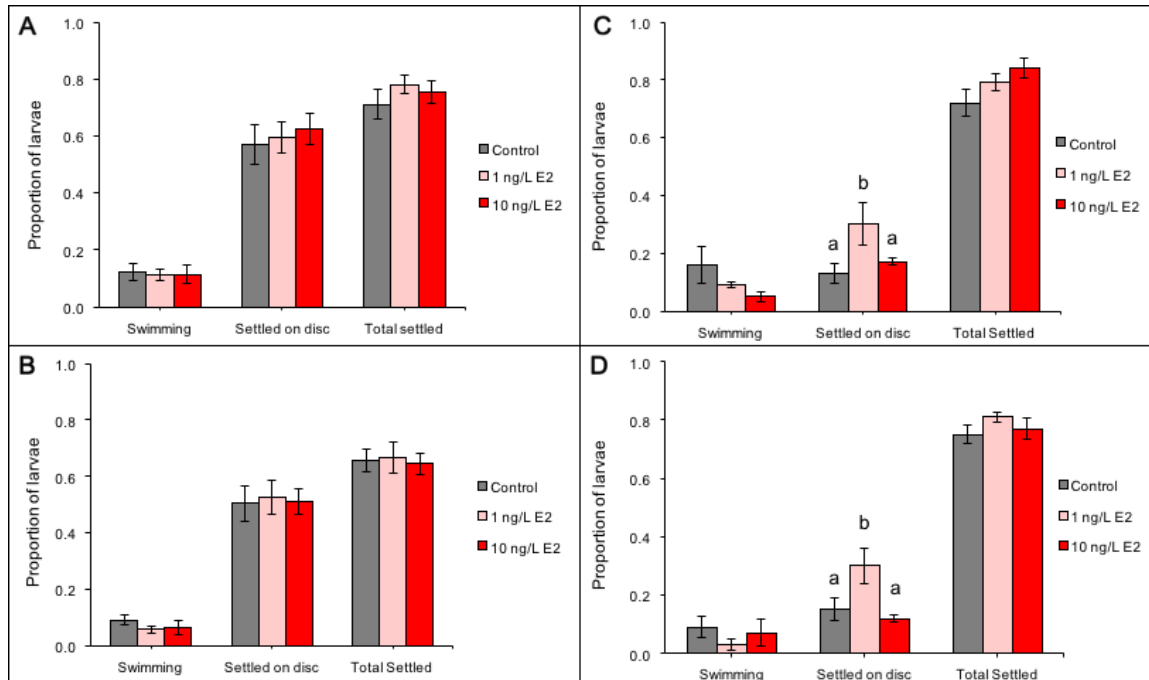

Figure 2. Proportion of larvae (mean ± SE) swimming, settled on disc, and total settled exposed to estradiol treatments. (A) Broward County day 1, (B) Broward County day 4, (C) lower Keys day 1, and (D) lower Keys day 4.

For the Broward County larval experiments (n = 13), there were no significant differences in the proportion of larvae settled on the disc or total settled for the estradiol (Fig. 2 A,B) and progesterone (Fig. 3 A,B) treatments (p>0.05). Also, there were no significant differences in the proportion of swimming larvae across estradiol treatments, but there were significantly fewer swimming larvae for the low progesterone treatment in comparison to the control on day 4 (Kruskal-Wallis, $\chi^2 = 43.744$, p = < 0.0001, Fig. 3 B). Throughout the 10-day Broward survival experiments, there were no significant differences between the controls and estradiol treatments (p>0.05, Fig. 4A). In the progesterone treatments, survival was significantly lower for the low progesterone treatment in comparison to the control and high progesterone treatments (Wilcoxon, $\chi^2 = 15.666$, p = 0.003, Fig. 4B).
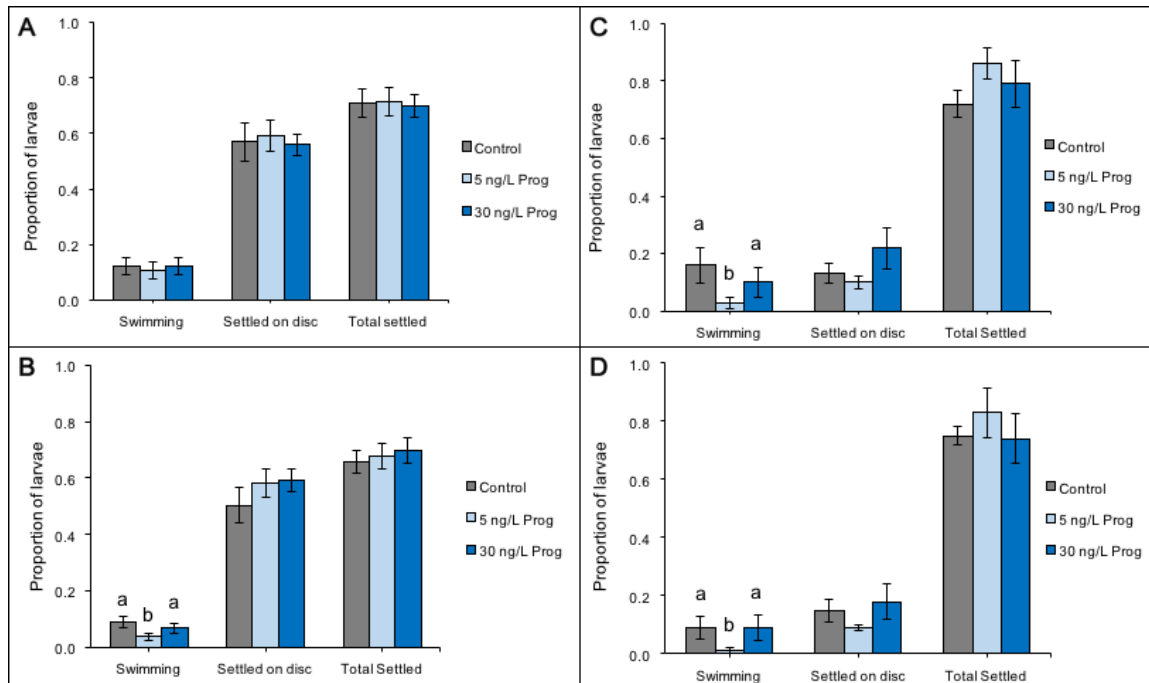
Figure 3. Proportion of larvae (mean ± SE) swimming, settled on disc, and total settled exposed to progesterone treatments. (A) Broward County day 1, (B) Broward County day 4, (C) lower Keys day 1, and (D) lower Keys day 4.

The low estradiol treatments (n = 5) in the lower Keys (Fig. 2 C,D) had significantly greater settlement on the disc than the control and high estradiol treatment (Kruskal-Wallis, $\chi^2 = 28.294$, p = 0.003), while the progesterone treatments (Fig. 3 C,D) had no differences for disc settlement (p>0.05). There were no significant differences in total settlement for the estradiol (Fig. 2 C,D) or progesterone (Fig. 3 C,D) treatments (p>0.05). There was no differences in the amount of swimming larvae for the estradiol treatments (p>0.05, Fig. 2 C,D), but there significantly fewer swimming larvae in the low progesterone treatments (Kruskal-Wallis, $\chi^2 = 23.383$, p = 0.016, Fig. 3 C,D). There were no significant differences in survival throughout the ten-day experiments for the estradiol (Fig. 4A) and progesterone (Fig. 4B) treatments (p>0.05).
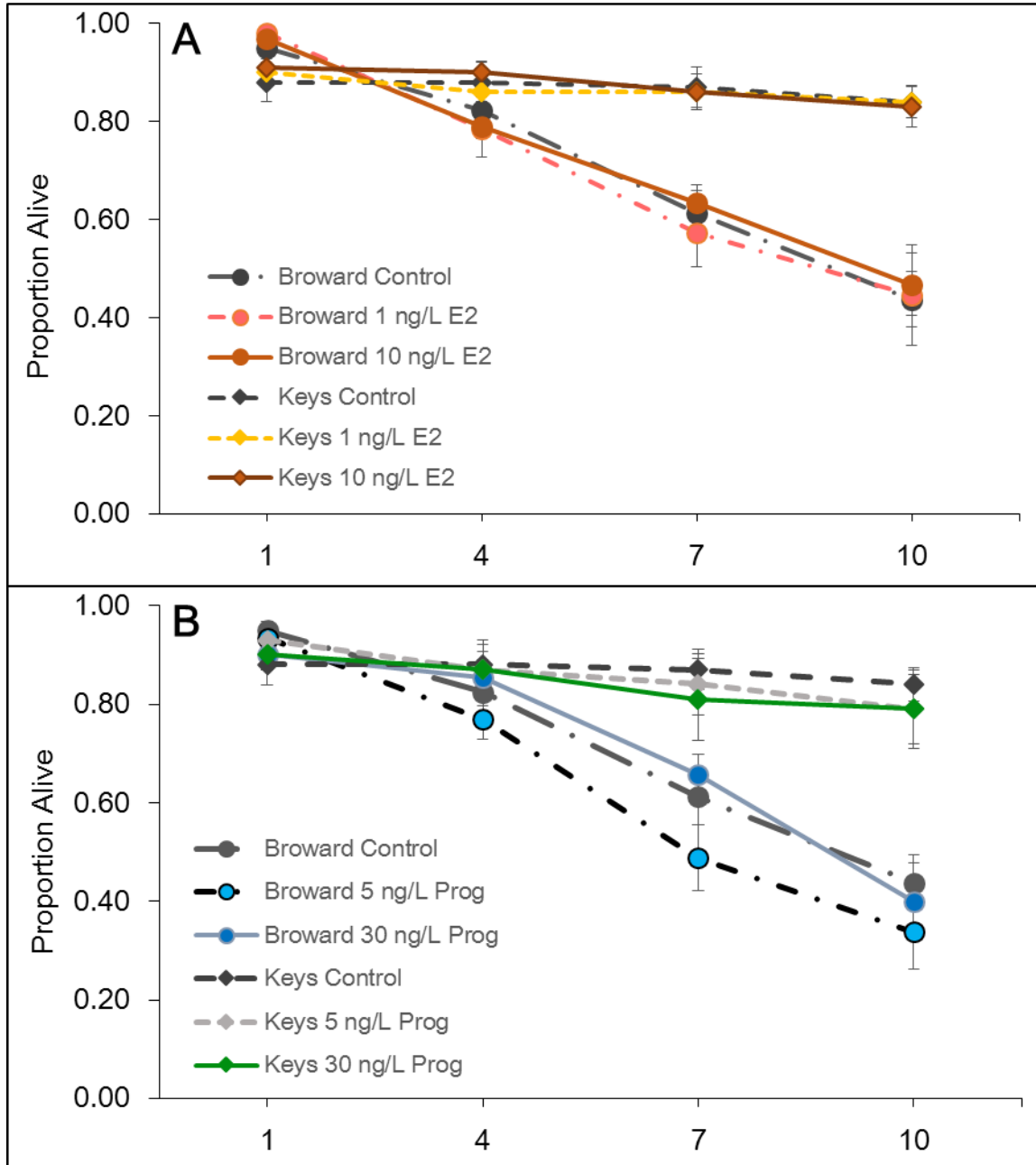
Figure 4. Proportion of larvae (mean ± SE) alive throughout the 10-day experiment. (A) Broward County and lower Keys estradiol treatments, (B) Broward County and lower Keys progesterone treatments.

Throughout the 10-day experiments there were statistically significant differences in settlement on disc, total settled, and survival for the estradiol and progesterone treatments between Broward County and lower Keys larvae. There was significantly greater on-disc settlement for Broward County larvae through the estradiol (Kruskal-Wallis, $\chi^2 = 25.941$, $p < 0.0001$, Fig. 4A) and progesterone (Kruskal-Wallis, $\chi^2 = 28.789$,

p < 0.0001, Fig. 4B) treatments. For total settlement, there were no significant differences between locations (p>0.05) except in the low estradiol treatment where lower Keys larvae had greater total settlement than Broward County larvae (Welch ANOVA, $F(1,14) = 6.378$, $p = 0.0238$). The lower Keys had greater overall survival between the low estradiol (Kruskal-Wallis, $\chi^2 = 40.549$, $p < 0.0001$, Fig. 4A), high estradiol (Kruskal-Wallis, $\chi^2 = 52.159$, $p < 0.0001$, Fig. 4A), low progesterone (Kruskal-Wallis, $\chi^2 = 8.26$, $p = 0.004$, Fig. 4B), and high progesterone (Kruskal-Wallis, $\chi^2 = 39.777$, $p < 0.0001$, Fig .4B) treatments.

2.3.3 Skeletal growth measurements

Acropora cervicornis fragments were buoyantly weighed and photographed immediately before and after two separate 3-week dosing experiments (represented as trial 1 and 2) to determine skeletal and linear growth for Broward County and lower Keys fragments. Each location was analyzed separately and combined, represented as both, to determine treatment effects. No significant estradiol or progesterone treatment effects were found for trials 1 and 2 from buoyant wet weight (p>0.05, Fig. 5) or linear growth analysis (p>0.05, Fig. 6). Additionally, there were no statistically significant differences in growth between Broward County and lower Keys fragments for trials 1 and 2 (p>0.05). Great variation was evident within genotypes, treatments, and trials. Each treatment had corals with minimal growth and others that exceeded average growth rates in wild populations.

Despite finding no significant differences among treatments for trial 1 or 2, there were differences in skeletal growth from buoyant wet weight between locations for each trial. In trial 1, *Acropora cervicornis* fragments in the high progesterone treatment from the lower Keys had greater skeletal growth than fragments from Broward County (ANOVA, $F(1,9) = 6.176$, $p = 0.0347$, Fig. 5B). There were no significant differences between location in the low estradiol, high estradiol, and low progesterone treatments for trial 1 (p > 0.05). In trial 2, *A. cervicornis* fragments from the lower Keys had greater skeletal growth than Broward County in the low estradiol treatments (ANOVA, $F(1,9) = 5.182$, $p = 0.0489$, Fig. 5C). There were no significant differences between location in the high estradiol, low progesterone, and high progesterone treatments for trial 2 (p > 0.05).

Additionally, there were no significant differences in linear growth between Broward County and lower Keys fragments for all estradiol and progesterone treatments (p > 0.05).
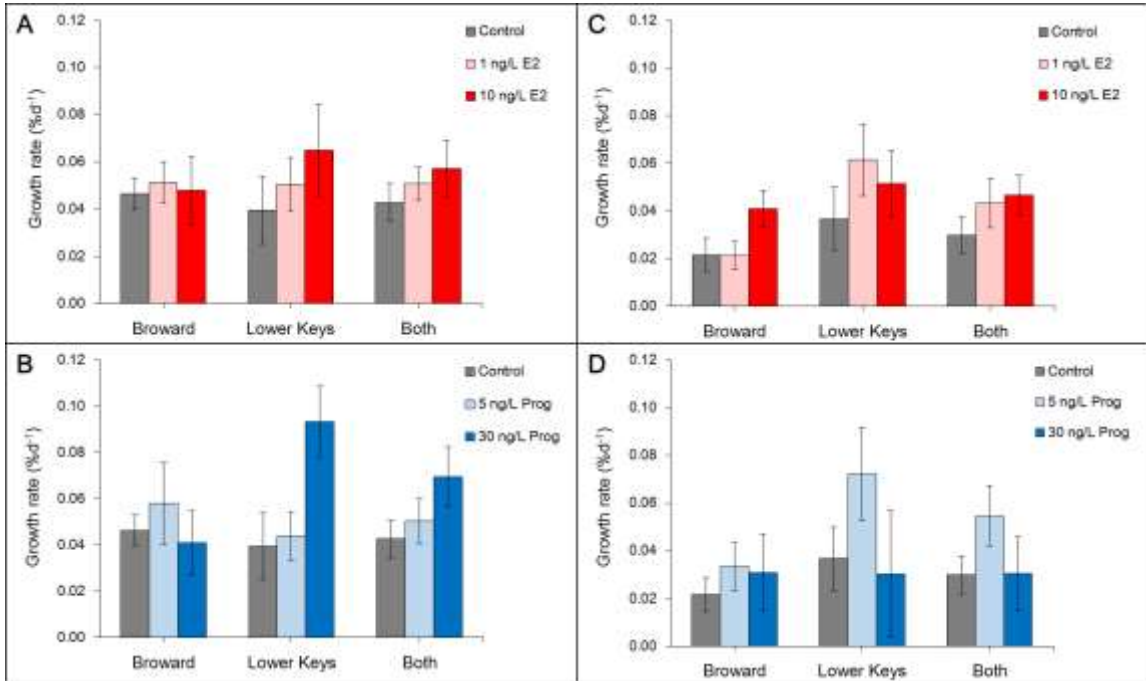

Figure 5. Normalized growth rate from buoyant wet weight (mean ± SE) for *Acropora cervicornis* fragments (n=11). Values expressed as percent change per day (% d^{-1}). (A) Trial 1 estradiol treatments. (B) Trial 1 progesterone treatments. (C) Trial 2 estradiol treatments. (D) Trial 2 progesterone treatments.

Figure 6. Normalized linear growth (mean ± SE). Normalized values expressed as percent of change per day (% d^{-1}) for each *Acropora cervicornis* fragment (n=11). (A) Trial 1 estradiol treatments. (B) Trial 1 progesterone treatments. (C) Trial 2 estradiol treatments. (D) Trial 2 progesterone treatments.
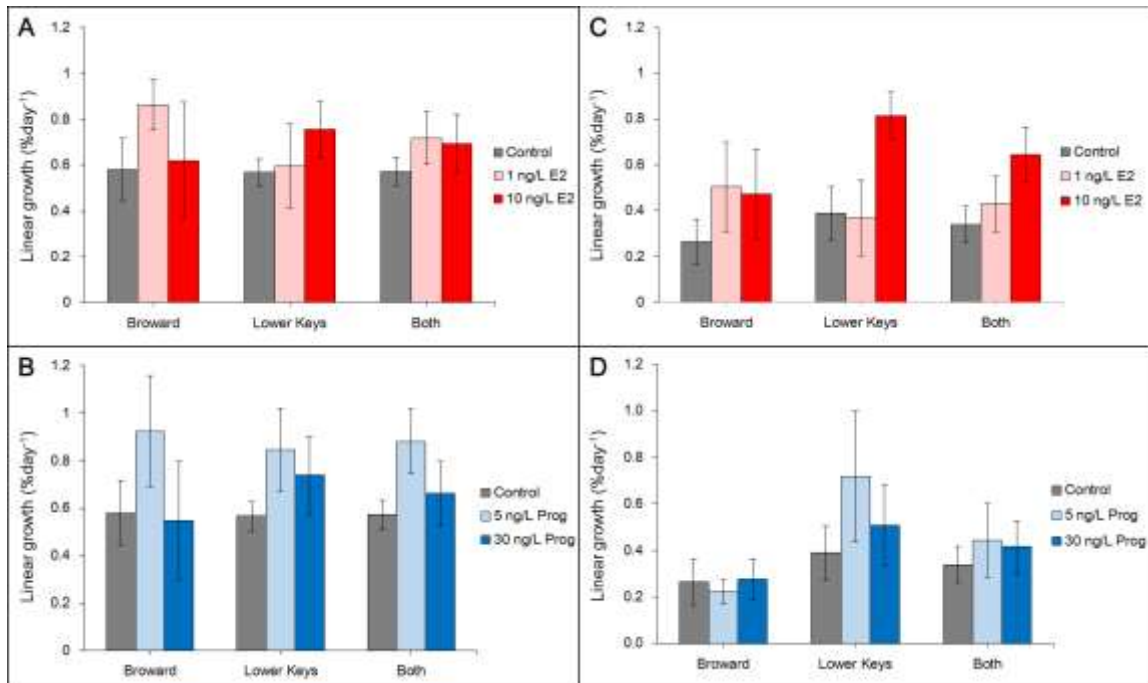
2.3.4   *Zooxanthellae densities*

Zooxanthellae densities were measured prior to (n=4) and following (n=4) the 3week dosing experiment for *A. cervicornis* fragments, while *P. astreoides* zooxanthellae densities were only measured after the dosing experiment with equal sample sizes of Broward (n=4) and lower Keys (n=4) colonies. A one-way ANOVA was used for parametric data, and the Kruskal-Wallis test for non-parametric data; estradiol and progesterone treatments were analyzed independently for each species. For *P. astreoides*, there were no significant differences between location for the estradiol (Fig. 7A) or progesterone (Fig. 7B) treatments (p>0.05). There were also no significant differences in *A. cervicornis* fragments in the estradiol (Fig. 7C) and progesterone (Fig. 7D). In addition, there were no significant differences in the zooxanthellae densities between pre- and post-treatment fragments in *A. cervicornis* fragments for the estradiol (Fig. 8A) and progesterone (Fig. 8B) treatments (p>0.05). Using a one-way ANOVA, there were no significant differences between Broward County and lower Keys *P. astreoides* colonies

and *A. cervicornis* fragments between the low estradiol, high estradiol, low progesterone, and high progesterone treatments (p>0.05, Fig. 7).
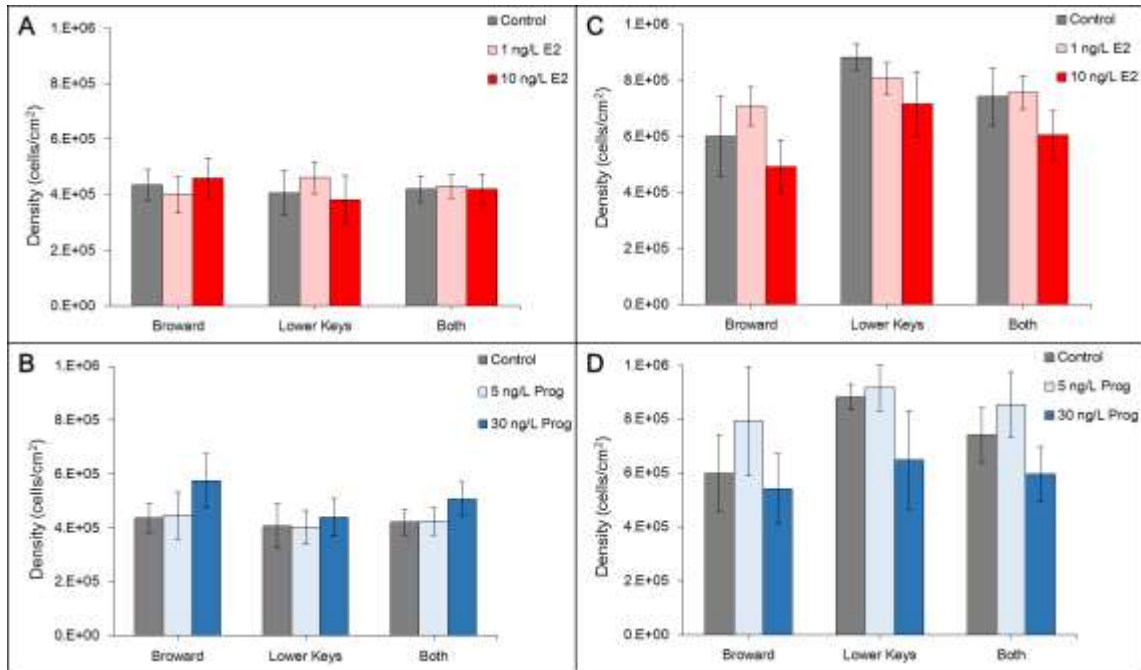
Figure 7. Zooxanthellae densities (mean ± SE) expressed as cells per square cm. *Porites astreoides* cell densities for Broward County (n=4), lower Keys (n=4), and both locations (n=8) post-exposure to (A) estradiol and (B) progesterone treatments. *Acropora cervicornis* cell densities for Broward County (n=2), lower Keys (n=2), and both locations (n=4) post-exposure to (C) estradiol and (D) progesterone treatments.

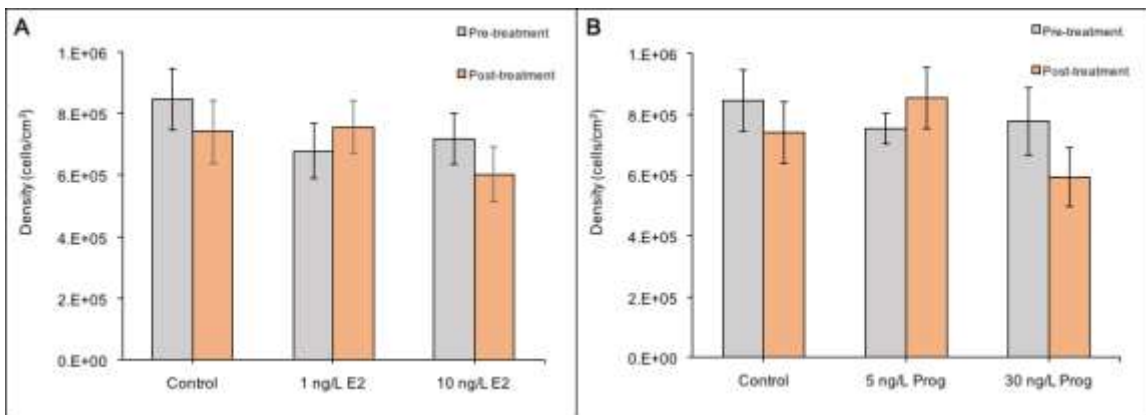Figure 8: *Acropora cervicornis* zooxanthellae densities (mean ± SE) expressed as cells per square cm for pre-treatment (n=4) and post-treatment (n=4) fragments.

2.3.5 *Histological analysis*

Histological analysis of *A. cervicornis* and *P. astreoides* was performed to assess the condition of the epidermis, gastrodermis, zooxanthellae, gonads, and calicodermis

after the 3-week dosing experiment. Overall coral health was analyzed using a semi-quantitative rubric that incorporated the severity and extent for each category, and the scores in all categories were added to provide a total health score for each sample (Table A1). When applicable, gonad counts were also recorded. Kruskal-Wallis tests were used to determine treatment effects on the histology scores and number of gonads. There were no significant differences in the total histology scores for estradiol and progesterone treatments in *A. cervicornis* pre-treatment (n=4) and post-treatment (n=4) fragments (p>0.05). In addition, there were no significant differences between the number of oocytes between treatments for pre- and post-treatment *A. cervicornis* fragments (p>0.05); however, there were a greater number of oocytes in pre-treatment fragments (Kruskal-Wallis, χ^2 = 13.431, p = 0.0002). No statistical differences were noted in the number of spermaries between estradiol and progesterone treatments in pre- and post-treatment fragments (p>0.05). Significantly more spermaries were detected in pre-treatment fragments (Kruskal-Wallis, χ^2 = 4.014, p = 0.045); however, spermaries were only observed in 11 of 20 pre-treatment samples and one post-treatment sample. In post-treatment *Porites astreoides* samples, there were no significant differences in total histological scores for estradiol and progesterone treatments (p>0.05).

Overall, most pre- and post-treatment *A. cervicornis* fragments appeared to be fairly healthy, having minimal to moderate degradation of epidermal and gastrodermal architecture in the surface and basal body walls. In the surface body wall there was slight atrophy, attenuation of epidermis over septal ridges, and slight atrophy of mucocytes with some release of mucus. The scoring of the basal body wall was due to less than one quarter of gastrodermal cells being lysed, mucus filling gastrovascular canals, slight atrophy of mucocytes with some release, and slight atrophy of the calicodermis. Zooxanthellae appeared healthy, abundant, and stained properly in nearly all samples. Most oocytes appeared to be healthy and intact between all treatments and spermaries, when present, were in early stages of development.
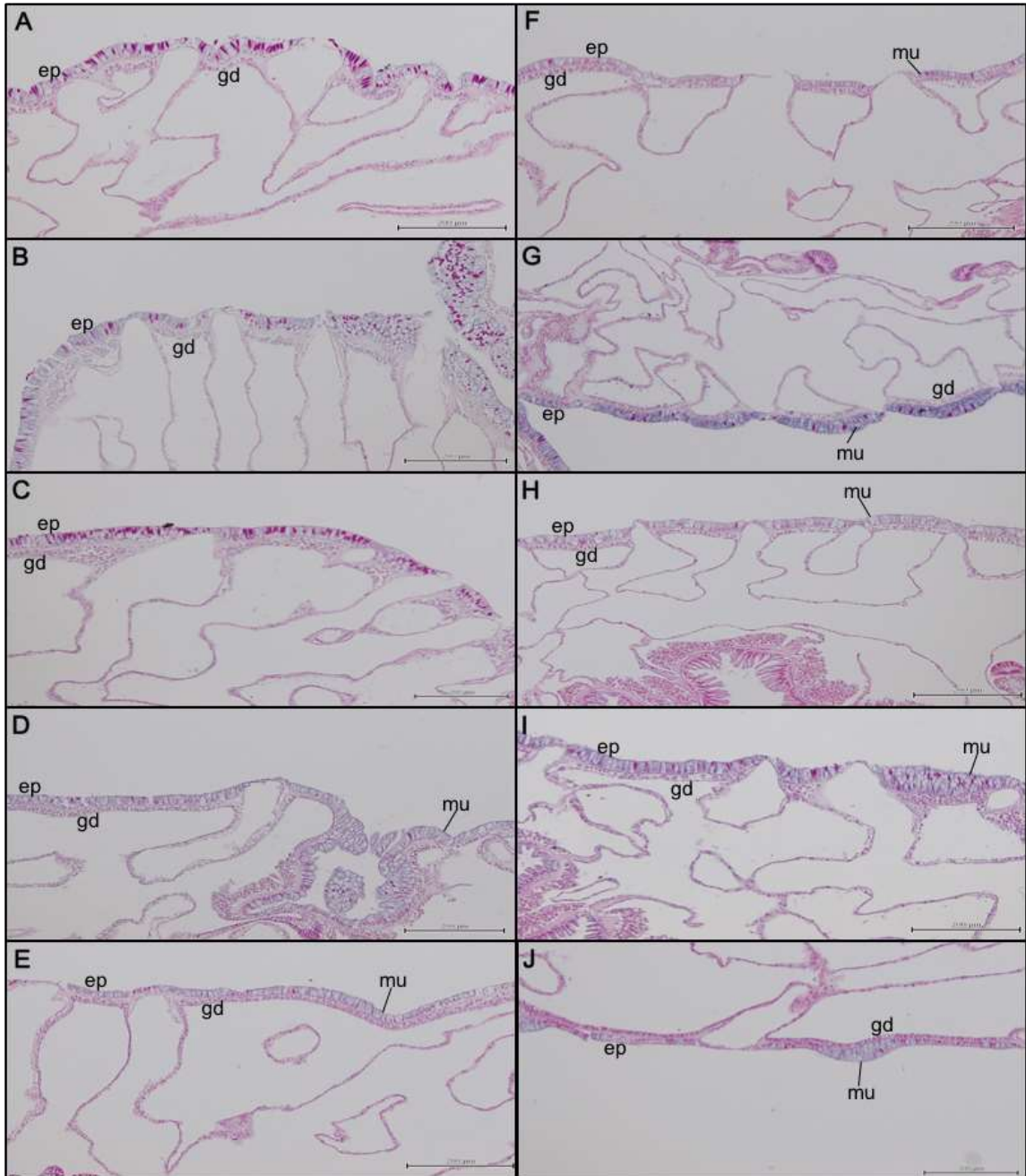
Figure 9. Histological sections of *Acropora cervicornis* coenenchyme and basal body wall at 10X magnification. (A) Pre-treatment control. (B) Pre-treatment 1 ng/L estradiol. (C) Pre-treatment 10 ng/L estradiol. (D) Pre-treatment 5 ng/L progesterone. (E) Pre-treatment 30 ng/L progesterone. (F) Post-treatment control. (G) Post-treatment 1 ng/L estradiol. (H) Post-treatment 10 ng/L estradiol. (I) Post-treatment 5 ng/L progesterone. (J) Post-treatment 30 ng/L progesterone. Scale bars = 200 μm. ep = epidermis, gd = gastrodermis, mu = mucocyte.

Most *P. astreoides* samples had mild to moderate degradation of surface and basal body walls among all post-treatment samples. In the surface body wall, severe thinning of tissue occurred over septal ridges, as well as atrophy and uneven appearance of mucocytes, and moderate lysing and atrophy with one-quarter to one-half of the epidermis and gastrodermis having ruptures and mucus discharge. One-quarter to one-half of the gastrodermis in the basal body wall had lysed cells, mucus filling gastrovascular canals, atrophy of mucocytes, and abundant mucus release. The calicodermis had variable thinning and lysis with fewer acidophilic granules. Zooxanthellae were healthy, abundant, and stained properly in all treatments. Oocytes and spermaries were degraded throughout all treatments, since both trials immediately followed the reproductive period for these corals.

2.4   Discussion

Estradiol and progesterone levels varied between the two regions, as did their effects on coral larvae and adults. Although both estradiol and progesterone were detected in water samples collected from Broward County and the lower Keys reefs sites, only estradiol was measured with confidence. The estradiol concentrations measured are within the effect level range (0.1–10 ng/L) for sensitive species (Purdom et al. 1994, Rotchell and Ostrander 2003, Langston et al. 2005, Auriol et al. 2006). There were substantial within-treatment and genotypic variances for the larval and adult experiments and interesting differences between the two regions of the Florida Reef Tract. The larval experiments had variable results. Estradiol treatments had no effect on settlement on disc, total settlement, or survival of Broward County larvae, but low estradiol treatments had increased disc settlement in the lower Keys larvae. The lower Keys larvae were not influenced by progesterone for settlement on the disc, total settlement, or survival. However, the low progesterone treatment had significantly fewer swimming larvae for both regions and reduced survival for Broward County larvae. There were also differences in the results between locations for each treatment. Although Broward County larvae had greater settlement on the disc for the estradiol and progesterone experiments, the lower Keys larvae had greater total settlement and survival in the low estradiol, high estradiol, low progesterone, and high progesterone treatments. In the adult experiments,

lower Keys fragments had increased skeletal growth in the low estradiol and high progesterone treatments for *A. cervicornis* (Table 2). There were no differences between location and each treatment in zooxanthellae density, reproduction, and overall histological scores for *A. cervicornis and P. astreoides*. Increased settlement and survival of lower Keys *P. astreoides* larvae within the effect level range suggests they may be healthier than Broward County corals or have adapted to higher levels of sex steroid concentrations and therefore were less likely to be affected by the dosing experiments.

Table 2. Comparison of results using a (+), (-), or NS (no significant difference) to signify which location and treatments had greater or less larval settlement, larval survival, skeletal growth, zooxanthellae densities, and total histological score.

	Estradiol				Progesterone			
	Broward		Keys		Broward		Keys	
Results	Low	High	Low	High	Low	High	Low	High
Larval settlement on disc	+	+	-	-	+	+	-	-
Total settlement of larvae	-	NS	+	NS	NS	NS	NS	NS
Larval survival	-	-	+	+	-	-	+	+
Skeletal growth	NS	NS	NS	NS	NS	NS	NS	NS
Zooxanthellae densities	NS		NS		NS		NS	
Histological score	NS		NS		NS		NS	

The various methodologies used to determine sex steroid concentrations in organisms or water have some limitations. Enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) use vertebrate antibodies to detect specific steroids and have cross-reactivity with other steroids (Markov et al. 2009). ELISA and RIA are accurate techniques but are not infallible (Lafont and Mathieu 2007). For example, vertebrate-type steroids detected in a sea-lamprey through RIA were later identified as 15α-hydroxylated steroids using high performance liquid chromatography (Kime and Larsen 1987, Lowartz et al. 2003). More sensitive methods such as gas chromatography-mass spectrometry (GC-MS) should be used as definite proof of any vertebrate-like steroid precursor, enzyme, or sex steroids in invertebrates or cnidarians (Lafont and Mathieu 2007, Markov et al. 2009). For this study, it is likely that estradiol

concentrations detected in off-shore reef environments are slightly inflated due to cross-reactivity to other steroids using ELISA. Progesterone was detected in surface and at depth samples at greater concentrations than estradiol; however, these results are not reliable since they are below the detection limit and lowest calibration standard. This can result in increased error, especially the farther it gets below or above the detection range.

Here, estradiol was detected at concentrations ranging from 0.55–3.38 ng/L at offshore reefs sites in the lower Florida Keys. Previous research using RIA found that E2 concentrations were not detectable at an offshore reef site, while Key Largo harbor concentrations ranged from ND–1.8 ng/L (Singh et al. 2010). Estrone concentrations have ranged from ND–0.88 ng/L at offshore reef sites, while near-shore sites (canal, harbor, channel, and shore) ranged from 0.6–5.2 ng/L (Atkinson et al. 2003, Singh et al. 2010). Surprisingly, lower Keys reefs had significantly greater concentrations of estradiol than Broward County reefs that ranged from 0.08–0.58 ng/L. There were no significant differences between the Hollywood open ocean outfall and either reef site. However, there is a large difference in depth between the sites, the open ocean outfall water samples were collected at 20 m whereas lower Keys samples ranged from 5–8 m. For all sites, surface samples typically had greater estradiol concentrations than at depth samples taken near corals. On average, the lower Keys had similar estradiol concentrations at depth to water samples taken from the city of Hollywood open ocean outfall.

I expected Broward County reefs, which are in close proximity to open ocean outfalls in a heavily populated region, to have greater values. Although septic tanks and cesspits are now banned throughout the Florida Keys, it is likely old sewage systems can be leaching into coastal environments, as well as deep injection wells (Lapointe et al. 2004). Deep injection wells are used throughout the Keys at centralized sewage treatment facilities. In the 2013 Status of Wastewater Implementation, approximately 72% of Keys residents (City of Marathon not included) are tied into their service areas' sewage system. In this 2013 report, 100% of Key West residents near our study area were tied into the sewage system the year before I collected my water samples. Perhaps ground water flow from tidal pumping through the porous substrate is allowing treated sewage into reef environments. Our water samples were collected in close proximity north of Key West along the Florida Current. Key West is a populated city with a large sewage treatment

facility that utilizes deep injection wells. My samples were collected at two sites in close proximity on one day in 2014, more extensive sampling throughout the Keys would be necessary to determine the sex steroid concentrations corals are routinely exposed to annually. To my knowledge, progesterone has not been detected in any coastal or marine environment and estradiol has not been previously quantified from any open ocean outfall or reef site in Broward County.

In a similar study looking into the effects of sex steroid pollution on corals in the environment, estradiol concentrations at contaminated sites ranged from approximately 1–9 ng/L along the Israeli-Mediterranean coast (Armoza-Zvuloni et al. 2012). These concentrations are very similar to our low (1 ng/L) and high (10 ng/L) estradiol treatments. In their study, colonies exposed to acute municipal pollution for one year had incomplete or disrupted gametogenesis, characterized by smaller oocyte diameter and a lower proportion of fully developed testes compared to the reference and chronically contaminated sites (Armoza-Zvuloni et al. 2012). Sites with chronic exposure to these contaminants (several decades) had normal gametogenesis, despite having significantly greater concentrations of estradiol, progesterone, and testosterone in the tissue. Armoza-Zvuloni et al. (2013) was the first to find high concentrations of estradiol, progesterone, and testosterone in scleractinian coral colonies from sewage-contaminated sites that indicated bioaccumulation. It is possible that acute stressors may initiate adaptation processes favoring hardy genotypes that can withstand chronic pollution (Armoza-Zvuloni et al. 2012). My research supports this hypothesis by finding that *P. astreoides* larvae from the Keys—in an environment with greater estradiol concentrations—had increased settlement and survival. Although no reproductive effects were detected in adult corals, *A. cervicornis* fragments from the Keys exposed to low estradiol and high progesterone treatments had greater skeletal growth.

This experiment used corals from two distinct regions with significantly different concentrations of estradiol but did not observe any disruption in gametogenesis from either treatment. Histological analysis did not indicate any significant effects from estradiol or progesterone on overall health or reproduction for estradiol and progesterone. In contrast, Tarrant et al. (1999) detected estrone and estradiol in *Montipora verrucosa* tissue throughout a year, finding daily and monthly variations coinciding with

gametogenesis. Estradiol peaks preceding estrone peaks provide evidence that corals may metabolize estrone from estradiol and may play a role in the late stages of gametogenesis and/or spawning events. *Montipora capitata* colonies exposed to high estradiol concentrations (20+ ng/L) for three weeks prior to spawning had a 29% decrease in the release of gamete bundles (Tarrant et al. 2004). However, these concentrations are more than a two-fold increase over the high treatments in this study, which are greater than any reported environmental concentration. In our experiment, there were significantly fewer oocytes and spermaries in post-treatment *A. cervicornis* fragments compared to pre-treatment fragments across all treatments, including the control. This was unexpected since the post-treatment fragments were three weeks closer to the spawning period and should have more developed gonads. Post-treatment *P. astreoides* samples also had degraded oocytes and spermaries across all treatments.

The difference in gametogenesis could have several likely explanations, including: (1) experimental stress may have led to resorption of the oocytes and spermaries; (2) location of sample collected from the branch due to the polyps being immature, need to repair the cut surface, or oocyte resorption, and (3) damage from fragmentation can decrease fecundity in nearby tissues (Harrison and Wallace 1990). The process of oocyte resorption during gametogenesis is not well understood, but has been reported in *Acropora* and *Porites* species (Harrison and Wallace 1990). Breaking down the lipid-rich vesicles in oocytes may provide nutrition for nearby oocytes and tissue (Harrison and Wallace 1990, Lueg et al. 2012). Metabolically, gametogenesis is demanding and the allocation of resources may be prioritized to repair, maintenance, and growth (Harrison and Wallace 1990, Lueg et al. 2012). All samples were less than ~7 cm, therefore growth from the apical polyp and tissue repair from the cut margins may have led to oocyte resorption. Various stressors and pollutants have been shown to reduce gametogenesis and lead to resorption in corals (Loya and Rinkevich 1979, Harrison and Wallace 1990). Collection, fragmentation, water quality, and the presence of cyanobacteria in the acclimation and experimental tanks are all stressors preceding this experiment. Water temperate, salinity, and nutrients remained stable in the acclimation tanks and throughout both 3-week dosing experiments, and therefore are unlikely to have had detrimental effects on the coral. Sampling location within the colony is also likely to

have had an effect on the reproductive results in this experiment. In *A. cervicornis*, the coral is more reproductive farther from the apical tip of the polyp. Efforts were made to sample as far from the apical tip as possible, however, pre-treatment samples were at least 1 cm further from the tip than post-treatment samples that ranged 3–5 cm from apical tip. Previous research indicates that *A. cervicornis* is does not produce gonads within 2–6 cm from the apical tip of branches (Szmant 1986, Soong and Lang 1992). All of our fragments were within this range, especially post-treatment fragments that were already clipped for pre-treatment analysis. Despite this, oocytes were detected in most of the samples. Far fewer spermaries were detected in *A. cervicornis* samples; however, spermaries generally do not fully develop until July for this species (Soong 1991), but may begin development in late May like its congener, *Acropora palmata* (Szmant 1986). Therefore, a critical time for reproductive development before spawning would have occurred during the experiment. The growth rates of *A. cervicornis* fragments and the zooxanthellae densities measured in both corals also indicated that they were stressed prior to or during the experiments.

Skeletal growth rates for *A. cervicornis* were comparable between treatments and collection locations. Growth rates for Broward County *A. cervicornis* fragments ranged from 0.04%–0.06% day^{-1} and 0.02%–0.04% day^{-1} for trials 1 and 2, respectively. For lower Key's fragments, growth rates ranged from 0.04%–0.09% day^{-1} and 0.04%–0.07% day^{-1} for trials 1 and 2, respectively. In comparison, *A. cervicornis* fragments used in an experiment by Fernandez (2012) and obtained from the Smithsonian Marine Station in Fort Pierce had increased growth rates. Mean skeletal growth rates of 0.23% day^{-1} in fed high light and 0.36% day^{-1} in unfed high light conditions were reported after their four-month experiment (Fernandez 2012). The high light treatments were based on photosynthetically active radiation (PAR) values recorded on a reef at 10 m depth in the Florida Keys and are comparable to the corals and shading used in this experiment. In contrast to our experiment, *Porites compressa* fragments treated with estrone (2 ng/L) for 2–8 weeks had a 13% and 24% decrease in skeletal growth in two replicated seasonal experiments at similar estrogen concentration to our low treatment (Tarrant et al. 2004). The reduced growth rates for these corals suggest they may have been affected by experimental, acclimation, or environmental stressors prior to the experiments. Tarrant et

al. (2004) reported estrone reducing growth rates while increasing tissue thickness in *P. compressa* nubbins, hypothesizing that estrogens may increase cellular proliferation while suppressing skeletal growth in corals. However, there was no evidence of this from histological analysis of *A. cervicornis* and *P. astreoides* samples.

Mean zooxanthellae densities between all treatments for *A. cervicornis* ranged from 0.68–0.85 x 10^6 cells cm^{-2} and 0.60–0.85 x 10^6 cells cm^{-2} for pre- and post-treatment fragments, respectively. Fernandez (2012) had much greater zooxanthellae densities across all of their *A. cervicornis* treatments. They reported densities of 1.1 x 10^6 cells cm^{-2} in fed high light, 2.2 x 10^6 cells cm^{-2} in unfed high light, 1.9 x 10^6 cells cm^{-2} in fed low light, and 2.0 x 10^6 cells cm^{-2} in unfed low light experimental conditions after the four-month experiment (Fernandez 2012). *Porites astreoides* zooxanthellae densities ranged from 0.4–0.57 x 10^6 cells cm^{-2} and 0.38–0.44 x 10^6 cells cm^{-2} for Broward County and lower Keys colonies, respectively, across all treatments. These densities are also much lower than densities reported in the literature for this species. Nagelkerken and Bak (1998) reported an average zooxanthellae density of 3.1 x 10^6 cells cm^{-2} for Caribbean *P. astreoides* colonies. The reduced zooxanthellae densities and growth rates reported further suggest that they were affected by experimental, acclimation, or environmental stressors. There is no evidence that the corals were exposed to thermal stress or any abnormal environmental stressor prior to the experiment. However, cyanobacteria were prevalent in the acclimation tanks, the experimental tanks, and were found on the tissue and exposed skeleton of some *P. astreoides* fragments. Cyanobacteria can negatively affect coral health and even lead to lysing of tissue (Charpy et al. 2012); it not clear what impact, if any, this had on coral growth or zooxanthellae densities.

Unlike the adult dosing experiment, there were treatment effects observed in the *P. astreoides* larval experiments with variable results between Broward County and the lower Keys larvae. To my knowledge, research investigating the effects of sex steroids on larval settlement and survival had not been performed. Interestingly, the only observable effects were with the two low steroid treatments at environmentally relevant concentrations. Unexpectedly, the lower Keys larvae had far greater settlement on the petri dish than the pre-conditioned settlement tile. This may be due to the low sample size and the larvae used in this experiment were collected several days before being used and

abandoned normal settlement cues. However, all settlement rates exceeded 60% and most rates reported in numerous studies that have ranged from approximately 12.5–85% (Albright et al. 2008, Sharp et al. 2015, Olsen et al. 2016). After 24 hours, survival in both experiments was similar to another experiment that reported approximately 80% survival after 24 hours (Olsen et al. 2016). Lower Keys larvae had greater survival (>80%) compared to Broward County larvae that dropped below 50% after the 10-day experiment and greater total settlement for controls, estradiol, and progesterone treatments. This indicates that the larvae used in these experiments were healthy and experimental conditions did not negatively affect the larvae. The reduced survival rates in the Broward County larvae were unexpected since they were used immediately. Although the Broward County larvae appeared healthy, it is possible that larvae from this region may be less viable since, over the course of this experiment *P. astreoides* colonies from Broward County released fewer larvae than colonies in the Florida Keys, an indication of poorer health and/or reduced reproductive potential.

Few studies have examined the effects of exogenous estrogens and progesterone on coral growth and reproduction, but some evidence exists that impacts vary among many factors, such as concentration, form of steroidal hormone, time of year, and coral species (Twan et al. 2003, Tarrant et al. 2004, Armoza-Zvuloni et al. 2012). There is evidence supporting the hypothesis that the concentrations detected in our study have the potential to negatively affect corals. These results indicated that estradiol and progesterone at environmentally relevant and elevated concentrations do not have an effect on the growth, reproduction, and overall health of these coral species. The reduced growth, zooxanthellae densities, and the minimal to moderate semi-quantitative histological scores suggest these corals were unhealthy prior to the experiment. However, it appears that low levels of estradiol and progesterone have the potential to reduce larval settlement and survival. Other studies have found more concrete evidence that sex steroids can detrimentally affect corals experimentally and naturally in coastal environments.

Further research is needed to determine the many synergistic effects of various sex steroids and pollutants to establish the dose-response effects these endocrine-disrupting compounds may have on important coral species. Previous research has

provided evidence that sex steroids are biologically active and can affect growth and reproduction (Tarrant et al. 2004, Armoza-Zvuloni et al. 2012). To determine these effects, it is important to quantify the concentration and extent of endocrine-disrupting compounds, especially sex steroids in marine environments and invertebrates using sensitive methods such as HPLC and GC-MS. I detected progesterone in surface and at-depth water samples from Broward County and lower Keys reefs. Like progesterone, the concentrations of testosterone and ethinylestradiol are unknown in reef environments but are potent endocrine-disrupting compounds. It is important that future research establishes the concentrations, effects, and metabolic pathways in scleractinian corals to determine whether or not they play a role in the current degradation of reefs worldwide.

Chapter 3: Discussion

Temperature, salinity, alkalinity, and nutrient levels (phosphate, nitrate, and nitrite) remained relatively stable over the course of the 21-day experiments for trials 1 (Table A2) and trial 2 (Table A3) and are unlikely to have had an impact on the results. In addition, nursery water quality for nitrate, nitrite, and phosphate remained stable for the duration of both experiments when used for the 50% water changes. There were no significant differences between the control and treatment tanks for temperature and salinity in both trials ($p<0.05$). The high recordings of 27.6 for the 10 ng/L estradiol tank and 26.9 for the 5 ng/L progesterone tank in trial 2 were caused by chiller malfunctions and were corrected within a couple of hours. Cyanobacteria was pervasive in the coral nursery for acclimation of *Acropora cervicornis* fragments and *Porites astreoides* colonies. Since nursery water was used for the experiment, cyanobacteria were pervasive in the experimental tanks throughout the experiment along the walls and equipment in the experimental tanks.

Cyanobacteria were prevalent on the tissue and exposed skeleton of most *P. astreoides* fragments. No cyanobacteria were found growing on any *A. cervicornis* fragments, however it did grow on the travertine tile bases. A toothbrush was used to gently brush the cyanobacteria off exposed skeleton along the cut margins. In addition, fragments were gently shaken and lightly turkey basted outside of the tanks using nursery water to remove cyanobacteria from tissue without causing damage. This proved to be difficult and failed to remove all cyanobacteria from the colonies. Harsher methods to remove the cyanobacteria from living coral tissue (e.g., toothbrush) would have caused tissue damage. Cyanobacteria can negatively affect coral health and even lead to lysing of tissue (Charpy et al. 2012).

All trial 3 fragments were exposed to an alkalinity increase of 135 ppm–170 ppm prior to day 1 of the experiment. This alkalinity spike culminated in the death of nearly all trial 3 fragments. Throughout the 3-week experiment, rapid tissue loss eliminated nearly all *A. cervicornis* in all treatments. In all *P. astreoides* colonies, tissue loss from the alkalinity spike was compounded by the pervasive cyanobacteria that proceeded to take over all living tissue and smother out the coral. The alkalinity spike was corrected in the nursery before using the water for dosing and water changes in trials 1 and 2.

This project could be improved in multiple ways. It is important to use healthy organisms that are not exposed to numerous stressors or disease. Broward County and lower Keys reefs have been subjected to several bleaching events and disease outbreaks over the past decade and unknown stressors may impact experimental studies. Assessing the condition of tissue histologically prior to an experimental study would be beneficial. It is also important to limit the amount of cyanobacteria in the coral nursery and experimental systems. Simply scrubbing off and siphoning does not remove all of the cyanobacteria from the water column and various surfaces it is attached to, allowing it to grow back quickly. While no chemicals or techniques are known to remove cyanobacteria, especially in a large-scale system, performing outdoor experiments in the late fall and winter months would significantly reduce or remove this stressor. Outdoor experiments performed during these months would also reduce heat stress on the chillers. Analytical methodologies could also be improved in several ways.

The buoyant wet weight methodology could have been improved by weighing the corals without the travertine tile before and after the experiment. Eliminating the base would help reduce error in the measurements. It would be beneficial to develop an alternative method using wires or an easily removable base to reduce this error. However, this would be difficult to achieve for *A. cervicornis* since the stable base was needed for acclimation in the nursery and placement in the experimental tanks. Gluing large branching corals is difficult and time consuming, and they are susceptible to breaking away from the base. For the linear growth rate analysis, error could have been reduced by not using branching or curved fragments.

For *A. cervicornis* histology, it would be beneficial to use longer fragments that allow for sampling >5 cm from the base when investigating reproductive characteristics, especially when comparing pre- and post-treatment fragments, since at least 1 cm of tissue is needed for zooxanthellae density measurement and histological analysis. For studies similar to this, it would be beneficial to use branching corals with a thick enough diameter that it is possible to use one-half (cut vertically) of a 1-cm pre- or post-treatment fragment for histological analysis and the other half for zooxanthellae density analysis. In this situation, it would be important to cut the samples vertically after decalcification to minimize polyp damage. Also when examining reproduction, it would be beneficial to

use two species that have similar reproductive cycles coinciding with the dosing experiment.

For the larval assays, it would have been beneficial to increase the sample size and replicate experiments, especially for the lower Keys (n=5). Broward County *P. astreoides* colonies did not consistently release thousands of larvae as anticipated, therefore, we were unable to run the experiment for multiple months and just used larvae collected in May. For the dose-response experiments, it would be important to sample the concentration of relevant sex steroid before, during, and after the experiment to validate results, calculations, and methods used for dosing. The concentrations detected in reef environments and results from other studies highlight the need for more research, especially considering the current state of reefs worldwide and future climate, urbanization, and reef predictions.

Interestingly, this study detected within-treatment differences in *P. astreoides* larvae and *A. cervicornis* fragments between Broward County and the lower Keys. Larvae from the Keys had greater settlement and survival across all treatments, while the adult fragments from the Keys had greater growth in the low estradiol and high progesterone treatments. This occurred despite water samples from the Keys having significantly greater estradiol concentrations at the surface and depth of corals. Corals potentially adapting to chronic stress from the sex steroids estradiol, progesterone, and testosterone has also been reported by Armoza-Zvuloni et al. (2012). It is important to determine how various coral species from larvae to adults are affected by acute and chronic sex steroid stress. To do this, it is necessary to determine sex steroid concentrations in coastal environments at various depths throughout an entire year. It is also important to do this research with more sensitive methods such as HP-LC and GC-MS. However, these techniques are very expensive and it is important to determine the variance between these more sensitive methods and ELISA or RIA that could be more widely utilized.

Future research should also incorporate synergistic effects that multiple sex steroids or EDCs may have on coral reproduction and health. It seems likely that estrone, estradiol, and progesterone are prevalent in reef ecosystems at relevant concentrations. More research is needed to determine the extent to which these sex steroids and others

(e.g. testosterone, ethinylestradiol) occur and their source. This field of research should assess the potential effects these compounds have individually and synergistically across all life stages of many different species of corals. It is also unclear what impacts inactive conjugated steroids (i.e., 17β estradiol glucuronide) have on corals and other marine organisms. These more water-soluble compounds have been detected in the tissue of coral and water column during mass spawning events (Twan et al. 2003). However, concentrations in the environment and effects are relatively unknown. Finally, steroid metabolism in cnidarians and scleractinian corals need to be fully mapped out and understood. While the endogenous bio-synthesis of these compounds appears to be plausible in several species, many gaps remain in understanding the numerous pathways and mechanisms of action. As reefs continue to decline, it will be increasingly important to fill the many gaps in knowledge regarding the physiological pathways, receptors, and effects these compounds may have on scleractinian corals.

Appendix

Table A1. Histology Scoring Rubric

Parameter	0 Normal	1 Minimal	2 Mild	3 Moderate	4 Marked	5 Severe
Zooxanthellae **40X magnification**	Gastro-dermal cells packed with well-stained symbionts in surface body wall and tentacles	Similar to controls; slightly fewer and scattered symbionts in gastrodermis of surface body wall and tentacles	Layer of well stained symbionts but less abundant than controls	Fewer symbionts in gastroder-mis, which is mildly atrophied; most still stain appropriate-ly, but some degenerating	Even fewer symbionts in gastrodermis of surface body wall and tentacle; some loss of acidophilic staining	No symbionts present in gastrodermis of colony (bleached)
Epidermal Architecture: SBW	Short columnar cells, uniform distribu-tion and not taller than ciliated support-ing cells, pale mucus	Slightly atrophied, particularly over septal ridge with minimal lysing	Many cells atrophied, severe atrophy over septal ridges with moderate lysing	Moderate lysing and fragmenta-tion; cells more squamous	Abundant fragmenta-tion and lysing	Epidermis severely atrophied to at least half normal thickness or more
Ruptures: SBW	Epithelia and mesoglea intact	One or few breaks in epithelia	One-quarter of epithelial area has structural gaps, mucus discharge	One-half of epithelial area has structural gaps, mucus discharge	Three-quarters of epithelial area has structural gaps, mucus discharge	Entire surface body wall dissociated; full thickness ablation evident
Mucocytes: SBW	Similar to controls, pale staining mucus	Slight atrophy and frothy mucus with some release	Atrophy of mucocytes and abundant release	Uneven appearance of mucocytes, some atrophied, darker staining mucus	Some epidermal foci lack mucocytes entirely, darker staining and stringy mucus	Loss of mucocytes

Granular Amoebocytes in SBW (for Porites astreoides)	None present	Presence of one to a few cells	One quarter of area contains these cells	Half of the area contains these cells	Three-quarters of the area contains these cells	Epidermis and gastrodermis heavily infiltrated by these cells
Gastrodermal Architecture: SBW	Gastro-dermis of surface body wall intact	One or few areas of gastrodermal cells lysed, mucus filling gastrovas-cular canals / cavity	One-quarter of the area of gastrodermal cells lysed, mucus filling gastrovas-cular canals / cavity	One-half of the area of gastrodermal cells lysed, mucus filling gastrovas-cular canals / cavity	Three-quarters of the area of gastrodermal cells lysed, mucus filling gastrovas-cular canals / cavity	All gastrodermal cells lysed, mucus filling gastrovas-cular canals / cavity
Gastrodermal Architecture: BBW	Gastro-dermis of basal body wall intact	One or few areas of gastrodermal cells lysed, mucus filling gastrovas-cular canals / cavity	One-quarter of the area of gastrodermal cells lysed, mucus filling gastrovas-cular canals / cavity	One-half of the area of gastrodermal cells lysed, mucus filling gastrovas-cular canals / cavity	Three-quarters of the area of gastrodermal cells lysed, mucus filling gastrova-scular canals / cavity	All gastrodermal cells lysed, mucus filling gastrovas-cular canals / cavity
Mucocytes: BBW	Similar to controls; pale staining mucus	Slight atrophy of mucocytes; frothy mucus with some release	Atrophy of mucocytes; abundant release of mucus	Mucocytes present with no staining	Mucocytes lysed and degraded	Loss of mucocytes
Granular Amoebocytes in Mesenteries and BBW (for Porites astreoides)	None present or all dead	Presence of one to a few cells	One quarter of area contain these cells	Half of the area contain these cells	Three-quarters of the area contain these cells	Epidermis and gastrodermis heavily infiltrated by these cells
Calicodermis	Squamous to low columnar cells, fine acidophil-ic granules of organic matrix	Slight atrophy of calicodermis with fewer acidophilic granules	Variable thinning of calicoder-mis, fewer acidophilic granules, and more areas affected	Squamous calicodermis, fewer acidophilic granules, lysing in some areas	Squamous calicoder-mis, necrotic or lysing, no acidophilic granules	Loss of calicoblasts along mesoglea, necrotic or lysing

Table A2. Trial 1 water quality data. Low, high, and average recorded temperatures (°C) along with average salinity (ppt) for each treatment.

	Temperature (°C)			Salinity (ppt)
Treatment	Low	High	Mean	Mean
Control	24.8	26.3	25.8	34.3
1 ng/L E2	25.0	26.4	25.8	34.4
10 ng/L E2	24.8	26.5	25.8	34.4
5 ng/L Prog	23.9	26.6	25.9	34.4
30 ng/L Prog	24.9	26.6	25.9	34.3

Table A3. Trial 2 water quality data. Low, highest, and average recorded temperatures (°C) along with average salinity (ppt) for each treatment.

	Temperature (°C)			Salinity (ppt)
Treatment	Low	High	Mean	Mean
Control	24.9	26.0	25.5	34.5
1 ng/L E2	24.5	26.1	25.5	34.3
10 ng/L E2	25.2	27.6	25.7	34.3
5 ng/L Prog	24.8	26.9	25.7	34.3
30 ng/L Prog	25.0	26.3	25.8	34.4

Literature Cited

Albright, R., Mason, B., & Langdon, C. (2008). Effect of aragonite saturation state on settlement and post-settlement growth of *Porites astreoides* larvae. *Coral Reefs, 27*(3), 485-490. doi:10.1007/s00338-008-0392-5.

Andersen, H., Siegrist, H., Halling-Sorensen, B., & Ternes, T. A. (2003). Fate of estrogens in a municipal sewage treatment plant. *Environmental Science & Technology, 37*(18), 4021-4026. doi:10.1021/es026192a.

Andrew, M. N., Dunstan, R. H., O'Connor, W. A., Van Zwieten, L., Nixon, B., & MacFarlane, G. R. (2008). Effects of 4-nonylphenol and 17α-ethynylestradiol exposure in the Sydney rock oyster, *Saccostrea glomerata*: vitellogenin induction and gonadal development. *Aquatic Toxicology, 88*(1), 39-47. doi:http://dx.doi.org/10.1016/j.aquatox.2008.03.003.

Andrew, M. N., O'Connor, W. A., Dunstan, R. H., & MacFarlane, G. R. (2010). Exposure to 17 alpha-ethynylestradiol causes dose and temporally dependent changes in intersex, females and vitellogenin production in the Sydney rock oyster. *Ecotoxicology, 19*(8), 1440-1451. doi:10.1007/s10646-010-0529-5.

Armoza-Zvuloni, R., Kramarsky-Winter, E., Rosenfeld, H., Shore, L. S., Segal, R., Sharon, D., & Loya, Y. (2012). Reproductive characteristics and steroid levels in the scleractinian coral *Oculina patagonica* inhabiting contaminated sites along the Israeli Mediterranean coast. *Marine Pollution Bulletin, 64*(8), 1556-1563. doi:10.1016/j.marpolbul.2012.05.020.

Aronson, R., Bruckner, A., Moore, J., Precht, B., & Weil E. (2008). *Acropora cervicornis. IUCN Red List of Threatened Species* (Version 2012.2).

Aronson, R. B., & Precht, W. F. (2001). White-band disease and the changing face of Caribbean coral reefs. *Hydrobiologia, 460*, 25-38. doi:10.1023/a:1013103928980.

Atkinson, S., & Atkinson, M. J. (1992). Detection of estradiol-17-beta during a mass coral spawn. *Coral Reefs, 11*(1), 33-35. doi:10.1007/bf00291932.

Atkinson, S., Atkinson, M. J., & Tarrant, A. M. (2003). Estrogens from sewage in coastal marine environments. *Environmental Health Perspectives, 111*(4), 531-535. doi: 10.1289/ehp.5233.

Auriol, M., Filali-Meknassi, Y., Tyagi, R. D., Adams, C. D., & Surampalli, R. Y. (2006). Endocrine disrupting compounds removal from wastewater, a new challenge. *Process Biochemistry, 41*(3), 525-539. doi:10.1016/j.procbio.2005.09.017.

Baker, M. E. (2002). Albumin, steroid hormones and the origin of vertebrates. *Journal of Endocrinology, 175*(1), 121-127. doi:10.1677/joe.0.1750121. doi:10.1016/j.jenvman.2005.04.006.

Belfroid, A. C., Van der Horst, A., Vethaak, A. D., Schäfer, A. J., Rijs, G. B. J., Wegener, J., & Cofino, W. P. (1999). Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. *Science of The Total Environment, 225*(1–2), 101-108. doi:http://dx.doi.org/10.1016/S0048-9697(98)00336-2.

Blomquist, C. H., Lima, P. H., Tarrant, A. M., Atkinson, M. J., & Atkinson, S. (2006). 17 beta-hydroxysteroid dehydrogenase (17 beta-HSD) in scleractinian corals and zooxanthellae. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 143*(4), 397-403. doi:10.1016/j.cbpb.2005.12.017.

Boulon, R., Chiappone, M., Halley, R., Jaap, W., Keller, B., Kruczynski, B., Rogers, C. (2005). *Acropora* Biological Review Team. Atlantic *Acropora* Status Review Document. Report to National Marine Fisheries Service, Southeast Regional Office. March 3, 2005. 152 p + App.

Brinton, R. D., Thompson, R. F., Foy, M. R., Baudry, M., Wang, J., Finch, C. E., Nilsen, J. (2008). Progesterone receptors: form and function in brain. *Frontiers in Neuroendocrinology, 29*(2), 313-339. doi:10.1016/j.yfrne.2008.02.001.

Cesar, H., Burke, L., & Pet-Soede, L. (2003). The economics of worldwide coral reef degradation.

Charpy, L., Casareto, B. E., Langlade, M. J., & Suzuki, Y. (2012). *Cyanobacteria* in Coral Reef Ecosystems*:* A Review (Vol. 2012). doi: 10.1155/2012/259571

Cooper, Wade T. (2009). Assessing the early life stage processes that regulate recruitment in the brooding coral, *Porites astreoides*. *Open Access Dissertations, Paper 325*, 1-207.

Corcoran, J., Winter, M. J., & Tyler, C. R. (2010). Pharmaceuticals in the aquatic environment: A critical review of the evidence for health effects in fish. *Critical Reviews in Toxicology, 40*(4), 287-304. doi: 10.3109/10408440903373590.

Darden, Heather. (2000). Wastewater in the Florida Keys: a call for stricter regulation of nonpoint source pollution. *Journal of Land Use & Environmental Law, 16*(2), 199-224.

Davies, P. S. (1989). Short-term growth measurements of corals using an accurate buoyant weighing technique. *Marine Biology, 101*(3), 389-395.

Desbrow, C., Routledge, E. J., Brighty, G. C., Sumpter, J. P., & Waldock, M. (1998). Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in Vitro biological screening. *Environmental Science & Technology, 32*(11), 1549-1558. doi:10.1021/es9707973.

Edinger, E. N., Jompa, J., Limmon, G. V., Widjatmoko, W., & Risk, M. J. (1998). Reef degradation and coral biodiversity in Indonesia: effects of land-based pollution, destructive fishing practices and changes over time. *Marine Pollution Bulletin, 36*(8), 617-630.

Fernandez, C. (2012). Effects of light and feeding on growth and biochemical responses of two Caribbean corals, *Acropora cervicornis* and *Porites porites*. UMI Dissertation Publishing, 1-111.

Ferrier-Pages, C., Gattuso, J. P., Dallot, S., & Jaubert, J. (2000). Effect of nutrient enrichment on growth and photosynthesis of the zooxanthellate coral *Stylophora pistillata*. *Coral Reefs, 19*(2), 103-113. doi:10.1007/s003380000078.

Futch, J. C., Griffin, D. W., Banks, K., & Lipp, E. K. (2011). Evaluation of sewage source and fate on southeast Florida coastal reefs. *Marine Pollution Bulletin, 62*(11), 2308-2316. doi:10.1016/j.marpolbul.2011.08.046.

Gassman, N. J., & Kennedy, C. J. (1992). Cytochrome-P-450 content and xenobiotic metabolizing enzyme-activities in the scleractinian coral, *Favia fragum* (Esper). *Bulletin of Marine Science, 50*(2), 320-330.

Harrison, P., & Wallace, C. (1990). Reproduction, dispersal and recruitment of scleractinian corals. *Ecosystems of the world, 25*, 133-207.

Hoegh-Guldberg, O., Mumby, P. J., Hooten, A. J., Steneck, R. S., Greenfield, P., Gomez, E., Hatziolos, & M. E. (2007). Coral reefs under rapid climate change and ocean acidification. *Science, 318*(5857), 1737-1742.

Fauth, J. E., Calestani, C., Dustan, P., Wham, D., Banks, K., Moulding, A., & Downs, C. (2011). Biomarker study to identify and trace coral reef contaminants. Florida Department of Environmental Protection, Coral Reef Conservation Program. Miami, FL, iv + 46 pages.

Jackson, J., Donovan, M., Cramer, K., & Lam, V. (2014). Status and trends of Caribbean coral reefs*: 1970-2012*. Global Coral Reef Monitoring Network, IUCN, Gland, Switzerland.

Janer, G., LeBlanc, G. A., & Porte, C. (2005). A comparative study on androgen metabolism in three invertebrate species. *General and Comparative Endocrinology, 143*(3), 211-221. doi:10.1016/j.ygen.2005.03.016.

Janer, G., & Porte, C. (2007). Sex steroids and potential mechanisms of non-genomic endocrine disruption in invertebrates. *Ecotoxicology, 16*(1), 145-160. doi:10.1007/s10646-006-0110-4.

Jokiel, P., Maragos, J., & Franzisket, L. (1978). Coral growth: buoyant weight technique. *Coral reefs: research methods. UNESCO, Paris*, 529-541.

Jones, J., Mosher, W., & Daniels, K. (2012). Current contraceptive use in the United States, 2006–2010, and changes in patterns of use since 1995. *National Health Statistics Reports, 60*, 1-25.

Ketata, I., Denier, X., Hamza-Chaffai, A., & Minier, C. (2008). Endocrine-related reproductive effects in molluscs. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology, 147*(3), 261-270. doi: 10.1016/j.cbpc.2007.11.007.

Kime, D. E., & Larsen, L. O. (1987). Effect of gonadectomy and hypophysectomy on plasma steroid-levels in male and female Lampreys (*Lampetra-Fluviatilis,L*). *General and Comparative Endocrinology, 68*(2), 189-196. doi:10.1016/0016-6480(87)90028-1.

Koopman, B., Heaney, J.P., Cakir, F.Y., Rembold, M., Indeglia, P., & Kini, G. (2006). Ocean outfall study. Florida Department of Environmental Protection, Tallahassee, Florida.

Lafont, R. (2000). The endocrinology of invertebrates. *Ecotoxicology,* 9(1-2), 41-57. doi:10.1023/a:1008912127592.

Lafont, R., & Mathieu, M. (2007). Steroids in aquatic invertebrates. *Ecotoxicology, 16*(1), 109-130. doi: 10.1007/s10646-006-0113-1.

Lai, K. M., Johnson, K. L., Scrimshaw, M. D., & Lester, J. N. (2000). Binding of waterborne steroid estrogens to solid phases in river and estuarine systems. *Environmental Science & Technology, 34*(18), 3890-3894. doi:10.1021/es9912729.

Langston, W. J., Burt, G. R., Chesman, B. S., & Vane, C. H. (2005). Partitioning, bioavailability and effects of oestrogens and xeno-oestrogens in the aquatic environment. *Marine Biological Association of the United Kingdom. Journal of the Marine Biological Association of the United Kingdom, 85*(1), 1-31.

Lapointe, B. E., Barile, P. J., & Matzie, W. R. (2004). Anthropogenic nutrient enrichment of seagrass and coral reef communities in the lower Florida Keys: discrimination

of local versus regional nitrogen sources. *Journal of Experimental Marine Biology and Ecology, 308*(1), 23-58. doi:10.1016/j.jembe.2004.01.019.

Le Curieux-Belfond, O., Fievet, B., Seralini, G. E., & Mathieu, M. (2005). Short-term bioaccumulation, circulation and metabolism of estradiol-17 beta in the oyster *Crassostrea gigas. Journal of Experimental Marine Biology and Ecology, 325*(2), 125-133. doi:10.1016/j.jembe.2005.04.027.

Legler, J., Jonas, A., Lahr, J., Vethaak, A. D., Brouwer, A., & Murk, A. J. (2002). Biological measurement of estrogenic activity in urine and bile conjugates with the in vitro ER-CALUX reporter gene assay. *Environmental Toxicology and Chemistry, 21*(3), 473-479. doi:10.1897/1551-5028(2002)021<0473:bmoeai>2.0.co;2.

Lowartz, S., Petkam, R., Renaud, R., Beamish, F. W. H., Kime, D. E., Raeside, J., & Leatherland, J. F. (2003). Blood steroid profile and in vitro steroidogenesis by ovarian follicles and testis fragments of adult sea lamprey, *Petromyzon marinus. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology, 134*(2), 365-376. doi:10.1016/s1095-6433(02)00285-4.

Loya, Y., & Rinkevich, B. (1979). Abortion effect in corals induced by oil pollution. *Marine Ecology Progress Series, 1*(1), 77-80.

Lueg, J. R., Moulding, A. L., Kosmynin, V. N., & Gilliam, D. S. (2012). Gametogenesis and spawning of *Solenastrea bournoni* and *Stephanocoenia intersepta* in southeast Florida, USA. *Journal of Marine Biology, 2012, 1-12.* doi: 10.1155/2012/370247.

Markov, G. V., Tavares, R., Dauphin-Villemant, C., Demeneix, B. A., Baker, M. E., & Laudet, V. (2009). Independent elaboration of steroid hormone signaling pathways in metazoans. *Proceedings of the National Academy of Sciences of the United States of America, 106*(29), 11913-11918. doi:10.1073/pnas.0812138106.

Moberg, F., & Folke, C. (1999). Ecological goods and services of coral reef ecosystems. *Ecological Economics, 29*(2), 215-233.

Nagahama, Y. (1994). Endocrine regulation of gametogenesis in fish. *International Journal of Developmental Biology, 38*(2), 217-229.

Olsen, K., Sneed, J. M., & Paul, V. J. (2016). Differential larval settlement responses of *Porites astreoides* and *Acropora palmata* in the presence of the green alga *Halimeda opuntia. Coral Reefs, 35*(2), 521-525.

Pascoe, D., Carroll, K., Karntanut, W., & Watts, M. M. (2002). Toxicity of 17 alpha-ethinylestradiol and bisphenol A to the freshwater cnidarian *Hydra vulgaris. Archives of Environmental Contamination and Toxicology, 43*(1), 56-63. doi:10.1007/s00244-001-0016-3.

Pernet, V., & Anctil, M. (2002). Annual variations and sex-related differences of estradiol-17 beta levels in the anthozoan *Renilla koellikeri. General and Comparative Endocrinology, 129*(1), 63-68. doi:10.1016/s0016-6480(02)00513-0.

Peters, E. C., Gassman, N. J., Firman, J. C., Richmond, R. H., & Power, E. A. (1997). Ecotoxicology of tropical marine ecosystems. *Environmental Toxicology and Chemistry, 16*(1), 12-40. doi:10.1897/1551-5028(1997)016<0012:eotme>2.3.co;2.

Porte, C., Janer, G., Lorusso, L. C., Ortiz-Zarragoitia, M., Cajaraville, M. P., Fossi, M. C., & Canesi, L. (2006). Endocrine disruptors in marine organisms: approaches

and perspectives. *Comparative Biochemistry and Physiology C—Toxicology & Pharmacology, 143*(3), 303-315. doi:10.1016/j.cbpc.2006.03.004.

Precht, W. F., Gintert, B. E., Robbart, M. L., Fura, R., & van Woesik, R. (2016). Unprecedented disease-related coral mortality in southeastern Florida. *Scientific Reports, 6*. doi: 10.1038/srep31374.

Purdom, C. E., Hardiman, P. A., Bye, V. J., Eno, N. C., Tyler, C. R., & Sumpter, J. P. (1994). Estrogenic effects of effluents from sewage treatment works. *Chemistry and Ecology, 8*(4), 275-285. doi:10.1080/02757549408038554.

Rotchell, J. M., & Ostrander, G. K. (2003). Molecular markers of endocrine disruption in aquatic organisms. *Journal of Toxicology and Environmental Health-Part B-Critical Reviews, 6*(5), 453-495. doi:10.1080/10937400390223430.

Rougee, L. R. A., Richmond, R. H., & Collier, A. C. (2014). Natural variations in xenobiotic-metabolizing enzymes: developing tools for coral monitoring. *Coral Reefs, 33*(2), 523-535. doi:10.1007/s00338-014-1136-3.

Rougee, L. R. A., Richmond, R. H., & Collier, A. C. (2015). Molecular reproductive characteristics of the reef coral *Pocillopora damicornis*. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology, 189*, 38-44. doi:10.1016/j.cbpa.2015.07.012.

Sandor, T. (1981). Steroids in invertebrates. *Developments in Endocrinology, 11*, 81-96.

Sharp, K. H., Sneed, J. M., Ritchie, K. B., McDaniel, L., & Paul, V. J. (2015). Induction of larval settlement in the reef coral *Porites astreoides* by a cultivated marine Roseobacter strain. *Biological Bulletin, 228*(2), 98-107.

Shinn, E. A. (1976). Coral reef recovery in Florida and the Persian Gulf. *Environmental Geology, 1*(4), 241-254.

Singh, S. P., Azua, A., Chaudhary, A., Khan, S., Willett, K. L., & Gardinali, P. R. (2010). Occurrence and distribution of steroids, hormones and selected pharmaceuticals in south Florida coastal environments. *Ecotoxicology, 19*(2), 338-350. doi: 10.1007/s10646-009-0416-0.

Slattery, M., Hines, G. A., Starmer, J., & Paul, V. J. (1999). Chemical signals in gametogenesis, spawning, and larval settlement and defense of the soft coral *Sinularia polydactyla*. *Coral Reefs, 18*(1), 75-84. doi:10.1007/s003380050158.

Slattery, M., Hines, G. A., & Watts, S. A. (1997). Steroid metabolism in Antarctic soft corals. *Polar Biology, 18*(1), 76-82. doi:10.1007/s003000050161.

Soong, K. (1991). Sexual reproductive patterns of shallow-water reef corals in Panama. *Bulletin of Marine Science, 49*(3), 832-846.

Soong, K. Y., & Lang, J. C. (1992). Reproductive integration in reef corals. *Biological Bulletin, 183*(3), 418-431.

Sorensen, P. W., Scott, A. P., Stacey, N. E., & Bowdin, L. (1995). Sulfated 17,20-Beta-dihydroxy-4-pregnen-3-one functions as a potent and specific olfactory stimulant with pheromonal actions in the goldfish. *General and Comparative Endocrinology, 100*(1), 128-142. doi:10.1006/gcen.1995.1141.

Szmant, A. M. (1986). Reproductive ecology of Caribbean reef corals. *Coral Reefs, 5*(1), 43-53. doi:10.1007/bf00302170.

Tarrant, A. M. (2005). Endocrine-like signaling in cnidarians: current understanding and implications for ecophysiology. *Integrative and Comparative Biology, 45*(1), 201-214. doi:10.1093/icb/45.1.201.

Tarrant, A. M. (2007). Hormonal signaling in cnidarians: do we understand the pathways well enough to know whether they are being disrupted? *Ecotoxicology, 16*(1), 5-13. doi:10.1007/s10646-006-0121-1

Tarrant, A. M., Atkinson, M. J., & Atkinson, S. (2001). Uptake of estrone from the water column by a coral community. *Marine Biology, 139*(2), 321-325.

Tarrant, A. M., Atkinson, M. J., & Atkinson, S. (2004). Effects of steroidal estrogens on coral growth and reproduction. *Marine Ecology-Progress Series, 269*, 121-129. doi: 10.3354/meps269121.

Tarrant, A. M., Atkinson, S., & Atkinson, M. J. (1999). Estrone and estradiol-17 beta concentration in tissue of the scleractinian coral, *Montipora verrucosa*. *Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology, 122*(1), 85-92. doi:10.1016/s1095-6433(98)10155-1.

Tarrant, A. M., Blomquist, C. H., Lima, P. H., Atkinson, M. J., & Atkinson, S. (2003). Metabolism of estrogens and androgens by scleractinian corals. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 136*(3), 473-485. doi:10.1016/s1096-4959(03)00253-7

Tarrant, A. M., Reitzel, A. M., Blomquist, C. H., Haller, F., Tokarz, J., & Adamski, J. (2009). Steroid metabolism in cnidarians: insights from *Nematostella vectensis*. *Molecular and Cellular Endocrinology, 301*(1-2), 27-36. doi:10.1016/j.mce.2008.09.037.

Ternes, T. A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R. D., & Servos, M. (1999). Behavior and occurrence of estrogens in municipal sewage treatment plants—I. Investigations in Germany, Canada and Brazil. *Science of The Total Environment,*

Thornton, J. W., Need, E., & Crews, D. (2003). Resurrecting the ancestral steroid receptor: ancient origin of estrogen signaling. *Science, 301*(5640), 1714-1717. doi:10.1126/science.1086185.

Twan, W. H., Hwang, J. S., & Chang, C. F. (2003). Sex steroids in scleractinian coral, *Euphyllia ancora*: implication in mass spawning. *Biology of Reproduction, 68*(6), 2255-2260. doi: 10.1095/biolreprod.102.012450.

Twan, W. H., Hwang, J. S., Lee, Y. H., Wu, H. F., Tung, Y. H., & Chang, C. F. (2006). Hormones and reproduction in scleractinian corals. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology, 144*(3), 247-253. doi: 10.1016/j.cbpa.2006.01.011.

Wang, C., & Croll, R. P. (2004). Effects of sex steroids on gonadal development and gender determination in the sea scallop, *Placopecten magellanicus*. *Aquaculture, 238*(1–4), 483-498. doi:http://dx.doi.org/10.1016/j.aquaculture.2004.05.024.

Worm, B., & Lenihan, H. S. (2014). Threats to marine ecosystems overfishing and habitat degradation. Sunderland: Sinauer Assoc.

Wright-Walters, M., & Volz, C. (2009). Municipal wastewater concentrations of pharmaceutical and xeno-estrogens: wildlife and human health implications. New York: Springer.

Young, C. N., Schopmeyer, S. A., & Lirman, D. (2012). A review of reef restoration and coral propagation using the threatened genus *Acropora* in the Caribbean and western Atlantic. *Bulletin of Marine Science, 88*(4), 1075-1098.

Zaneveld, J. R., Burkepile, D. E., Shantz, A. A., Pritchard, C. E., McMinds, R., Payet, J. P., Thurber, R. V. (2016). Overfishing and nutrient pollution interact with

temperature to disrupt coral reefs down to microbial scales. *Nat Commun, 7*. doi: 10.1038/ncomms11833.

Zhu, B. T., & Conney, A. H. (1998). Functional role of estrogen metabolism in target cells: review and perspectives. *Carcinogenesis, 19*(1), 1-27. doi:10.1093/carcin/19.1.1.