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Variations of Ghrelin, Growth Hormone, and Insulin-Like Growth Factor I in the West Indian Manatee (*Trichechus manatus*)

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VARIATIONS OF GHRELIN, GROWTH HORMONE, AND INSULIN-LIKE GROWTH FACTOR I IN THE WEST
INDIAN MANATEE (*Trichechus manatus*)

by

Rachel Lynn Cimino

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ABSTRACT

The metabolic hormones ghrelin, growth hormone, and insulin-like growth factor I are influenced by developmental age, sex, and nutritional status in domestic and free-ranging species. However the role these hormones play has not previously been explored in sub-tropical/ tropical mammals. Furthermore, the seasonality of species with less dynamic environmental cues, such as the West Indian manatee, have not been studied. The West Indian manatee is an endangered species distributed in the southeastern United States and throughout the Caribbean basin, and its nutritional physiology is influenced by environmental factors. Understanding the hormone response to nutritional status in this species and its seasonality will enhance our knowledge of the links between season, nutrition, and life history.

The purpose of this research is to understand the biology and seasonal patterns of metabolic hormones in free-ranging manatees which will allow us to assess the nutritional status of wild populations. The research objectives include validation assays to accurately quantify hormone concentrations in manatees. Hormones were quantified in manatee serum using heterologous radioimmunoassay. Hormones were then evaluated between summer, fall, and winter and compared to body composition. Developmental patterns were also assessed. Lastly, hormones were examined between Florida and Antillean manatee populations.

Manatees exhibited differences in GH, IGF-I, and body composition demonstrating seasonality similar to other species. Manatees exhibited differences between age classes suggesting decreased growth rate as the animals age. Differences were detected between populations. This research suggests that ghrelin, GH, and IGF-I can be used to indicate nutritional status and detect seasonality and developmental age in the manatee. This could prove to be a valuable tool in rehabilitation facilities and during captures and health assessments to provide better veterinary care and further improve overall health and body condition to better manage the survival of the species.

CHAPTER ONE: Scope of Study

Mammals live in diverse environments and are confronted with consistent seasonal environmental changes in light, temperature, and food availability. Seasonal environmental cues may stimulate a physiological response in an animal. Physiological responses initiated by the endocrine system, function to maintain homeostasis. Environmental stimuli may elicit a response of the organism to adjust homeostatic set points. These adjustments result in acclimatization and may include responses, such as changes in food intake, body weight, fat content, and feed efficiency (Jeanniard du dot et al. 2008; Florant and Healy 2012). The purpose of this research is to understand the biology and seasonal patterns of metabolic hormones in free-ranging manatees, *Trichechus manatus latirostris*, which will allow us to assess the nutritional status of wild populations in context with their normal life history patterns.

Goal: Seasonal changes in body composition are commonly observed in sub-arctic species that exhibit strong seasonality (seasonal physiological response) driven by dynamic environmental cues (Jeanniard du Dot et al. 2008; Richmond et al. 2010). However, little is known about the seasonality of tropical or sub-tropical species with limited environmental cues (Florant and Healy 2012). The goal of this research is to investigate seasonality in tropical/subtropical species compared with arctic species and determine how metabolic hormones play a role in regulating seasonal change in body composition and

influence metabolism. The physiological regulation of seasonal changes in nutrient intake on body mass gain and composition of gain can be assessed by investigating endocrine factors that link energy regulation, nutrient intake, growth, and tissue specific nutrient allocation.

Objectives: To accomplish this goal, six objectives were established for this thesis research:

(1) Validate assays that accurately and precisely quantify serum concentrations of metabolic hormones in manatees. (2) Compare hormone concentrations between short (< 12 hours of daylight) and long (> 12 hours of daylight) photoperiods. (3) Determine the seasonal pattern of these hormones and quantify their relationship to body composition in manatees. (4) Evaluate sex differences in the hormones. (5) Assess the ontogeny of components of the somatotrophic axis in manatees. (6) Assess hormone differences between free-ranging manatee populations in Florida, Belize, and Puerto Rico.

CHAPTER TWO: Literature Review

LIFE HISTORY

Distribution and Habitat

West Indian manatees (*Trichechus manatus*), from the Order Sirenia, are aquatic mammals that inhabit shallow rivers, estuaries, coastal waters, and saltwater bays throughout the southeast United States and the Caribbean (Domning 1996). West Indian manatees were well known to Indigenous people in tropical and subtropical environments on the eastern Atlantic Coast and the rivers of North and South America (Domning 1996; 2005). The West Indian manatee received its scientific name, *Trichechus manatus*, in 1758 by Linnaeus (Linnaeus 1758). Two sub-species of West Indian manatee exist, the Florida manatee (*T.m. latirostris*) which inhabit the east coast of the United States from Massachusetts to Florida and the states bordering the Gulf of Mexico, and the Antillean manatee (*T.m. manatus*) that inhabit Belize and Puerto Rico and other Caribbean coasts of Central and South America (Powell and Rathburn 1984; Deutsch et al. 1998; Fertl et al. 2005; Beck 2006; Hunter et al. 2010; 2012). The two subspecies can be distinguished based on their distinct geographical distribution, genetic diversity, and slight differences in skeletal morphology (Garcia-Rodriguez et al. 1998; Vianna et al. 2006; Hunter et al. 2010; Nourisson et al. 2011; Hunter et al. 2012). Generally, body length is less in Antillean manatees compared with Florida manatees (Odell 1982; Converse et al. 1994). Florida manatees are environmentally constrained; their distribution exists at the upper most thermal limits and requires winter migration to warm water sites. In contrast, Antillean manatees, specifically, Belize and Puerto

Rico populations inhabit tropical waters that maintain at relatively constant temperatures throughout the year (Irvine 1983; Worthy et al. 2000; Bossart et al. 2003).

Regionally, manatees exhibit different foraging habits. The Florida manatee is a generalist primary consumer eating terrestrial, freshwater, estuarine, and marine vegetation (Hartman 1979; Best 1981; Bengtson 1983). Populations in Belize and Puerto Rico spend more time in marine environments and therefore consume a greater volume of seagrass than other types of vegetation (Mignucci-Giannoni and Beck 1998; Alves-Stanley et al. 2010). Availability, quantity, and quality (nutrient richness) of forage are reduced in Belize and Puerto Rico compared with Florida (Alves-Stanley et al. 2010).

Anatomy and Physiology

The West Indian manatee is a large mammal that has gray to tan epidermis and is sparsely covered with hairs (Reep et al. 2002; Reep and Bonde 2006). The head has external nares located on the snout with muscles that expand and contract allowing the manatee to take a breath when breaking the water's surface. Their muzzle has prehensile lips and highly innervated bristles used for tactile discrimination when foraging (Marshall et al. 1998). Manatees have two pectoral flippers with nails used to manipulate food and other objects. The pectoral flippers are also used to "pec walk" through sediment and extend the manatees reach on shorelines for improved foraging (O'Shea 1986). A large round tail is used like a paddle to propel the animals through the water. Manatees are polyphyodonts (teeth continuously replaced); their hypsodont dentition (high crowned molars) are pushed forward and replaced as they wear down due to their plant-based diets and intake of sand (Reynolds and Odell 1991; Reep and Bonde 2006).

West Indian manatees digest cellulose they ingest in their plant-based diet using microbial bacteria during the process of hindgut fermentation (Reynolds and Rommel 1996). West Indian

manatees spend approximately 6 to 8 hours a day feeding and can consume about 4 to 9% of their body weight in wet vegetation (Etheridge et al. 1985). They are generalist herbivores, feeding on over 60 species of aquatic plants, including submerged, emergent, and floating vegetation (Domning 1980; Lefebvre et al. 2000).

Marine mammals have a unique adipose storage site in comparison to terrestrial species known as blubber. Blubber is hypodermal fat that contains more blood vessels than adipose found in terrestrial animals (Young 1976; Whittow 1987). The most significant role of blubber is to provide insulation for existence in aquatic environments (Young 1976; Whittow 1987). In addition to thermal regulatory functions, blubber plays a role in buoyancy, streamlining body shape, and providing storage for energy reserves (Iverson 2002). In many marine mammal species, blubber consists of multiple subcutaneous lipid layers. The Florida manatee has alternating layers of blubber and paniculus muscle; however, their blubber layers are less dense and thick compared with other marine mammals; this modification makes manatee blubber less efficient in thermoregulation (Irvine 1983).

Thermoregulation

In addition to their unique blubber structure, the West Indian manatee has a low metabolic rate and a reduced capacity to retain body heat in comparison to other marine mammals (Irvine 1983). Manatees' low metabolic rate reduces their ability to maintain their core body temperature during the winter season, which can contribute to cold susceptibility and induce a physiological reaction referred to as cold-stress syndrome, which is characterized by emaciation, fat store depletion, and other responses (Irvine 1983; Bossart et al. 2003).

To avoid cold stress syndrome, many manatees in the southeastern United States exhibit seasonal migration to warmer waters in the winter once water temperatures fall below 20°C (Deutsch

et al. 2003; Reep and Bonde 2006). Warm water refuges may include natural springs and artificial power plant discharge canals (Reynolds and Wilcox 1986; Reynolds and Odell 1991). Individuals often show fidelity to particular winter refuge areas most likely based upon behaviors learned as calves (Deutsch et al. 2003). Most warm water refuges, such as power plants, tend to have limited availability of forage (Reynolds 2000). Decreased intake further exacerbates manatee reduced thermoregulatory capacity because nutrients stored in blubber are mobilized and therefore blubber thickness is reduced (Young 1976; Whittow 1987; Iverson 2002). A reduction in blubber thickness causes the insulated capacity to decrease (e.g. Ortiz et al. 1978; Best 1981).

Mating and Reproduction

Generally, adult males tend to have larger home ranges compared with adult females. Males travel greater distances likely in search of receptive females for mating (Hartman 1979). Manatees can mate throughout the year but peak breeding is during spring and summer (Hartman 1979). A group of males forming a mating herd, may consort a female in estrous for up to four weeks (Hartman 1979; Reep and Bonde 2006). Mating herds can be composed of over 20 males. Females have been seen to mate with more than one male (Hartman 1979).

Females tend to be larger than males in both weight and length (Odell 1982). Females reach sexual maturity around 3 to 4 years of age (Hartman 1979). Typically males produce viable sperm around 2-3 years of age, but are not usually successful in competing for a female until 9 – 10 years of age (Hartman 1979). The West Indian manatee has a gestation period of 11-14 months (Rathburn et al. 1995). Generally, females give birth to a single calf with a 2.5 year inter-birth interval to a single calf but on rare occasions twins do occur (Kendall et al. 2004). Typically calves nurse for 1-2 years before being

weaned (Rathburn et al. 1995). Mother and calf pairs are known to communicate underwater using individually distinct vocalizations at 1-18 kHz range (Gerstein et al. 1999; O'Shea and Poche 2006).

Population Status

Life history of distinct populations and the overall species is determined by carcass recovery and necropsy and long term photo identification of individual manatees based on distinct scars (Langtimm et al. 2004). The lifespan of a West Indian manatee is approximately 60-70 years (Langtimm et al. 2004; Reep and Bonde 2006). Both subspecies of the West Indian manatee are listed as endangered. The Florida manatee is protected in the U.S. and Puerto Rico by both the Endangered Species Act (ESA 1973) and the Marine Mammal Protection (MMPA 1972). Manatees are protected in Belize by the Wildlife Protection Act of 1981. In the past, the West Indian manatee population was threatened by over hunting which is no longer prevalent. Nevertheless, the animals are still faced with other human-related threats such as vessel strikes and impact, entanglements, entrapments, exposure to pollution, loss and degradation of habitat, and harassment (Jimenez 2002; Lightsey et al. 2006). Natural causes of increased mortality may include cold stress, disease, and dinoflagellate blooms (red tide) (O'Shea et al. 1991).

Population estimates of Antillean manatees have been conducted since 1987 in Belize and since the 1970s in Florida with the utilization of range-wide synoptic surveys (Bengtson and Magor 1979; Hartman 1979; Irvine et al. 1982; O'Shea and Salisbury 1991). Although Antillean manatee numbers are thought to be increasing with approximately 2,500 adults in the population, results are based on surveys that used different techniques and were executed during different seasons in different geographical areas (Colmenero and Zarate 1990; Percival et al. 1995; Mignucci-Giannoni et al. 2000; De Meirelles 2008; Castelblanco-Martinez et al. 2009). Therefore, population counts may not be an accurate

measure due to inconsistency in methods and the proportion of manatees located outside of the survey areas, animals in visually disrupted areas (i.e. murky water), and observer sighting error (Lefebvre et al. 2000; Edwards et al. 2007). Population numbers of Florida manatees are denser than Antillean manatees particularly in Belize and Puerto Rico (FWC unpublished; Slone et al. 2006; Quintana-Rizzo and Reynolds 2007; Hunter et al. 2010, 2012; Tucker et al. 2012). Florida manatee populations are stable with approximately 5,000 individuals (FWC unpublished).

Population numbers are affected by numerous environmental factors including climate, forage, habitat, and unpredicted seasonal changes. Although, the Florida manatee migrates to warm water refuge areas during cold temperatures and experience seasonal changes in blubber thickness (Iverson 2002; Deutsch et al. 2003), little is known about the hormonal mechanisms that contribute to this change. In addition, understanding the role hormones play in regulating body composition and indicating nutritional status in manatees in different regional areas may provide knowledge that will contribute to the management and protection of the species. Therefore, the goal of this research was to investigate seasonality of the Florida manatee and determine if metabolic hormones play a role in regulating seasonal changes in body composition and metabolism. In addition, this research evaluated if hormonal differences could be detected among populations and further, if these differences can be used to indicate nutritional status. This was assessed in the manatee by investigating endocrine factors that link energy regulation, nutrient intake, growth, and tissue specific nutrient allocation.

THE SOMATOTROPIC AXIS AND GHRELIN

Energy Regulation and Nutrition

Homeostasis is maintained by multiple hormones and growth factors within the endocrine system such as components of the somatotrophic axis and ghrelin. Tissue specific energy allocation is

regulated by ghrelin and the components of the somatotrophic axis that include growth hormone (GH), insulin-like growth factor (IGF)-I, and IGF binding proteins (IGFBP) (Lawrence and Fowler 1997; Kojima et al. 1999). In addition to energy allocation the hormones indicate nutritional status within individuals (Breier 1999; Ortiz et al. 2003; Cammisoto et al. 2010; Castenda et al. 2010).

Ghrelin is a peptide hormone predominantly produced by enteroendocrine cells of the stomach and released into the blood (Date et al. 2000; Nakazato et al. 2001). Ghrelin receptors can be found in the liver, pancreas, kidney, the arcuate nucleus of the hypothalamus, and the pituitary (Kojima et al. 1999; Date et al. 2000). Ghrelin has been identified as the endogenous ligand for the GH secretagogue receptor (GHS-R; Kojima et al. 1999; Date et al. 2002). Ghrelin has been shown to stimulate the release of GH, decreases fat oxidation, and stimulates food intake, body weight gain, and adiposity in humans and rats (Tschop et al. 2000; Nakazato et al. 2001; Wren et al. 2001a, 2001b; Theander-Carrillo et al. 2006). The secretion of ghrelin by the stomach is dependent upon nutritional status. Not only do ghrelin concentrations increase pre-prandially and decrease postprandially (Ariyasu et al. 2001; Cummings et al. 2001; Tschop et al. 2001), but concentrations also demonstrate diurnal variations which can be influenced by GH and body mass (Korbonits et al. 2004). Concentrations of GH can negatively feedback on ghrelin concentrations and inhibit adiposity (Korbonits et al. 2004).

Growth hormone is regulated by growth hormone releasing hormone (GHRH) and somatostatin which are released from the hypothalamus and stimulate or inhibit growth hormone release from the anterior pituitary, respectively (Enright et al. 1987). The majority of GH secretion occurs nocturnally in association with slow wave sleep (Van Cauter et al. 1992). Growth hormone regulates energy deposition and mobilization through its direct action on adipose tissue (Renaville et al. 2002). During normal physiological conditions with adequate nutrient intake, GH stimulates protein and adipose accretion (Eigenmann et al. 1985; Breier et al. 1999; Carrel and Allen 2000).

When energy intake is maintained, GH stimulates the production and release of IGF-I from the

liver and stimulates protein accretion. Insulin-like growth factor-I regulates somatic growth, cell proliferation, differentiation, and apoptosis (Renaville et al. 2002). Protein synthesis, bone formation, and glucose uptake are stimulated by IGF-I (Etherton 1991). There are at least seven IGFBPs that regulate the bioavailability and therefore activity of circulating IGF-I (Clemmons and Underwood 1991; Estivariz and Ziegler 1997). However, IGFBP2 and IGFBP3 are most responsive to nutrient intake and correlated to whole body growth rate (Schwarz et al. 1992; Skaar et al. 1994; Breier 1999; Rausch et al. 2002; Govoni et al. 2003).

Malnourishment and Fasting

When food intake is altered and mammals are either fasting or in a malnourished state, metabolic hormones respond. Ghrelin increases with fasting stimulating the release of GH, and promoting increased adiposity and food intake; a typical response observed in most mammals (Cummings et al. 2001; Tschop et al. 2001; Ortiz et al. 2003). Growth hormone concentrations are normally elevated as a result of restricted food intake resulting in increased lipolysis (Breier 1999), whereas, IGF-I decreases which then reduces anabolic effects of the somatotrophic axis (Phillips and Young 1976; Branham et al. 1977; Amatayakul et al. 1978; Froesch et al. 1982; Clemmons et al. 1983; de Bruijne et al. 1985; Burleigh et al. 1987; Breier et al. 1988; Elsasser et al. 1989; Straus and Takemoto 1990; Buonomo and Baile 2011). Similar to IGF-I, IGFBP-3 decreases in a fasted or malnourished state whereas, IGFBP-2 increases and binds to IGF-I causing the hormone to become unavailable in some peripheral tissues (Smith et al. 1995; Renaville et al. 2000).

Age and sex related patterns

Ghrelin and the somatotrophic axis have age-related patterns and sex differences that result in differential growth rates. Ghrelin hormone concentrations are inconsistent with age; a study in mice revealed an increase in ghrelin with age from birth to maturity, whereas other research found no differences (Chanoine et al. 2006; Akimoto et al. 2011). Generally, GH and IGFBP-2 concentrations decrease as animals age, whereas, IGF-I and IGFBP-3 increase with age associated with decreased growth rate (Schwarz et al. 1992; Skaar et al. 1994; Gerrard et al. 1999; Harrell et al. 1999; and Govoni et al. 2000).

Sex differences in ghrelin and the somatotrophic axis have been found in ungulates such as the red tailed deer, mule deer, and cattle (Webster et al. 1999; Govoni et al. 2003; Comeau et al. 2009, unpublished). The limited studies of sex differences in ghrelin concentrations have been contradictory with some studies finding no sex differences (Purnell et al. 2003; Vilarrasa et al. 2005; Weiss et al. 2006), whereas other research reported greater concentration of ghrelin in females to stimulate food intake for reproductive needs (Barkan et al. 2003; Greenman et al. 2004; Salbe et al. 2004). Both serum GH and IGF-I concentrations are greater in males compared with female cattle, sheep, and pigs (Gatford et al. 1996; Owens et al. 1999; Govoni et al. 2003), and are associated with increased growth rate in males (Rausch et al. 2002).

Seasonality and Body Composition

Seasonal changes in body composition may be signaled by environmental variables (light, temperature, food availability) and regulated by the somatotrophic axis or other metabolic hormones (Fuglei et al. 2004). Some mammals that exhibit seasonal changes in body composition are generally leaner during long photoperiods (greater than 12 hours of daylight, summer) with greater adiposity

during short photoperiods (less than 12 hours of daylight, winter) (Zinn et al. 1986; Genin and Perret 2000). Ghrelin and the somatotrophic axis work together to regulate body composition (i.e. the relative portion of lean vs. adipose tissue).

Ghrelin can stimulate the secretion of GH and therefore, indirectly lipolysis (Tschop et al. 2000). During the short photoperiod ghrelin may be elevated to stimulate food intake to maintain body fat stores or increase food uptake because resources are limited (Tschop et al. 2000; Nakazato et al. 2001). In opposition to the typical ghrelin response to reduced intake, ghrelin concentrations in fasting arctic mammals decrease with a decrease in food intake, perhaps due to limited food availability (Mustonen et al. 2005). In some species, concentrations of GH are greater during short photoperiods to allow fatty acids to be mobilized from adipose stores to provide energy for maintenance requirements when nutritional intake is reduced (Fuglei et al. 2004; Mustonen et al. 2006). Concentrations of IGF-I are reduced in cattle and red tailed deer during short photoperiod to minimize protein and presumably conserve energy (Zinn et al. 1986; Webster et al. 1999). During short photoperiod, IGFBP-3 is positively correlated with growth rate and increases in moose to stimulate protein accretion (Parillo 2010). Concentrations of IGFBP-2 are negatively associated with growth rate and concentrations have been found to be reduced during short photoperiods in some ungulates (Parillo 2010). Hormone concentrations in conjunction with species specific body composition patterns support that animals typically have greater adiposity during short photoperiod.

During long photoperiod ghrelin concentrations in some terrestrial ruminant species are negatively correlated with food intake presumably to reduce adiposity (Nieminen et al. 2002). In a species where reduced intake is part of a long overwintering period, decreased GH in summer and fall facilitates adipose accumulation (Fuglei et al. 2004). Alternatively, in some ungulates increased GH in long photoperiods facilitates lean tissue accretion mediated by IGF-I (Rosenfeld and Hwa 2009). In the red tailed deer, IGF-I is elevated in long photoperiod to promote protein accretion (Zinn et al. 1986;

Webster et al. 1996; Gautsch et al. 1998; Hornick et al. 2000). Similar to GH, IGFBP-3 decreases during long photoperiod whereas IGFBP-2 increases (Parillo 2010). The differential pattern of hormones in species during different seasons indicates animals have different acclimation methods.

Seasonality in Marine Mammals

Limited studies on the seasonal effects of the somatotrophic axis, ghrelin, and body composition have been conducted in marine mammals. Some marine mammal species are naturally subject to seasonal variation in prey availability and intake (Raum-Suryan et al. 2004; Perrin et al. 2009) and exhibit traits that include extended periods of fasting on shore during the summer breeding season (Gentry 1974; Maniscalco et al. 2006; Perrin et al. 2009). Therefore, subarctic marine mammals start building adequate adipose stores during the fall season to maintain homeostasis during short photoperiod when food is scarce and temperatures are cold. During the long photoperiod marine mammals, particularly males, may build lean tissue, perhaps to compete for females during breeding (Gentry 1974; Perrin et al. 2009).

In a previous study performed in a sub-arctic sea lion, blubber was greater during short photoperiods and decreased during long photoperiods (Richmond et al. 2010). Concentrations of GH, and IGFBP-2 decreased during long photoperiods and increased during short photoperiods, whereas, IGF-I and IGFBP-3 increased during long photoperiod and decreased during short photoperiod (Richmond et al. 2010). Greater blubber stores during short photoperiods assists with thermoregulatory requirements (Jeanniard du dot et al. 2008). In addition to blubber providing insulation during cold temperatures, it also serves as an energy reserve during times of food availability (Irvine 1983).

In the northern hemisphere many terrestrial species feed on high quantity of available food in warmer months to create energy stores in order to maintain energy needs during colder months when

food may be scarce and animals fast (Mustonen et al. 2005; Asikainen et al. 2004). Although, photoperiodic changes in ghrelin concentrations have not been previously explored in marine mammals it is likely that ghrelin concentrations would be reduced due to their fasting strategy. Ghrelin generally increases with fasting, a typical response observed in most mammal models (Cummings et al. 2001; Tschop et al. 2001; Ortiz et al. 2003).

Importance

The West Indian manatee is considered a sentinel species that provides insight as to how other species in the ecosystem may respond to acute or chronic changes in their climate, habitat, and forage (Bonde et al., 2004; Bossart 2006). The mechanisms responsible for the physiological response to seasonal change in subtropical and tropical marine mammals may provide new information on energetics, fasting metabolism, dietary intake, and seasonal patterns in body composition (Strandberg et al. 2007). In addition, understanding the role of ghrelin and the somatotrophic axis in the West Indian manatee is important for developing a method to evaluate energy balance, nutritional status, and health status of free-ranging populations.

CHAPTER 3: The Seasonal Response of Ghrelin, Growth Hormone, and Insulin-like Growth Factor I in the Free-ranging Florida Manatee (*Trichechus manatus latirostris*)

INTRODUCTION

Mammals live in diverse environments and are confronted with consistent seasonal environmental changes in light, temperature, and food availability. Seasonal environmental changes may stimulate a physiological response in an animal. Physiological responses initiated by the endocrine system, function to maintain homeostasis. Environmental stimuli may elicit a response of the organism to adjust homeostatic set points. These adjustments result in acclimatization and may include responses, such as changes in food intake, body weight, fat content, and hormonal functions (Florant and Healy 2012). Hormones within the endocrine system such as ghrelin, growth hormone (GH), and insulin-like growth factor (IGF)-I function to regulate tissue specific energy allocation and together link growth physiology, developmental age, energy balance (the amount of calories consumed vs. calories burned), and nutritional status (negative or positive nutrient intake) in domestic animals (Lawrence and Fowler 1997; Breier 1999; Ortiz et al. 2003; Cammisoto et al. 2010; Castenda et al. 2010).

The secretion of the metabolic hormone ghrelin in the stomach is dependent upon nutritional status (Cummings et al. 2001). The increase of ghrelin during fasting or reduced nutrient intake stimulates the release of GH, fat oxidation, and food intake, and inhibits body weight gain and adiposity (Tschop et al. 2000). Growth hormone regulates energy deposition and mobilization through its direct action on adipose tissue (Breier et al. 1999). During energy deficiency or periods of low nutritional status, GH stimulates lipolysis and inhibits lipogenesis (Fuglei et al. 2000). The mobilization of fat from

adipose tissue, or lipolysis, provides energy for maintenance (Vijayakumar et al. 2010). When nutritional intake is adequate for energy requirements of maintenance, GH stimulates the production and release of IGF-I from the liver. Insulin-like growth factor-I regulates somatic growth, cell proliferation, differentiation, and apoptosis (Renaville et al. 2002). During normal physiological conditions IGF-I stimulates protein synthesis, bone formation, and glucose uptake (Etherton 1991).

Ghrelin, GH, and IGF-I have age related patterns and sex differences that result in differential growth rates or tissue-specific nutrient allocation. A recent study of mice, ghrelin concentrations increased with age (Chanoine et al. 2006). Previous studies on domestic species, mice, and humans had found that GH concentrations generally decrease as animals age, whereas, IGF-I concentrations increase initially and then plateau after puberty (Skaar et al. 1994; Schwarz et al. 1992; Harrell et al. 1999; Govoni et al. 2000).

Sex differences in ghrelin, GH, and IGF-I concentrations have been found in ungulates such as the red tailed deer, mule deer, and cattle (Webster et al. 1999; Govoni et al. 2003; Comeau et al. 2009, unpublished). The limited studies of sex differences in ghrelin concentrations have been contradictory with some studies reporting no sex differences (Weiss et al. 2006; Purnell et al. 2003; Vilarrasa et al. 2005), whereas other research documented greater concentration of ghrelin in females (Greenman et al. 2004; Salbe et al. 2004; Barkan et al. 2003). Both serum GH and IGF-I concentrations are greater in males when compared with female cattle, sheep, and pigs (Gatford et al. 1996; Owens et al. 1999; Govoni et al. 2003), and are associated with increased growth rate (Rausch et al. 2002).

Changes in nutrient intake or seasonal abiotic variables elicit an endocrine response resulting in a change in growth rate and tissue-specific nutrient priorities (Gerrard and Grant, 2006). Mammals that exhibit seasonal changes in body composition, such as bears, cattle, deer, lemurs, tree shrews, and marmots, are generally leaner during long photoperiod (summer, greater than 12 hours of daylight) with greater adiposity during the short photoperiod (winter, less than 12 hours of daylight; Zinn et al. 1986;

Webster et al. 1999; Florant and Healy 2012; Zhang et al. 2012; Zhu et al. 2012). Some mammals demonstrate weight gain, with increased adiposity, during a transitional period (fall) in preparation for the winter when food may be scarce, ambient temperature declines, and additional energy is needed for thermoregulation (Webster et al. 1999; Fuglei et al. 2004; Florant and Healy 2012; Zhang et al. 2012, Zhu et al. 2012). During winter, some mammals minimize lean tissue accretion to conserve body protein (Prestrud and Nilssen 1992; Nunes et al. 1998; Webster et al. 1999). Previous studies in mice found that during winter ghrelin stimulates lipogenesis and food intake (Tschop et al. 2000). During fall GH was reduced in the arctic fox to facilitate adipose accumulation in preparation for short photoperiod (Fuglei et al. 2004). In contrast, summer GH concentrations were increased in the red-tailed deer to facilitate lean tissue accretion mediated by IGF-I (Rosenfeld and Hwa 2009). In previous studies of domestic and free-ranging ungulates, IGF-I concentrations were greater in summer compared with winter and were correlated with increased lean tissue accretion (Barenton et al. 1988; Webster et al. 1996).

Most research on the role of metabolic hormones in the regulation of seasonal change in body composition has been examined in species living at middle to high latitudes that exhibit strong seasonal patterns in food intake and body composition. However, the physiological mechanism for seasonal body composition is unknown in sub-tropical or tropical species with less pronounced seasonal patterns. The free-ranging Florida manatee (*Trichechus manatus latirostris*), a sub-tropical marine mammal, seasonally change their habitat preference due to their narrow thermoneutral zone (Reynolds and Wilcox 1986). Manatees tend to remain relatively inactive in warm water areas during cold air or ambient temperatures to reserve energy (Reynolds and Wilcox 1986; Reynolds and Rommel 1996). Therefore, manatees feed and forage less frequently to maintain body temperature resulting in negative energy balance and blubber loss (Gallivan et al. 1983; Reynolds 2000). While seasonal changes in blubber thickness have been observed, the physiological mechanisms that contribute to seasonal changes in body composition have not been explored in the Florida manatee. The primary objective of this

research was to evaluate the response of ghrelin, GH, and IGF-I to nutritional seasonality of the free-ranging Florida manatee and to assess if these hormones were related to seasonal changes in body composition. In addition, hormone concentrations were evaluated between age class and sex to determine if manatees exhibit the typical mammalian developmental and sex-related pattern in metabolic hormones.

MATERIALS AND METHODS

Sample Collection

Research was conducted as part of routine manatee health assessments performed by the United States Geological Survey (USGS) Sirenia Project and the Florida Fish and Wildlife Conservation Commission (FWC) from 2005 – 2010. If individual manatees were captured in multiple seasons, only data collected in one season was used for this study. Age class was categorized for 59 manatees (33 male; 26 female) by standard length (tip of nose to tip of paddle), as follows: adult >260 cm; juvenile 236-260 cm; and calf <236 cm (Table 1, O'Shea et al. 1985). During long photoperiod (summer; June-August) and the transitional period (fall; October), samples were collected from the Everglades, Florida using nets in an open water boat capture method (Wong et al. 2012). Samples during short photoperiod (winter; December-February) were collected from Crystal River, Florida using nets in a land based capture method (Bonde et al. 2012). During land and boat based captures, a No. 30 or 56 braided seine twine net 122 m long and up to approximately 8 m deep, with a 10- to 17-cm stretch knotted nylon mesh was deployed from a motorized modified mullet skiff with a removable transom. The net encircled the manatee, and the capture team pulled the net and the manatee onto the capture boat or river bank (Bonde et al. 2012; Wong et al. 2012). Manatees were then transported to the health assessment base on-shore for a medical examination by a team of biologists and veterinarians.

Blood Handling and Hormonal Analysis

Blood samples were collected within 20 minutes of capture from the brachial bundle on the medial aspect of the pectoral flipper (Bonde et al. 2012; Stamper and Bonde 2012) using serum separator tubes and stored in a cooler with ice (Bossart et al. 2001; Bonde et al. 2012; Stamper and Bonde 2012). Within 1 hour of collection, whole clotted blood was centrifuged at 6,000g for 10 minutes. Serum was collected and archived frozen at -80°C (Bonde et al 2012; Wong et al. 2012). A physical exam was also conducted which included general appearance, body temperature, body mass, morphometrics (standard length and axillary girth), and blubber thickness measured via ultrasound (Ward-Geiger 1997; Bonde et al. 2012).

Ghrelin concentrations were determined by a heterologous radioimmunoassay (RIA) kit (GHRT-89HK, Millipore, Billerica, MA). Manufacturer protocols were precisely followed with one modification: all reagent quantities were halved. Radioactivity of the final pellet was measured using a gamma counter (Wizard Walla 1470, Perkin Elmer, MA, USA). Serum concentrations of GH and IGF-I were also determined by heterologous RIA (Richmond and Zinn 2009). Antisera and standards were purchased from A.F. Parlow, National Hormone & Peptide Program (Torrance, CA). Rabbit-anti-porcine antisera for GH (AFP422801) was used with goat secondary antisera against rabbit γ -globulin and porcine GH (AFP10864B) standard to quantify manatee GH concentrations. Serum concentrations of IGF-I were quantified with a rabbit-anti-human IGF-I antibody (AFP4892898) and purified human IGF-I standard (Lot #1). Serially diluted pooled serum from free-ranging West Indian manatees was used to validate heterologous ghrelin, GH, and IGF-I RIA. Standard validation parameters including percent recovery, precision of the assay, parallelism, and dilution linearity were assessed (Richmond and Zinn 2009).

Statistical Analysis

The homogeneity of variance (HOV) was calculated using the Bartlett test to determine normality and variance of hormone and morphometric measures between season and age classes. To evaluate seasonal differences, measurements of serum ghrelin, GH, and IGF-I and blubber thickness were analyzed in a general linear model using an analysis of variance (ANOVA) to determine significant differences (SAS Inst. Inc., Cary, NC). The initial statistical model included sex and the season*sex interaction; however, there were no significant differences ($p > 0.05$) observed between sex with the exception of blubber thickness which was greater in females ($p=0.04$). In addition there was no significant interaction between the season*sex interaction and therefore, sex and the season*sex interaction were removed from the model. The final model included season. A Fisher's Least Significant Difference (LSD) post-hoc test was used to determine differences among seasons (summer, fall, winter). Two outliers were removed from the winter season group for IGF-I. Outliers were identified based on high variability. To test the effect of age, ghrelin, GH, and IGF-I concentrations were analyzed using an ANOVA, separate from season. The LSD post-hoc test was used to determine differences among age classes (calf, juvenile, adult). Two outliers were removed from the adult group and two outliers were removed from the juvenile group for GH. The results were presented as the mean \pm standard error (SE). Differences were considered significant at $p\text{-value} \leq 0.05$ and a trend if $p\text{-value}$ was > 0.05 and ≤ 0.10 .

RESULTS

Percentage recovery of pooled manatee serum spiked with human ghrelin standard RIA was $105.7 \pm 9.8 \%$. Dilution series of pooled manatee serum was parallel to the ghrelin standard curve (dilution linearity $R^2 = 0.97$). The intraassay and interassay coefficient of variation (CV) for ghrelin were 10.0 and 8.3, respectively. Percentage recovery of pooled manatee serum spiked with porcine GH standard RIA was $92.5 \pm 7.9 \%$. Dilution series of pooled manatee serum was parallel to the GH standard

curve (dilution linearity $R^2 = 0.98$). The intraassay and interassay CV were 6.3 and 15.6, respectively. Percentage recovery of pooled manatee serum spike with human IGF-I standard RIA and high and low manatee serum was $93.1 \pm 5.7\%$. Dilution series of pooled manatee serum was parallel to the IGF-I standard curve (dilution linearity $R^2 = 0.95$). The intraassay and interassay CV were 10.8 and 10.9, respectively.

Seasonal Differences

As expected, blubber thickness differed between seasons ($p = 0.03$; Fig 1A). Summer manatees had reduced blubber thickness (5.7 ± 0.9 mm) compared with the winter (8.7 ± 0.7 mm) and fall (8.4 ± 1.0 mm) animals. Blubber thickness was similar between fall and winter seasons ($p = 0.82$). Ghrelin concentrations (Fig 1B) were similar across all seasons ($p = 0.66$): summer (1255.1 ± 93.2 pg/ml), fall (1272.8 ± 120.4 pg/ml), or winter (1359.2 ± 78.8 pg/ml). There was a seasonal trend in GH concentrations ($p = 0.08$). Summer had greater GH concentrations (14.5 ± 2.3 ng/ml) compared with winter at (8.1 ± 1.6 ng/ml; $p = 0.03$; Fig 1C) while there were no differences between fall (9.1 ± 2.4 ng/ml) and either summer or winter ($p = 0.12$ or 0.71 , respectively). Concentrations of IGF-I were different among seasons ($p < 0.01$; Fig 1D) with greater concentrations during summer (164.0 ± 13.2 ng/ml) and winter (147.2 ± 11.7 ng/ml) compared with fall (91.3 ± 14.6 ng/ml). There were no differences in IGF-I concentrations between summer and winter ($p = 0.35$).

Age-class Differences

Serum ghrelin concentrations did not exhibit significant differences between age class ($p = 0.12$; Fig 2A) and variance was similar among age categories (HOV, $p = 0.12$, 1265.7 ± 281.8). Serum concentrations of GH were highly variable (HOV, $p = 0.03$, 8.0 ± 4.3) among age categories, and GH

concentrations tended ($p = 0.07$; Fig2B) to be greater in calves (10.3 ± 1.8 ng/ml) and juveniles (10.1 ± 1.6 ng/ml) compared with adults (6.4 ± 1.0 ng/ml). There was no significant difference in IGF-I concentrations ($p = 0.28$; Fig 2C) between age class and variability was similar (HOV, $p = 0.29$, 138.7 ± 51.2).

DISCUSSION

The response of ghrelin, GH, and IGF-I concentrations to changes in season, growth, development, energy, and protein metabolism and their role in tissue-specific nutrient allocation have been found in arctic and ruminant species (Webster et al. 1996; Fuglei et al. 2004; Iqbal et al. 2006); however, it was unknown if similar physiological mechanisms exist in sub-tropical or tropical species with less pronounced seasonal patterns. Therefore, the primary objective of this research was to evaluate the response of ghrelin, GH, and IGF-I to seasonality of the free-ranging Florida manatee and to assess if these hormones were related to seasonal changes in body composition. In addition, hormone concentrations were evaluated between age classes to determine if manatees exhibit the typical mammalian developmental pattern in metabolic hormones.

The Florida manatee exhibited physiological and hormonal differences among seasons. As previously reported, blubber layers were thicker during the fall and winter. Ghrelin concentrations were consistent among seasons, however, GH and IGF-I exhibited differences. That is, GH was greater in the summer compared with winter, and IGF-I concentrations were greater in summer and winter compared with fall. The Florida manatee may have similar seasonality patterns compared with temperate species, revealing that although they are in a warmer climate, their physiological demands are still effecting hormone concentrations. Therefore, these data can provide a better overall understanding of the seasonality of species in sub-tropical environments to further monitor and protect their ecosystems. In

addition, the age-related hormonal patterns were not as predicted; there were no differences among age classes unlike previous research.

Seasonal Differences

The Florida manatee experiences relatively minor seasonal variations in temperature, light, and food availability that may lead to physiological responses to cope with environmental pressures. They are a subtropical species that have a narrow thermoneutral zone and low metabolic rate influencing their need to migrate to warm water sites in order to seek refuge from cold temperatures (Reynolds and Wilcox 1986; Reynolds and Odell 1991). Like other marine mammals, manatees have blubber, multiple subcutaneous lipid layers that assist with their thermoregulatory needs during cold temperatures (Irvine 1983). Unique to manatees is their thin alternating layers of blubber and panculus muscle when compared with other marine mammals (Irvine 1983). In the current study, manatees had reduced blubber thickness during summer compared with fall and winter. Previous studies on domestic and wild species found that animals are typically leaner in the summer perhaps for advantageous competitive mating with greater adiposity in the fall and winter to maintain positive energy balance due to cold temperatures and the lack of food (Fuglei et al. 2004; Genin and Perret 2000; Zinn et al. 1986). The tropical tree shrew has body mass gain in fall, with the greatest body mass during winter and reduced body mass during the summer (Zhang et al. 2012; Zhu et al. 2012). In the manatee, greater blubber thickness in the fall may suggest adipose accumulation for thermoregulatory needs and energy demands during the winter season as observed in other species. Thicker blubber in the winter may be used to maintain energy balance and for tissue-specific nutrient partitioning (Fuglei et al. 2004; Genin and Perret 2000; Zinn et al. 1986).

Ghrelin concentrations were consistent among seasons in the free-ranging Florida manatee. In monogastric species such as rodents and humans, ghrelin concentrations increase preprandially followed by a postprandial decrease (Cummings and Foster 2003; Kojima et al. 1999). However, in a study by Iqbal and colleagues (2006), sheep, a ruminant species, were found to be unresponsive to the orexigenic properties of ghrelin because their gut is consistently full of food and not completely emptied between feedings (Sugino et al. 2002; Sugino et al. 2004). Ghrelin is produced in the rumen and cecum of ruminant species versus the proximal region of the stomach in monogastric species (Date et al. 2000; Hayashida et al. 2001). The cecum houses microorganisms that function to digest cellulose in the process known as hindgut fermentation (Reynolds and Rommel, 1996). In sheep, ghrelin concentrations are consistently maintained across seasons, resulting in similar food intake throughout the year (Harrison et al. 2007). Similar to sheep, The Florida manatee is a ruminant species that uses hindgut fermentation. Although, studies have not been performed in manatees to determine where the abundance of ghrelin-producing cells are located it can be hypothesized that it may be in the cecum, similar to other hindgut fermenters (Hayashida et al. 2001). The gastrointestinal tract, including the cecum, of a manatee is not fully emptied between meals. Therefore ghrelin concentrations may not differ significantly among seasons because of the continuous presence of food in the lumen of the gastrointestinal tract (Iqbal et al. 2006; Reynolds and Rommel, 1996).

In the current study, GH concentrations were greater during the summer compared with winter and no differences between fall and winter. Growth hormone plays a role in tissue specific nutrient partitioning (Breier et al. 1999). Elevated GH concentrations promote lipolysis and inhibit lipogenesis (Breier et al. 1999; Fuglei et al. 2000). Therefore, it was hypothesized that greater GH concentrations in association with reduced blubber thickness in the summer suggests GH is stimulating IGF-I and protein accretion. Whereas reduced GH in the winter and greater blubber thickness suggests blubber is being maintained or accumulated (Webster et al. 1996).

In previous studies, IGF-I responded to seasonal changes. Concentrations were greater in the summer compared with the winter and were positively correlated with tissue accretion (Barenton et al. 1988; Webster et al. 1996). Manatees were predicted to have a similar response. As expected, IGF-I concentrations were high during summer, however, they did not differ from winter concentrations suggesting lean and/or adipose stores or both are being maintained or accumulated during both of the seasons. In addition, IGF-I concentrations were reduced in the fall which was unexpected. Although, previous research suggests animals in temperate climates have greater IGF-I concentrations in the fall compared with winter for adipose accumulation (Webster et al. 1996; Fuglei et al. 2004), it is possible that manatees are not building and maintaining adipose stores until the winter season.

Insulin-like growth factor-I stimulates protein accretion and works in conjunction with GH to facilitate adipose and lean tissue accretion (Gautsch et al. 1998; Hornick et al. 2000). Greater concentrations of GH and IGF-I are positively correlated with growth rate and food intake in red deer during the spring season and are thought to produce rapid lean tissue accretion (Suttie et al. 1989; Adam et al. 1996; Webster et al. 1996). Alternatively, reduced GH concentrations in association with greater IGF-I concentrations during the fall and winter suggest the facilitation of lipogenesis (Rosenfeld and Hwa, 2009). In the present study, manatees had elevated GH and IGF-I concentrations in conjunction with reduced blubber, suggesting lean tissue accretion. This may be beneficial to manatees due to the peak in mating activity and reproductive demands during the summer and fall seasons (Hartman 1979; Reep and Bonde 2006; Rosenfeld and Hwa 2009). Although GH concentrations were not significantly different in fall compared with summer and winter, greater GH from summer to winter in conjunction with reduced IGF-I and greater blubber thickness suggest manatees may be accumulating adipose for the winter as seen in other species like the arctic fox and red tailed deer (Webster et al. 1996, 1999; Fuglei et al. 2004). Greater IGF-I, reduced GH, and greater blubber thickness during the winter may suggest lipogenesis is occurring likely to meet the thermoregulatory needs due to colder

temperatures. Changing hormone concentrations in association with blubber thickness among summer, fall, and winter seasons suggest seasonal changes in nutrient priorities are occurring in the Florida manatee.

Age-class Differences

Ghrelin is highly conserved across species (Kojima et al. 1999), is widely expressed in different tissues (Kojima et al. 1999), and is found primarily in the stomach (Ariyasu et al. 2001; Date et al. 2000). Although ghrelin is known to stimulate food intake, adiposity, and the release of GH (Nakazato et al. 2001; Tschop et al. 2000), the role ghrelin plays in development is still unclear (Akimoto et al. 2011). Although there are limited studies on the ontogeny of ghrelin; it was hypothesized that ghrelin concentrations may be constant between age classes in the ruminant species to maintain a positive nutritional status. In the present study, ghrelin concentrations exhibited no differences among age classes in the free-ranging Florida manatee. As previously mentioned, manatees are a ruminant species with food continuously in their gastrointestinal tract which may be contributing to consistent ghrelin concentrations between age classes (Iqbal et al. 2006; Reynolds and Rommel 1996). This may further suggest food intake is consistent with aging in manatees.

In most mammalian species including humans, cattle, and sheep, GH generally declines with age, with increased secretion during the perinatal and pubescent stages associated with periods of increased growth rate (Anderson et al. 1988; Zinn et al. 1989; Martha et al. 1992; Schwarz et al. 1992; Albertsson-Wikland et al. 1994; Blackman et al. 1994). Like these species, the free-ranging Florida manatee exhibited reduced GH concentrations in adults compared with calves and juveniles. Manatee calf growth rate is high the first few years of life during and after nursing. Calves range from 91 to 137 centimeters at birth and can reach 152 to 183 centimeters within two years (Odell 1982). As seen in

previous research, greater GH concentrations in calves and juveniles suggest a greater growth rate (Anderson et al. 1988; Zinn et al. 1989; Martha et al. 1992; Schwarz et al. 1992; Albertsson-Wikland et al. 1994; Blackman et al. 1994). In addition, the juvenile age class exhibited a wide range of GH concentrations demonstrating high variability. The juveniles used in this study may include prepubescent and pubescent individuals increasing the likelihood that some animals may be experiencing rapid growth which may also explain the wide range of GH concentrations; spiked GH concentrations in individuals experiencing puberty and therefore, growth, may contribute to the high variability observed in this group (Zinn et al. 1989; Schwarz et al. 1992; Marmontel 1993, Govoni et al. 2003).

Serum IGF-I concentrations are known to change significantly during the first few years of life and then begin to decreasing with age eventually plateauing after puberty (Schwarz et al. 1992; Blackman et al. 1994; Skaar et al. 1994; Gatford et al. 1996; Harrell et al. 1999; Govoni et al. 2003). Surprisingly, there were no differences in IGF-I concentrations among age classes in the free-ranging Florida manatee. Previous studies found humans, beef and dairy cattle, pigs, and lamb IGF-I concentrations were initially greater during the first year of life and later plateaued when individuals reached puberty (Schwarz et al. 1992; Blackman et al. 1994; Skaar et al. 1994; Gatford et al. 1996; Harrell et al. 1999; Govoni et al. 2002; Govoni et al. 2003). It is likely that the manatee calves used for the current study were no longer in their initial perinatal stage when captured; young calves are specifically not targeted during health assessments. In addition, the manatee calves used for the present study may no longer have been nursing and therefore, IGF-I concentrations had plateaued further explaining the consistent IGF-I concentrations among the age classes.

During normal physiological conditions, GH stimulates the release of IGF-I. Together the hormones are associated with growth rate and protein metabolism. Increased GH and IGF-I concentrations in the perinatal and pubescent stages of life in domestic species promote increased

growth rate (Anderson et al. 1988; Zinn et al. 1989; Martha et al. 1992; Schwarz et al. 1992; Albertsson-Wikland et al. 1994; Blackman et al. 1994). In the current study, greater GH concentrations in conjunction with consistent IGF-I and ghrelin concentrations in calf and juvenile manatees suggest greater growth rate. Although, IGF-I and ghrelin concentrations were consistent with the adult age class, greater GH may stimulate growth. Whereas reduced GH in adults and the consistency in IGF-I and ghrelin concentrations may no longer stimulate growth rate, but rather changes in body composition. The differences in GH between age classes suggest that there are age-related changes occurring; however, future research needs to be conducted to further understand how these hormones impact growth rate in the Florida manatee.

The Florida manatee is an endangered species protected by the Marine Mammal Protection Act (MMPA 1972) and the Endangered Species Act (ESA 1973). Managers and conservationists have conducted a plethora of research on the Florida manatee to further protect this endangered species. However, limited research has been done on the physiological mechanisms that are influenced by age and seasonal changes that ultimately effect tissue-specific nutrient partitioning. The Florida manatee has a low metabolic rate that reduces their ability to maintain their core body temperature during the winter season which contributes to their cold susceptibility and ultimately induces a physiological reaction contributing to clinical symptoms (i.e. emaciation, fat store depletion) and disease referred to as cold-stress syndrome (Irvine, 1983; Bossart, 2001). Determining the normal pattern of seasonality in the Florida manatee may provide a better understanding for managers to develop successful methods to determine population health status and further the survival of the species.

Based on previous seasonal research conducted in domestic, ruminant, and arctic species, manatees shared interesting similarities to domestic and ruminant animals. However, age related changes did not respond as predicted. Ghrelin, GH, and IGF-I profiles in conjunction with body composition changes between seasons indicated seasonal adjustments in manatee tissue-specific

nutrient partitioning. Ghrelin concentrations were similar to domestic ruminant species remaining consistent through changes in season. These data suggest manatees are prioritizing lean tissue accretion during the summer for potential mating and reproductive demands, whereas, manatees may be prioritizing adiposity in the fall and winter for energy maintenance and thermoregulatory needs. Although, this data contributes to the understanding of seasonal patterns and tissue-specific nutrient partitioning it does not consider nutritional status of individuals which may be important in determining the overall health of an individual. Future controlled nutrient intake research needs to be conducted to determine the role of ghrelin, GH, and IGF-I in relation to nutritional status in the manatee. However, the current measurements of ghrelin, GH, and IGF-I concentrations may prove to be a useful tool in evaluating the influence of seasonality and may later contribute to determining overall nutritional status of the Florida manatee.

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CHAPTER THREE: TABLES AND FIGURES

Table 3. 1 Sample size of manatees that were used to quantify hormone concentrations during photoperiod comparisons from Crystal River and Everglades, Florida from 2005 to 2010

Age Class	Winter		Summer		Fall	
	<i>Male</i>	<i>Female</i>	<i>Male</i>	<i>Female</i>	<i>Male</i>	<i>Female</i>
Adult	11	10	9	3	2	7
Juvenile	6	3	---	---	---	---
Calf	5	3	---	---	---	---

Relative age classifications were determined based on straight length measurements.

Age classes defined as adult >260cm, juvenile 236-260cm, calf <236cm.

Table 3. 2 Reported results for ghrelin, GH, and IGF-I validations for manatee serum including parallelism, dilution linearity, percent recovery, and intraassay and interassay coefficient of variation (CV)

Hormone	Standard Curve	Dilution Linearity	% Recovery (mean±SD)	Interassay CV (mean±SD)	Interassay CV (mean±SD)
Ghrelin (pg/ml)	$y = -0.6344x + 2.3416$ $R^2 = 0.95$	$y = 30.383x - 90.963$ $R^2 = 0.97$	105.7±9.8	9.9±139.0	8.3±110.2
GH (ng/ml)	$y = -0.6509x + 0.6731$ $R^2 = 0.94$	$y = 12.303x + 11.385$ $R^2 = 0.98$	92.5±7.9	6.3±0.9	15.6±2.5
IGF-I (ng/ml)	$y = -0.419x + 1.7748$ $R^2 = 0.98$	$y = 3.0958x + 0.7416$ $R^2 = 0.95$	93.1±5.7	12.8±10.1	10.3±25.0

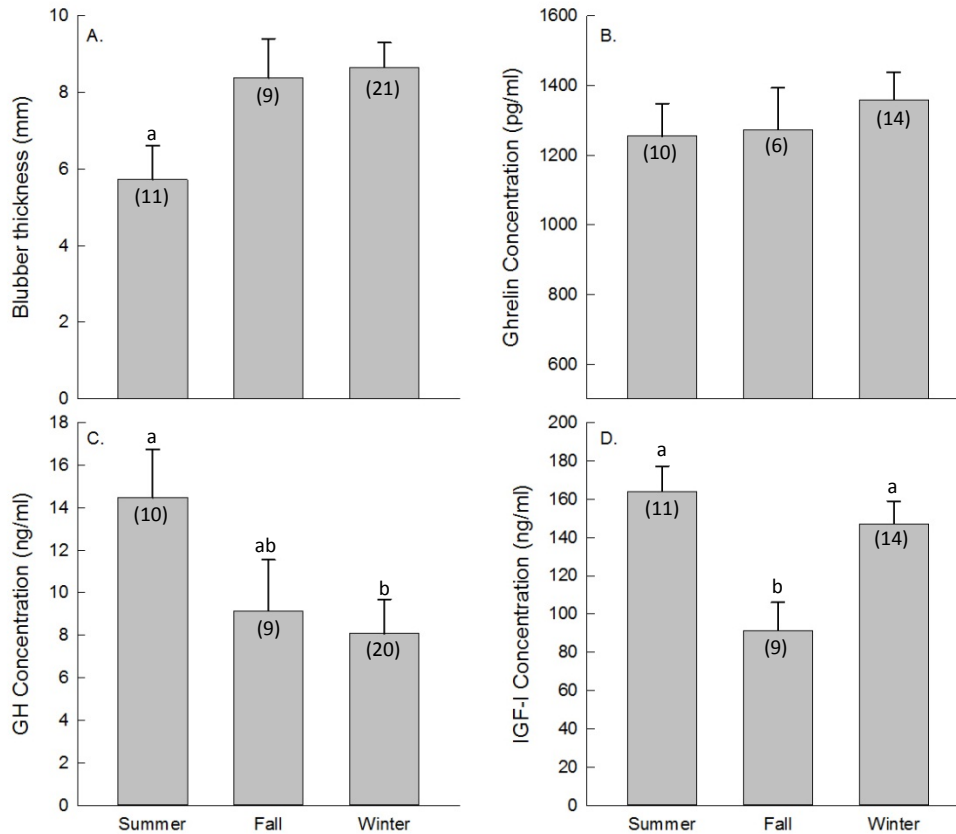


Figure 3. 3 (A – D) Differences among seasons (summer, fall, winter) presented as mean \pm SE in free-ranging manatees from 2005 to 2010. (A) Average blubber thickness (mm) (B) Average ghrelin concentrations (pg/ml) (C) Average GH concentrations (ng/ml) (D) Average IGF-I concentrations (ng/ml). Statistical significant differences among seasons were presented as letters (a, b). Sample size is within the column of each season.

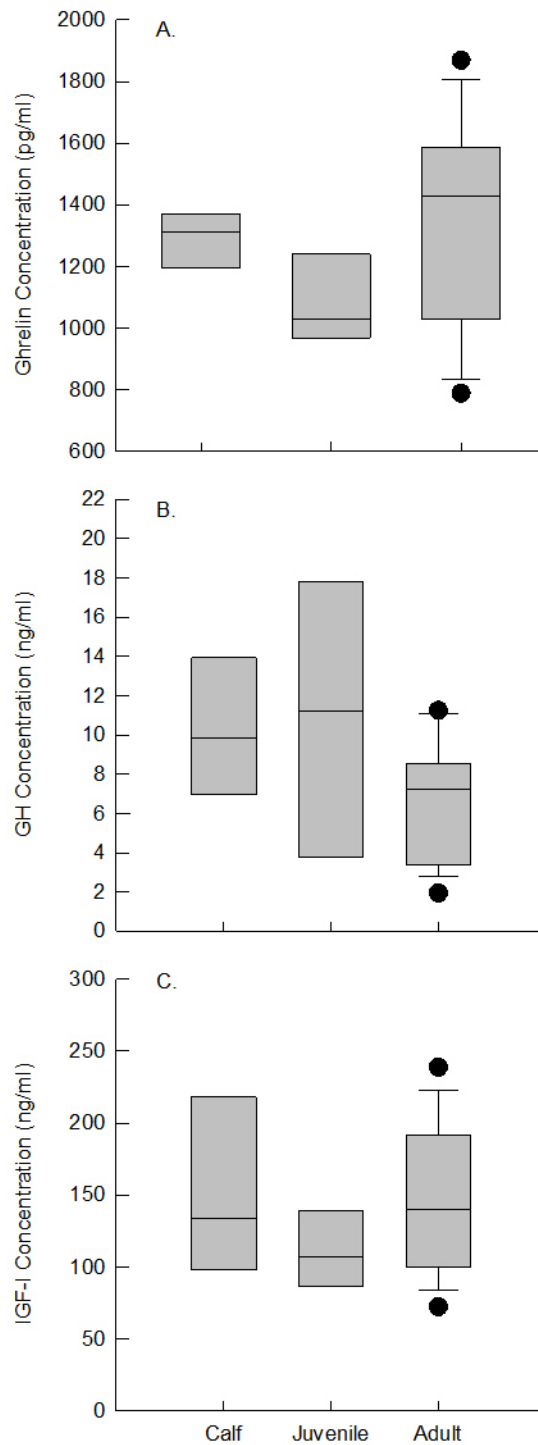


Figure 3. 4 Average hormone concentrations among age classes (calves, juveniles, adults) from 2005-2010 in the free-ranging Florida manatee as the mean \pm SE in (A) ghrelin (pg/ml) (B) GH (ng/ml) and (C) IGF-I (ng/ml).

CHAPTER FOUR: Assessing Nutritional Status in the West Indian Manatee (*Trichechus manatus*) in Florida, Puerto Rico, and Belize Populations Through Evaluation of Ghrelin, Growth Hormone, and Insulin-like Growth Factor I

INTRODUCTION

The West-Indian manatee (*Trichechus manatus*) is a sentinel species currently listed as endangered, and is protected by the Marine Mammal Protection Act (MMPA 1972) and the Endangered Species Act (ESA 1973; Bonde et al. 2004; Bossart 2006). There are two subspecies of the West-Indian manatee, the Florida manatee (*Trichechus manatus latirostris*) and Antillean manatee (*Trichechus manatus manatus*). Both sub-species inhabit marine, estuarine, and freshwater coastal habitats and require a freshwater source. Florida and Antillean manatees are distributed in different regions (Garcia-Rodriguez et al. 1998; Vianna et al. 2006; Hunter et al. 2010; Nourisson et al. 2011; Hunter et al. 2012; Tucker et al. 2012). Florida manatee distribution is from Florida to Rhode Island on the east coast, Florida to Texas on the west coast, and has been documented as far east to the Bahamas and south to Cuba. The Antillean manatee inhabits Belize, Puerto Rico, and other Caribbean coasts of Central and northeast South America (Powell and Rathbun 1984; Fertl et al. 2005; Beck 2006; Alves-Stanely et al. 2010; Hunter et al. 2010, 2012). Due to distribution differences, Florida and Antillean manatees inhabit regions with variable climates, habitats, and forage availability.

In the past, West Indian manatee populations were threatened by over hunting which is no longer prevalent throughout most of their range (Reynolds 1999). Nevertheless, manatees are faced with other anthropogenic threats such as vessel strike and impact, entanglement, entrapment, pollution, loss and degradation of habitat, and harassment (O'Shea 1988; O'Shea et al. 2001; Jimenez 2002; Lightsey et al. 2006). In addition, the Florida manatee is environmentally constrained and needs

to remain in water temperatures 20°C or warmer (Reynolds and Wilcox 1986; Reynolds and Odell 1991; Reep and Bonde 2006). Their low metabolic rate reduces the ability of manatees to maintain their core body temperature during cold temperatures (Irvine 1983). This can contribute to their susceptibility to the cold and ultimately induce a physiological reaction contributing to pathological symptoms (i.e. emaciation, fat store depletion); a disease referred to as cold-stress syndrome (Irvine 1983; Bossart 2001). To minimize the impact of cold temperatures, the Florida manatee migrates to warm water sites in the winter (Reynolds and Wilcox 1986). Since Antillean manatees inhabit tropical waters that maintain relatively constant temperatures above 20 °C throughout the year, migration is not necessary (Irvine 1983; Worthy et al. 2000; Bossart et al. 2003).

Habitat differences across the range of the Florida and Antillean manatees are influenced by the type of forage available. Antillean populations in Belize and Puerto Rico spend more time in marine environments compared with Florida manatees, which moves between freshwater, estuarine, and marine environments (Garcia-Rodriguez et al. 1998; Vianna et al. 2006; Hunter et al. 2010; Nourisson et al. 2011; Hunter et al. 2012; Tucker et al. 2012). The Florida manatee is a generalist herbivore feeding on freshwater, estuarine, and marine vegetation such as hydrilla, waterhyacinth (*Eichhornia crassipes*), water lettuce (*Pistia stratiotes*), and valisneria (*Vallisneria americana*) (Hartman 1979; Best 1981; Bengtson 1983). Florida manatees also consume terrestrial plants such as mangroves and overhanging branches by crawling closer to the shoreline and using their prehensile lips to grab vegetation (Hartman 1979; Best 1981; Bengtson 1983; O'Shea 1986). In contrast, Antillean populations in Belize and Puerto Rico spend more time in marine environments and therefore consume a greater volume of seagrass such as turtle grass (*Thalassia testudinum*), turtle grass is considered to be a low quality forage (Mignucci-Giannoni and Beck 1998; Alves-Stanley et al. 2010). In addition to habitat and forage differences, the subspecies exhibit moderate differences in body morphometrics; generally, body length is less in Antillean manatees of the same age (Converse et al. 1994). It is possible that forage type and

availability in Florida, Puerto Rico, and Belize manatee populations may contribute to differences in nutritional status and ultimately body size.

Although there are known differences in location, climate, and foraging habits between the Florida and Antillean manatee populations, differences in nutritional status (positive or negative nutrient intake) have not been previously explored. Nutritional status influences the metabolic hormones, ghrelin, growth hormone (GH), and insulin-like growth factor (IGF)-I often in predictable ways. Changes in energy and nutrient intake will result in hormonal response (Breier 1999, Cummings et al. 2001, Halaas et al. 1998). Under normal physiological conditions, ghrelin stimulates food intake, body weight gain, and adiposity (Nakazato et al. 2001; Theander-Carrillo et al. 2006; Tschop et al. 2000; Wren et al. 2001a, 2001b). If nutritional status is low, ghrelin concentrations are elevated and stimulate the release of GH (Tschop et al. 2000). Growth hormone regulates energy deposition and mobilization through its direct action upon adipose tissues (Breier et al. 1999). During low nutritional status, GH increases which stimulates lipolysis partitioning fat for other energy maintenance needs (Breier et al. 1999; Carrel and Allen 2000; Eigenmann et al. 1985). When nutritional status is maintained, GH stimulates the production and release of IGF-I from the liver. Insulin-like growth factor-I is transported from the liver to target tissues (Carter-Su et al. 1996; Elsassser et al. 1989). During positive nutritional status, IGF-I stimulates protein synthesis (lean tissue accretion), bone formation, and glucose uptake (Etherton 1991). However, during energy deficiency (low nutritional status) IGF-I decreases reducing its anabolic effects (Renaville et al. 2002).

Because ghrelin, GH, and IGF-I respond to energy and nutrient availability and regulate tissue-specific nutrient partitioning, hormone profiles in combination with body composition can serve as indicators of nutritional status. Therefore, the purpose of this research was to evaluate ghrelin, GH, and IGF-I concentrations in Florida, Puerto Rico, and Belize manatees to determine if differences can be detected between the Florida and Antillean sub-species at the population level. Furthermore, the

quantification of hormone concentrations and body composition will be used to indicate if nutritional status can be assessed between these populations.

MATERIALS AND METHODS

Animal capture and handling

Manatee health assessments were conducted by the U.S. Geological Survey (USGS) Sirenia Project in partnership with the Florida Fish and Wildlife Conservation Commission (FWC), and other organizations to understand health related issues confronting manatees (Bonde et al. 2012). Serum samples were collected during long photoperiod (>12 hours of daylight) as part of the manatee health assessments in 2005-2007 in the Everglades, Florida, in 2003-2005 in Puerto Rico, and in 2011-2012 in Belize. These serum samples were archived frozen at -80°C for subsequent analyses. Adult individuals were selected from Florida (male = 9; female = 3), Puerto Rico (male = 10; female = 10), and Belize (male = 10; female = 11).

Capture methods in Florida, Puerto Rico, and Belize were modified based on the geographical location (Reid et al. 1995; Deutsch et al. 1998; Auil et al. 2007). Manatees were captured using a specialized 24-foot skiff with an outboard engine located near the front of the vessel and a removable transom, which encircled the target manatee in a net after which a team onboard pulled the manatee onto the deck (Bonde et al. 2012; Wong et al. 2012). The net was stored on the aft deck prior to deployment. A 122 m long and 8 m deep number 30 or 56 braided seine twine net with a 10- to 17-cm stretch knotted nylon mesh was used for manatee captures (Bonde et al. 2012; Wong et al. 2012). The top of the net had football floats and the bottom was laced with lead lining to keep the net in contact with the sediment (Bonde et al. 2012). Once onboard, the manatee was transported to the assessment processing site, placed on a foam pad, and vital signs were continually monitored including heart rate, body temperature, and respiration rate (Bonde et al. 2012; Wong et al. 2012). Standard length and

blubber thickness measurements were assessed (Bonde et al. 2012; Wong et al. 2012). Blubber thickness was measured via ultrasound (Ward-Geiger 1997).

Blood Handling and Hormonal Analysis

Blood was collected from the medial aspect of the brachial vascular plexus bundle just beneath the pectoral flipper with a Vacutainer® system extension set (BD Co., Franklin Lake, NJ, USA; Bossart et al. 2001, Bonde et al. 2012; Stamper and Bonde 2012). Blood was centrifuged at 6,000 x g for 10 minutes to separate serum which was archived by USGS at -80°C within 1 hour of collection (Bonde et al. 2012; Wong et al. 2012).

Heterologous radioimmunoassays (RIA) were used to quantify GH, IGF-I, and ghrelin concentrations with methods previously validated for manatee serum (Chapter 2). All anti-sera and standards were purchased from A.F. Parlow, National Hormone & Peptide Program (Torrance, CA) with the exception of ghrelin which were analyzed using a commercially available kit (GHRT-89HK, Millipore, Billerica, MA). The manufacturer's protocols for the ghrelin kit were followed except for one modification: all reagent and sample volumes were halved. Methods for GH and IGF-I followed published protocols in Richmond and Zinn (2009). A rabbit-anti-porcine GH antibody (AFP422801) was used with porcine GH standard (AFP10864B) to quantify manatee serum. Concentrations of IGF-I were quantified using a rabbit-anti-porcine antibody (AFP4892898) and purified human IGF-I (Lot #1).

Statistical Analysis

Sensitivity, recovery of mass, assay precision, parallelism and dilution linearity was determined for each assay (Richmond and Zinn 2009; Chapter 2). Significant differences in serum hormone

concentrations between populations were analyzed using the general linear model, analysis of variance procedure (SAS Inst. Inc., Cary, NC). The original statistical model included sex and population as independent variables and hormone concentrations of GH, IGF-I and ghrelin as dependent variables. However, no differences were found between sex and the population*sex interaction and therefore were removed from the statistical model. A LSD post hoc test was completed to determine statistical differences among populations (Florida, Puerto Rico, Belize). A *p*-value of < 0.10 but > 0.05 was considered a trend and ≤ 0.05 was considered statistically significant. The results are presented as the mean ± standard error (SE).

RESULTS

Differences in standard length measurements (*p* = 0.06, Figure 1A) were found among the Florida, Puerto Rico, and Belize manatee populations. The Florida manatee was greater in length (290.8 ± 5.6 cm) compared with Puerto Rico (274.5 ± 4.3 cm, *p* = 0.03) and Belize (275.71 ± 4.2 cm, *p* = 0.04), while Puerto Rico and Belize populations (*p* = 0.84) were similar. Each population had differences in blubber thickness (*p* < 0.001, Figure 1B). The Puerto Rico population had the greatest blubber thickness (11.9 ± 0.7 mm) compared with Belize (9.5 ± 0.7 mm, *p* = 0.02) and Florida (5.7 ± 0.9 mm, *p* < 0.001). Manatees in Belize also had greater blubber thickness compared with Florida (*p* < 0.01). There were no differences in ghrelin concentrations between populations (*p* = 0.91, Figure 2A). However, concentrations of GH were statistically different between populations (*p* = 0.01, Figure 2B). Manatees in Puerto Rico had reduced GH concentrations (5.7 ± 1.9 ng/ml) compared with Florida (14.5 ± 2.4 ng/ml, *p* = 0.01) and Belize (13.8 ± 1.9 ng/ml, *p* < 0.01). There were no differences in GH concentrations between Florida and Belize populations (*p* = 0.83). Populations also exhibited differences in IGF-I concentrations (*p* = 0.03, Figure 2C). Florida manatees had greater IGF-I concentrations (164.0 ± 12.1 ng/ml) compared with Puerto Rico (121.7 ± 9.4 ng/ml, *p* = 0.01). Belize manatees exhibited a trend (144.5 ± 8.7 ng/ml) of

greater IGF-I when compared with Puerto Rico ($p = 0.08$). There were no differences between Florida and Belize manatee IGF-I concentrations ($p = 0.20$).

DISCUSSION

Florida and Antillean manatees exhibit slight differences in habitat, climate, quality, and availability of forage when compared with each other (Hartman 1979; Best 1981; Bengtson 1983). The Florida manatee migrates to warm water areas during colder temperatures, unlike Antillean manatees in Puerto Rico and Belize where the temperature remains relatively constant. These differences may cause the nutritional status of a population to differ from one another. Therefore, it was hypothesized that migratory behavior in conjunction with differences in climate, habitat, and dietary habits between Florida and Antillean manatees would result in differences in body composition, ghrelin, GH, and IGF-I at the population level (Hartman 1979; Mignucci-Giannoni and Beck 1998; Alves-Stanley et al. 2010). Further, it was predicted that body composition in conjunction with hormone profiles would indicate differences in nutritional status among the populations. Although our data exhibited differences among populations in standard length, blubber thickness, and hormone concentrations, the differences in nutritional status were not as expected.

Although colder temperatures occur more frequently in Florida, Florida manatees were longer and leaner compared with animals in both Puerto Rico and Belize. Blubber thickness was predicted to be greater in Florida manatees because of the colder climate and greater food availability; however, this was not the case (Irvine 1983; Worthy et al. 2000; Bossart et al. 2003). Although, blubber thickness differed for all three populations, the Florida manatee exhibited significantly less blubber than manatees from Puerto Rico and Belize. Manatees in Florida migrate further distances compared with Puerto Rico and Belize and may therefore benefit from greater lean tissue during the summer to assist with the energetic demands of migration and mating (Irvine 1983; Worthy et al. 2000; Bossart et al. 2003). To

further assess the nutritional status of the Florida, Puerto Rico, and Belize populations, hormone concentrations need to be considered in conjunction with body composition.

Ghrelin is secreted in the stomach and is dependent on nutritional status (Kojima et al. 1999). In previous studies on rats and humans, ghrelin stimulates food intake, body weight gain, and adiposity (Nakazato et al. 2001; Theander-Carrillo et al. 2006; Tschop et al. 2000; Wren et al. 2001a, 2001b). Not only has ghrelin been found to increase pre-prandially and decrease postprandially in humans, rats, and sheep (Cummings et al. 2001; Ariyasu et al. 2001; Tschop et al. 2001a), but concentrations also demonstrate diurnal variations which can be influenced by GH and body mass (Sugino et al. 2002; Korbonits et al. 2004; Sugino et al. 2004). Florida, Puerto Rico, and Belize manatee populations have slightly different foraging habits and access to food sources (Hartman 1979; Best 1981; Bengtson 1983; Mignucci-Giannoni and Beck 1998; Alves-Stanley et al. 2010). It was hypothesized that concentrations of ghrelin may be different between populations based on their dietary habits. Because ghrelin is usually elevated during low nutritional status, (Nakazato et al., 2001; Theander-Carrillo et al., 2006; Tschop et al., 2000; Wren et al., 2001a, 2001b) manatees in Puerto Rico and Belize were expected to have greater ghrelin concentrations due to the abundance of low quality forage available, Turtle grass (*Thalassia testudinum*) (Mignucci-Giannoni and Beck 1998; Alves-Stanley et al. 2010). However, ghrelin concentrations were found to be similar among Florida, Belize, and Puerto Rico populations.

Florida and Antillean manatees are ruminant species that use hindgut fermentation to digest the cellulose in the vegetation they ingest (Reynolds and Rommel 1996). Previous studies on ruminant species such as sheep, found that animals were unresponsive to the orexigenic properties of ghrelin because their gut is consistently full of food (Iqbal et al. 2006). Like sheep, manatees are grazers and forage throughout the day. Therefore, ghrelin concentrations may remain consistent between Florida, Puerto Rico, and Belize due to the individuals' consistent food intake regimen versus differences in quality of forage to maintain metabolic status.

Previous studies have demonstrated GH plays an important role in fat and protein metabolism (Breier et al. 1999). During times of positive nutritional status, GH is reduced allowing lipogenesis to occur (Breier et al. 1999; Carrel and Allen 2000; Eigenmann et al. 1985). However, animals experiencing caloric restriction (low nutritional status) have elevated GH concentrations that promote lipolysis in adipose tissue (Lawrence and Fowler 1997; Fuglei et al. 2000; Vijayakumar et al. 2010). Studies in humans have found that elevated GH concentrations may promote increased lipid metabolism, thereby partitioning nutrients to areas of the body in need of energy (Norrelund et al. 2003). Therefore, it was hypothesized that GH concentrations would be greater in Puerto Rico and Belize Antillean manatee populations compared with Florida due to their previously predicted low quality of available food (Mignucci-Giannoni and Beck 1998; Alves-Stanley et al. 2010). However, we found that manatees in Florida and Belize exhibited no differences compared to one another while both had greater GH concentrations when compared with Puerto Rico. Growth hormone plays an indirect role in protein accretion (Johnsson and Hart 1986, Breier 1999; Vijayakumar et al. 2010). In previous studies, elevated GH promoted lipolysis and stimulated the release of IGF-I from the liver (Smith et al. 1995; Renaville et al. 2002). Therefore, the elevated GH concentrations in Florida and Belize manatees may be stimulating lipolysis and the release of IGF-I further promoting lean tissue accretion.

Insulin-like Growth Factor I mediates the anabolic effects of GH on whole body growth and protein and lipid accretion (Lawrence and Fowler 1997; Breier 1999; Cammisoto et al. 2010; Castenda et al. 2010). In addition, concentrations of IGF-I can serve as an indicator of nutrient intake (Renaville et al. 2002). Under normal physiological conditions with adequate intake, IGF-I concentrations are stimulated by GH and may become elevated to stimulate protein accretion (Breier 1999; Renaville et al. 2002). However, animals that are nutrient deficient due to low quality and quantity of forage have reduced IGF-I concentrations (Rausch et al. 2002). Based on low quality and availability of forage in Puerto Rico and Belize, it was predicted that animals would have reduced IGF-I concentrations indicating nutrient

deficiency. However, Florida and Belize manatees had elevated IGF-I concentrations when compared with the Puerto Rico population. In a previous study on deer, elevated IGF-I concentrations promoted lean tissue accretion (Webster et al. 1996). Therefore, elevated IGF-I concentrations in Florida and Belize manatees may provide further support that protein anabolism is occurring. Although Puerto Rico manatees have reduced IGF-I concentrations, their blubber depth was similar to Belize animals and greater than Florida manatees, suggesting these animals were not nutrient deprived.

Together, GH and IGF-I can indicate nutritional status through their pattern and effect on tissue-specific nutrient partitioning. Both GH and IGF-I influence growth rate and directly act upon lean and adipose tissue accretion. In previous research during positive nutritional status concentrations of GH and IGF-I were elevated stimulating protein accretion (Breier 1999; Renaville et al. 2002). Therefore, the elevated GH and relatively elevated IGF-I concentrations in Florida and Belize animals and their blubber depth compared with Puerto Rico suggests positive nutritional status. In contrast, manatees in Puerto Rico had reduced GH and IGF-I concentrations in conjunction with the greatest blubber thickness when compared with Florida and Belize populations. Although, IGF-I concentrations were reduced in Puerto Rico manatees and previous research found reduced IGF-I may be an indicator of low nutritional status (Renaville et al. 2002), IGF-I coupled with GH concentrations and blubber thickness suggest otherwise. In previous research, reduced GH promoted lipogenesis which may explain the thick blubber in the current study when compared to Florida and Belize animals (Webster et al. 1996; Breier et al. 1999). Therefore, hormone profiles in conjunction with blubber thickness suggest Puerto Rico manatees are not nutritionally compromised.

This research may provide a baseline data for further studies to monitor changes in nutritional status within and among populations. In summary, these data indicate that Florida manatees were longer and leaner when compared to Puerto Rico and Belize manatee populations. However, body composition in combination with hormone profiles suggests that Florida, Puerto Rico, and Belize

populations are not nutritionally compromised as previously suggested (Breier et al. 1999). Previously, there were limited data available on Florida and Antillean manatees and the hormonal mechanisms that regulate body composition and nutrient intake. The current data provides the first values of ghrelin, GH, and IGF-I in relation to body composition and nutritional status within the West Indian manatee. Previous mammalian research found that nutritional status influenced ghrelin, GH, and IGF-I concentrations (Breier et al. 1999). Because these hormones function in a predictable manner with changes in body composition they could prove to be valuable tools in indicating nutritional status. Therefore, monitoring changes in ghrelin, GH, IGF-I, and body composition during future manatee capture and health assessments or rehabilitation could provide better veterinary care and further improve overall health and body condition. Overall, these data will provide managers with additional knowledge on the health and welfare of the West Indian manatee to continue to make progress in species recovery.

ACKNOWLEDGEMENTS

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CHAPTER FOUR: FIGURES

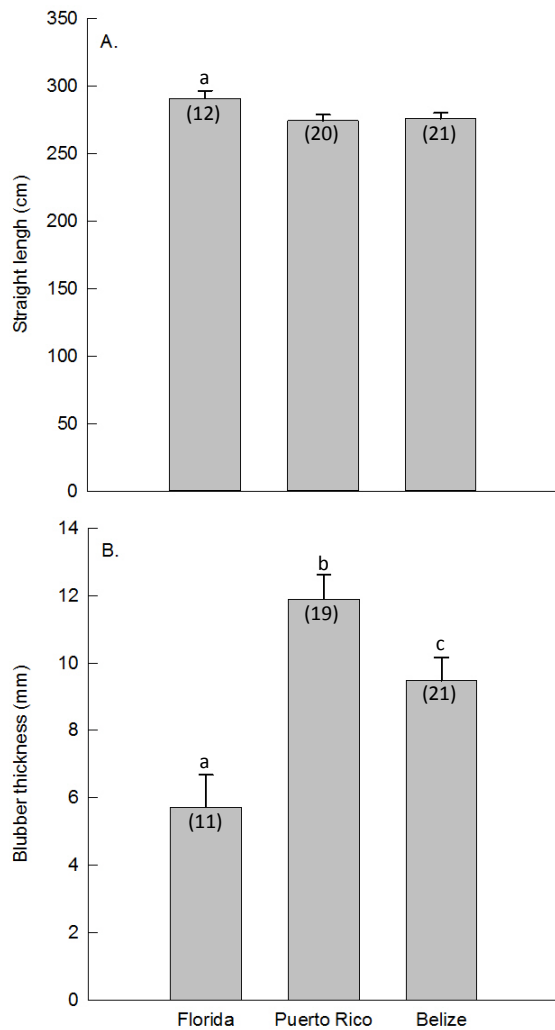


Figure 4. 3 Population differences among manatees from Florida, Puerto Rico, and Belize morphometrics. Gray bars delineate different manatee populations (Florida, Puerto Rico, Belize) with letters (a, b, c) representing significant differences between groups. Numbers in parenthesis at the top of each bar represent sample size for the group. Values for morphometrics including (A) straight length and (B) blubber thickness are presented as mean \pm standard error.

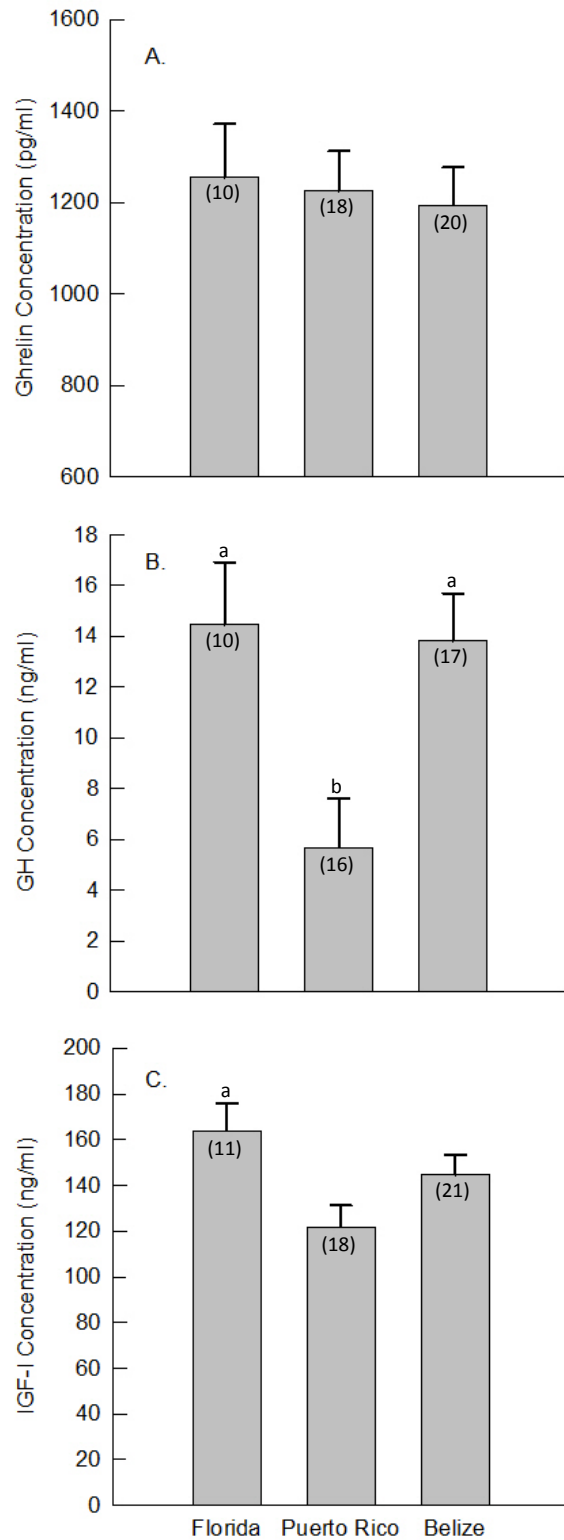


Figure 4. 4 Population differences of hormone profiles in manatees from Florida, Puerto Rico, and Belize. Gray bars delineate different manatee populations (Florida, Puerto Rico, Belize) with lower case letters (a, b) representing significant differences between groups. Numbers in parenthesis at the top of each

bar represent sample size for the group. Hormone concentrations include (A) ghrelin, (B) growth hormone (GH), and (C) insulin-like growth factor I (IGF-I) with values presented as mean \pm standard error.

CHAPTER 5: CONCLUSION

DISCUSSION AND APPLICATION OF RESEARCH

The West Indian manatee (*Trichechus manatus*) is currently listed as an endangered species and is protected under the Endangered Species Act and Marine Mammal Protection Act in the United States. In addition to their endangered status, manatees are considered a sentinel species; changes in their population status, mortality rate, and behaviors can be used to determine potential threats to the ecosystem and human health (Bonde et al. 2004; Bossart 2006). Changes in the environment can elicit an endocrine response in the manatee to adjust homeostatic set points. These adjustments may include responses such as changes in food intake, body weight, fat content, and feed efficiency (Breier 1999). Hormones within the endocrine system such as ghrelin, growth hormone (GH), and insulin-like growth factor (IGF)-I function to regulate tissue specific energy allocation and together link growth physiology, developmental age, energy balance, and nutritional status (negative or positive nutrient intake) in domestic animals (Lawrence and Fowler 1997; Breier 1999; Ortiz et al. 2003; Cammisoto et al. 2010; Castenda et al, 2010). Therefore, understanding the role metabolic hormones play in the manatee is of extreme importance. Hormone concentrations may serve as an indicator to environmental shifts that could result in individual or species mortality. Therefore, the purpose of this study was to establish baseline data for ghrelin, GH, and IGF-I concentrations and their relation to body composition in the West Indian manatee. The following objectives were established and addressed in this research. Hormone concentrations were compared among summer, fall, and winter to determine the seasonal

pattern of these hormones and quantify their relationship to body composition. Sex and developmental patterns of the hormones were assessed. In addition, hormone differences were considered among free-ranging manatee populations in Florida, Belize, and Puerto Rico to further determine their response to different environmental factors.

Validations were performed for ghrelin, GH, and IGF-I heterologous radioimmunoassays (RIA) to determine accuracy and precision of measurements in the West Indian manatee. Improper or failure to perform validations can lead to erroneous results. Serially diluted pooled serum from free-ranging West Indian manatees was used to validate the RIA. All assays demonstrated excellent parallelism and linearity quantifying hormone concentrations with R^2 values above 0.90 indicating manatee serum can be measured. Percentage recovery of pooled manatee serum was also good with more than 90% of ghrelin, GH, and IGF-I recovered. The intra assay coefficient of variation (CV) was low ($< 11\%$) for all assays, indicating precision and repeatability within an assay is high. Inter assay CV values were also low ($< 16\%$) and consistent, indicating a high degree of precision across repeated assays. All assay validations performed indicated that heterologous RIA for ghrelin, GH, and IGF-I could be used to quantify these hormone concentrations in West Indian manatee serum and further used to evaluate changes in physiological state.

The experiment in the free-ranging Florida manatee indicated that GH and IGF-I may be a useful predictor of seasonality in the manatee (Chapter 2). As expected, GH and IGF-I profiles in conjunction with body composition indicated seasonal adjustments in tissue-specific nutrient partitioning (Webster et al. 1996; Breier et al. 1999; Fuglei et al. 2004). During summer, greater GH and high concentrations of IGF-I in conjunction with reduced blubber suggests manatees are prioritizing lean tissue accretion for potential mating and reproductive demands. During winter, reduced GH, high concentrations of IGF-I and greater blubber suggests the prioritization of adipose accumulation for energy maintenance and thermoregulatory needs. Further, reduced GH and high concentrations of IGF-I in the winter may

indicate positive nutritional status associated with food intake because blubber was not diminishing (Breier et al. 1999; Renaville et al. 2002). This hormonal profile may provide a useful tool to predict nutritional status and seasonal changes in free-ranging individuals when nutrient intake is unknown.

Chapter 2 also assessed the ontogeny of the hormones. Similar to other mammals, GH concentrations were reduced in adults compared with calves and juveniles suggesting a reduction in growth rate as a manatee ages (Anderson et al. 1988; Zinn et al. 1989; Martha et al. 1992; Schwarz et al. 1992; Albertsson-Wikland et al. 1994; Blackman et al. 1994). However, IGF-I concentrations did not exhibit differences between age classes which was unexpected. Previous studies found that IGF-I is initially greater during the first year of life and later plateaus when individuals reach puberty (Schwarz et al. 1992; Blackman et al. 1994; Skaar et al. 1994; Gatford et al. 1996; Harrell et al. 1999; Govoni et al. 2002; Govoni et al. 2003). Age class was estimated by standard length which may not truly reflect actual age. Some manatee calves may experience rapid growth rate when compared to other individuals placing them in an incorrect age class altering the results. Due to the contradictory nature of the results, future studies should be performed to better understand the role IGF-I plays in age-associated changes in the manatee. Other suggested studies will be discussed further in the “Future Directions” section below.

Previous research found ghrelin, GH and IGF-I were different in males and females in some ungulate species (Webster et al. 1999; Govoni et al. 2003). Both serum GH and IGF-I concentrations are greater in males compared with female cattle, sheep, and pigs (Gatford et al. 1996; Owens et al. 1999; Govoni et al. 2003), and are associated with increased growth rate (Rausch et al. 2002). Similar results were predicted in the West Indian manatee; however, this was not the case. Although differences in body composition were observed between males and females, hormonal differences were not found. The power to detect differences was low due to the variability of these data. Future research should consider an increase in sample size with equal distribution. Although some differences may be

undetected due to the variability of data, it is also likely that ghrelin, GH, and IGF-I do not have sex related patterns in the manatee. In addition to understanding hormonal patterns associated with seasonal changes, development, and sex differences, differences in populations (Florida, Puerto Rico, Belize) and subspecies of the West Indian manatee (Florida and Antillean manatees) were explored. Differences in climate, habitat, and quantity/quality of forage between populations were predicted to produce dissimilar physiological responses. Ghrelin, GH, and IGF-I were assessed to determine nutritional status at the population level. Florida, Puerto Rico, and Belize populations exhibited differences in body composition and hormone concentrations. Manatees in Florida and Belize had elevated GH and IGF-I concentrations when compared with Puerto Rico. Their hormone profiles in conjunction with blubber thickness suggested lean tissue accretion may be occurring remaining consistent with Chapter 2 results for the summer season. However, Puerto Rico manatees had the greatest blubber thickness and reduced GH and IGF-I when compared with the other populations. Although, previous research found reduced IGF-I with increased GH may be an indicator of nutritional stress (Renaville et al. 2002), IGF-I coupled with GH concentrations and blubber thickness suggest otherwise. Reduced GH and IGF-I combined with thick blubber suggest adipose stores are being maintained. The current data suggests ghrelin, GH, and IGF-I can be used to indicate nutritional status in Florida, Puerto Rico, and Belize manatee populations. Although environmental factors (climate, habitat, forage) may be contributing to the differences in nutritional status between populations, further research needs to be performed to determine what specific factors are causing different physiological responses. Suggested research includes controlled feedings to determine if the quantity and quality of forage play a role in hormonal responses between populations. Samples should be collected from each population at consecutive times of the year. Studies on captive manatees could be provided as a potential control to assess the impact of variables (temperature, sex, habitat, forage) on hormone levels.

In Chapter 2 and 3 ghrelin concentrations were consistent among seasons, when comparing age classes and among populations in the West Indian manatee as seen in other ruminant species (Iqbal et al. 2006). Previous research found that sheep were unresponsive to the orexigenic properties of ghrelin due to their regularly full gut (Iqbal et al. 2006). The entire gastrointestinal tract of a manatee also is not fully emptied between meals because of long ingesta passage rates and unset foraging times. Therefore ghrelin concentrations may not differ significantly because of the continuous presence of food in the gut (Iqbal et al. 2006; Reynolds and Rommel 1996).

Previously, there was limited data available on the hormonal mechanisms that regulate body composition and nutritional status in the manatee. Overall, this research has established baseline data for ghrelin, GH, and IGF-I concentrations in the West Indian manatee. These data provide the first values of ghrelin, GH, and IGF-I in relation to body composition in manatee among seasons and populations. Understanding the mechanisms behind energy balance and tissue-specific nutrient partitioning can provide further knowledge on the ability of these species to cope with seasonal and environmental changes. In addition, age-related patterns of GH revealed manatees respond similarly to other mammals. Understanding the age-related patterns of these hormones in the manatee may establish a tool to further predict developmental changes associated with nutrient intake in rehabilitated and free-ranging individuals.

FUTURE DIRECTIONS

Hormonal Studies

Free-ranging manatees in the United States are brought into captivity for rehabilitation with the intent for release. Managers and conservationists use rehabilitation as a method to further conserve and protect the species; new techniques to effectively treat sick and injured manatees are often sought after. The combination of blubber depth, standard length, and hormone profiles can be used to indicate

not only body composition but nutritional and energy status of an individual manatee. This could prove to be a valuable tool in rehabilitation facilities and during captures and health assessments to provide better veterinary care and further improve overall health and body condition monitoring. This research focused on understanding seasonality and nutritional status in the West Indian manatee. Although ghrelin, GH, and IGF-I appear to be an effective tool in determining seasonal and nutritional changes, other binding proteins and hormones can further indicate a physiological response and provide a better overall assessment of an individual.

Although, IGF-I may be a partial indicator of nutritional status and plays a role in the regulation of body composition, IGF binding proteins (IGFBP) further regulate the bioavailability and therefore activity of circulating IGF (Clemmons and Underwood 1991; Estivariz and Ziegler 1997). In particular, the IGFBP2 and IGFBP3 have been found to be the most responsive to nutrient intake and correlated to whole body growth rate (Rausch et al. 2002; Govoni et al. 2003, 2004, 2010). It is well known that IGFBP3 stimulates IGF-I and increases with growth (Renaville et al. 2000; Govoni et al. 2003, 2004), whereas, IGFBP2 is inhibitory and typically follows a pattern opposite IGFBP3 (Rausch et al. 2002). Binding proteins may further indicate nutritional status based upon their relationship with IGF-I and their role in regulating whole body growth. Therefore, IGFBP2 and IGFBP3 should be considered in future research (Smith et al. 1995; Renaville et al. 2000; Rausch et al. 2002; Govoni et al. 2010).

In addition, the hormone leptin can provide more information on energy balance and tissue-specific nutrient partitioning (Zhang et al. 1994). Leptin is synthesized primarily from white adipose tissue (Ahima and Flier 2000) with multiple functions pivotal in regulating energy homeostasis and food intake (Zhang et al. 1994). These actions have been demonstrated in the mouse, sheep, cattle, pig, and horse (Zhang et al. 1994; Pfister-Genskow et al. 1997; Dyer et al. 1997; Buff et al. 2002). Studies in humans and mice have also shown that serum leptin concentrations are greater in individuals with greater body mass (Schwartz et al. 1996) suggesting leptin has a positive correlation with adiposity

(Frederich et al. 1995; Trayhurn et al. 1995; Kolaczynski et al. 1996). In previous research, leptin has also been found to be modified by season producing contradictory results. Although a positive correlation has been found between leptin concentrations and body mass during short photoperiods in some arctic terrestrial mammals (Soppela et al., 2008), a negative correlation between body mass and leptin during fasting in short photoperiods has been documented in Antarctic fur seals and mink and no correlation was found in the raccoon dog, blue fox, and Antarctic fur seals (Nieminen et al. 2001; Arnould et al. 2002). The differential response between species may indicate that animals are acclimating to environmental stimuli differently. Thus, investigating leptin in the West Indian manatee will not only provide a better understanding of body composition changes in individuals, but will provide additional support when determining seasonality and nutritional status.

Changes in day length and food availability are known to be confounding variables in determining what triggers a seasonal physiological response and causes changes in body composition. Does day length affect adiposity or is nutrition the sole cause of change during different seasons? To further investigate if changes in photoperiod illicit a physiological response in the West Indian manatee, melatonin concentrations can be measured. Melatonin is a hormone released by the pineal gland that is known to serve a central role in mammalian photoperiodism (Bartness and Goldman 1989). Melatonin is linked to a light/dark cycle with greater concentrations at night regardless of the species daily behaviors (e.g. diurnally or nocturnally active) (Reiter 1991). Thus, the duration of night is based on the duration of melatonin secretion which can ultimately trigger seasonal responses (Bartness et al. 1993). Numerous experiments have demonstrated that the effect of photoperiod on seasonal functions (body composition, energy balance, reproduction) is profoundly altered in melatonin treated animals (Malpaux et al. 2001). Although animals have exhibited changes in body composition when treated with melatonin during different seasons, this idea has not been previously explored in the West Indian manatee. Future melatonin research can provide a more precise understanding on the light vs. nutrition

controversy in the West Indian manatee and further explore how melatonin contributes to changes in body composition during different photoperiods.

In addition to day length, stress has also been associated with seasonal changes and nutritional status. The hormone cortisol has been found to be an indicator of chronic stress in manatees with concentrations greater in unhealthy animals (Tripp et al. 2011). Previous research has focused on stress associated with capture, injury, disease, and rehabilitation in manatees. Cortisol provides energy to cope with stress by stimulating lipolysis and glucose formation (Munck et al. 1984; Breazile 1987). Energy is often allocated from important biological processes disrupting circadian rhythms, metabolism, growth, gastrointestinal cycles, and immune response which can lead to sickness and in extreme cases, death (Turner et al. 2002; Cooke et al. 2004; Reeder and Kramer 2005). Although the role of cortisol in captured and rehabilitated manatees has been investigated, the seasonal and environmental stressors have not been previously explored (Tripp et al. 2011). Quantifying seasonal cortisol concentrations combined with morphometrics and other hormones like ghrelin, GH, IGF-I, IGF-BP, leptin, and melatonin can determine levels of chronic stress and further indicate a physiological response linked to changes in season and nutrient intake.

Nutritional Studies

Previous mammalian research found that nutritional status influenced ghrelin, GH, and IGF-I concentrations (Breier et al. 1999). Although, the data from the present study provides a better understanding of the roles these hormones play in regulating body composition, they do not fully reflect the influence of nutritional status. To fully understand the how the hormones fluctuate to cope with changes in nutritional status controlled intake studies would need to be conducted. A small select number of facilities house captive manatees making controlled studies challenging; however, if food

intake can be regulated in healthy captive manatees we can determine the effect nutrition has on metabolic hormones. This can be used as a tool to indicate nutritional status in free-ranging individuals which can assist in the management of manatee populations.

Age-related Studies

In the present study, adult manatees had reduced GH concentrations when compared to calf and juvenile age classes. However, IGF-I concentrations were consistent among age classes. Although GH followed a similar pattern seen in other mammals, controlled studies could provide additional results which may help us better understand the role hormones play in development (Anderson et al. 1988; Zinn et al. 1989; Martha et al. 1992; Schwarz et al. 1992; Albertsson-Wikland et al. 1994; Blackman et al. 1994). A longitudinal study following multiple individuals from birth to adulthood would be ideal, but also challenging. Healthy free-ranging calves would provide the best quality of data to reflect normal developmental changes. However, manatee calves are specifically avoided during captures to prevent high levels of stress and potential for separation from their mothers. Manatee calves that are taken into rehabilitation facilities often arrive malnourished which could skew the results for the first few months. However, calves are usually held for a few years in a facility which would provide the opportunity to collect data during the calf, juvenile, and early adulthood periods.

SUMMARY

The current measurements of ghrelin, GH, and IGF-I concentrations may prove to be a useful tool in evaluating the seasonality and nutritional status of the West Indian manatee. Based on previous seasonal research conducted in domestic, ruminant, and arctic species, manatees shared interesting similarities to domestic and ruminant animals. However, age related changes did not respond as predicted. Ghrelin, GH, and IGF-I profiles in conjunction with body composition changes between

seasons indicated seasonal adjustments in manatee tissue-specific nutrient partitioning. Ghrelin concentrations were similar to domestic ruminant species remaining consistent through season and population. Similar to free-ranging ruminant species, manatees had reduced blubber thickness, greater GH concentrations, and greater IGF-I concentrations in the summer compared with fall and greater blubber thickness, reduced GH, and greater IGF-I concentrations in the winter (Webster et al. 1996). These data suggest manatees are prioritizing lean tissue accretion during the summer for potential mating and reproductive demands, whereas, manatees may be prioritizing adiposity in the fall and winter for energy maintenance and thermoregulatory needs. Hormonal and body composition differences were also observed between Florida and Antillean manatees. Hormone concentrations in combination with body composition were able to indicate nutritional status in Florida, Puerto Rico, and Belize populations. Florida and Antillean manatees did not appear to be nutritionally deprived (Breier et al. 1999).

The West Indian manatee is considered a sentinel species that provides insight as to how other species in the ecosystem may respond to acute or chronic changes in their climate, habitat, and forage (Bonde et al. 2004; Bossart 2006). Monitoring the response of ghrelin, GH, and IGF-I to nutritional status and seasonal change in subtropical and tropical marine mammals may provide new information on energetics, fasting metabolism, dietary intake, and seasonal patterns in body composition (Strandberg et al. 2007). In addition, these hormones can be used to evaluate energy balance, nutritional status, and health status of free-ranging populations. In conclusion, this research provided baseline data for future studies to monitor changes in nutritional status and season within and among populations which will further assist in managing the survival of the species.

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VITA

Rachel L. Cimino received her Bachelors of Science at the University of Central Florida with a major in Psychology, a minor in American Humanics, and a certificate in Nonprofit Management. Rachel interned with the Florida Fish and Wildlife Conservation Commission Manatee Section in 2009 in which she performed duties including carcass recovery and salvaging, necropsy, rescues, photo ID, and public outreach. After completing her internship, Rachel was hired as an Educator at Sea World Orlando where she conducted frequent fact-based presentations. Rachel is currently employed as a marine mammal biologist with the Florida Fish and Wildlife Conservation Commission Marine Mammal Section. She has assisted with numerous manatee and cetacean rescues, over 100 marine mammal necropsies including two right whales, and manatee capture and health assessments. In addition, she has received Level 2 large whale disentanglement training, HAZWOPER training, and various boat and trailer safety training. Rachel has been able to participate in North Atlantic Right Whale biopsies, dolphin health assessments, and dolphin disentanglement efforts. In addition to her time spent at work, Rachel was able to present her graduate thesis research at multiple conferences including the Southeastern Estuarine Research Society, Society for Integrative and Comparative Biology, and Southeast Mid-Atlantic Marine Mammal Symposium.