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Assessment of Fertility Potential in Bottlenose Dolphins (*Tursiops truncatus*):

An ELISA-based Biomarker Analysis

by

Leslie Schwierzke Wade

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
College of Marine Science
University of South Florida

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Date of Approval:
October 31, 2011

Keywords: Anti-Müllerian hormone, Inhibin, Contaminants, Reproduction, Marine Mammal

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ACKNOWLEDGMENTS

This research was funded by the Florida Protect Wild Dolphins License Plate Fund through Harbor Branch Oceanographic Institute (HBOI) at Florida Atlantic University. I am grateful to Megan Davis, Stephen McCulloch, Greg O'Corry-Crowe, and many others at HBOI for their efforts in ensuring the funding of this research. The College of Marine Science Endowed Fellowship Program, specifically the John B. and Katherine Ann Lake Fellowship provided additional support for my graduate studies.

I would also like to thank the Sarasota Dolphin Research Program, the Chicago Zoological Society, Dolphin Quest, the Morris Animal Foundation, the Georgia Aquarium, the Health and Environmental Risk Assessment Program at HBOI, the National Oceanic and Atmospheric Administration (NOAA), the National Ocean Service Center for Coastal Environmental and Biomolecular Research (CCEBR), NOAA Fisheries Marine Mammal Health and Stranding Response Program, the Florida Protect Wild Dolphins License Plate Fund, and NOAA's Ocean and Human Health Initiative for their support of the Sarasota Bay, Indian River Lagoon, and southern Georgia sample collection. In addition, I would like to thank the numerous researchers, veterinarians and volunteers who participated in the dolphin capture and release studies.

Finally, I express my gratitude to Erin Pulster, for her incredible contaminant expertise and many laughs; to Bill Roudebush, for providing statistical input; to my husband, Ryan, for supporting my dreams; and to my advisors, John Reynolds, Dana Wetzel, Kendra Daly and Ted Van Vleet, for their guidance and support.

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ABSTRACT

As apex predators in coastal systems, bottlenose dolphins (*Tursiops truncatus*) are susceptible to persistent organic pollutant (POP) accumulation and retention over time, which has prompted continued interest in understanding the extent to which contaminant body burdens or other stressors are sufficient to cause adverse sublethal effects on energetic fitness, immune function, or reproduction. Increasing our knowledge of reproductive endocrinology in bottlenose dolphins may provide insight into changes in reproductive rates, thereby expanding the capacity to assess conservation status. This study used the Enzyme-Linked ImmunoSorbent Assay (ELISA) technique to examine peptide fertility hormones [inhibin A, inhibin B and anti-Müllerian hormone (AMH)] measured in serum of free-ranging dolphins (n = 129) of varying age, gender, and maturity status from three locations (Sarasota Bay, FL, Indian River Lagoon, FL, and southern Georgia). The primary research objectives were to establish hormone baselines, investigate AMH and inhibin use as reproductive biomarkers, and examine the potential use of these hormones as biomarkers of toxicant or other stressor effects on reproduction. AMH secretion differed significantly with gender ($p < 0.001$), where levels were approximately 1,000-fold higher in males than females (1,122 ± 427 ng mL^{-1} and 1.15 ± 1.25 ng mL^{-1}, mean ± SD). Male AMH levels were related to maturity status, and linear regression analysis revealed a significant, negative relationship between male AMH and age, body length, body weight, and maximum girth in all populations. Of the parameters

assessed, age was the best indicator of AMH levels in males. AMH concentrations in females did not vary significantly over time or with maturity status, but exhibited a decrease in some older individuals, potentially indicating an AMH decline in long-lived female dolphins. Inhibins did not differ significantly between age classes in males, but appeared to be an estrous cycle indicator in females, where inhibin peaks were likely related to follicular and luteal phases. These data provide new information on circulating serum AMH and inhibin levels in bottlenose dolphins, which appear to reflect a degree of gonadal function and show promise as reproductive biomarkers. Our findings suggest the possibility of toxicant effects on AMH and inhibin production, but not conclusively. Further investigation of mechanism(s) of action for contaminant-related reproductive toxicity will elucidate the diagnostic value of these hormones to assess the effects of POPs on fertility potential in bottlenose dolphins.

INTRODUCTION

Coastal ecosystem health is a topic of great concern, especially as human populations expand in coastal regions, where marine organisms are exposed to anthropogenic stressors. Due to their high trophic status and life histories, several marine mammal species, specifically bottlenose dolphins (*Tursiops truncatus*), are considered good indicators of stressors impacting coastal systems, as dolphins are increasingly exposed to environmental contaminants (Wells et al., 2004). Bottlenose dolphins are common inhabitants of coastal waters of the southeastern U.S. and several long-term, longitudinal research efforts have studied dolphin populations within the U.S., providing considerable knowledge of this species (Wells et al., 2004; Reif et al., 2008; Mazzoil et al., 2008).

Possible stressors have been identified for marine mammals, but their effects on vital biological functions are not fully understood. For example, it has been well established that many marine mammals contain elevated levels of lipophilic persistent organic pollutants (POPs), which bioaccumulate in marine food webs and biomagnify in top-trophic level species (O'Shea, 1999; Aguilar et al., 2002). Cetaceans are long-lived apex predators with extensive lipid-rich blubber layers, that are susceptible to contaminant accumulation and retention over time. Their limited capacity for pollutant degradation or biotransformation serves to heighten toxic effects (Tanabe et al., 1988), which has prompted continued interest in understanding the extent to which contaminant

body burdens are sufficient to cause adverse sublethal effects on biological functions, such as reproduction, energetic fitness, or immune function.

Substantial evidence now links chronic exposure to POPs [specifically polychlorinated biphenyls (PCBs)] with adverse effects on immune system function (Ross et al., 1995, 1996; De Swart et al., 1996), reproduction (Reijnders, 1986; Béland et al., 1993; Schwacke et al., 2002), infectious disease susceptibility (Kannan et al., 1993; Aguilar and Borrell, 1994; Schwacke et al., 2011), and skeletal growth (Zakharov and Yablokov, 1990; Bergman et al., 1992) in marine mammal populations worldwide. In addition, PCB studies have shown toxicological effects on neurological, developmental, immunological, reproductive, and endocrine systems of other mammals (Robertson and Hansen, 2001). Contaminant concentrations in several marine mammal species are consistently reported above the threshold PCB level (17 $\mu g\ g^{-1}$ lipid weight) known to cause adverse health effects in several other species, determined through experimental studies on reproductive and immunological effects in seals, otters, and mink (Kannan et al., 2000). However, there is substantial variation among mammals in sensitivities to toxic effects of PCBs, given differences in diet, selective biomagnification of PCB congeners, and biotransformation capacities (Kannan et al., 2000).

Previous research has shown that aquatic mammals are sensitive to toxicological effects of endocrine-disrupting compounds (EDCs), such as PCBs and organochlorine pesticides (OCPs), which target the reproductive tract (Fossi and Marsili, 2003). Dall's porpoise (*Phocoenoides dalli*) populations in the western north Pacific have demonstrated reduced testosterone concentrations with increased organochlorines (PCBs and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene [DDE, a major degradation product of DDT]),

which possibly indicated the causative effects of PCBs and DDE in affecting normal reproductive hormone concentrations and sexual function (Subramanian et al., 1987). Captive harbor seals (*Phoca vitulina*) harboring higher PCB concentrations have shown decreased pup production due to lower estradiol levels and impaired implantation (Reijnders, 1986). In addition, premature births in California sea lions (*Zalophus californianus*) were associated with high organochlorine (OC) levels (DeLong et al., 1973).

Reduced reproductive rates and reproductive failure could threaten the conservation status of affected populations. Hormones play key roles in reproductive rates and control many functions of the reproductive system. Measured hormonal concentrations may offer a better understanding of factors that might impair a species' reproductive viability or act as early warning signals for changes in reproductive rates (Kirby and Ridgway, 1984). It is difficult to understand the factors (such as potential effects of POPs and/or other stressors) that may be influencing reproduction in an individual or population, without knowledge of hormone cycling. A thorough understanding of reproductive biology and reproductive cycling will increase the capacity for assessing conservation status and possibly support the establishment or implementation of effective management and conservation strategies.

Mammalian Reproductive Endocrinology

The field of mammalian reproductive endocrinology began early in the 20th century, when physiologists started to question the role of the pituitary and gonads (Bronson, 1989). Hormonal identification and quantification have provided insight into

the function of the hypothalamic-pituitary-gonadal (HPG) axis, which is critical to gonadal differentiation and the development and regulation of the mammalian reproductive system. Ultimately, the HPG forms a functional endocrine axis that controls hormone production in the ovary and testes (Figure 1).

The principal tissues that synthesize polypeptide proteins and steroid hormones, which are most directly associated with mammalian reproduction, are the hypothalamus, anterior pituitary, ovaries, and testes. The hypothalamic central nervous system produces gonadotropin-releasing hormone (GnRH), which is transported to the anterior pituitary, where it induces secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH). These hormones then stimulate gonadal production of sex steroids (estrogens and androgens) and peptide hormones [anti-Müllerian hormone (AMH), inhibins, activins, etc.]. The reproductive axis is regulated by a negative feedback system, where increased hormone production in the gonads results in a decreased hormonal secretion from the hypothalamus and pituitary gland (Figure 1).

Both FSH and testosterone induce spermatogenesis, the process of male gamete production. LH stimulates the Leydig cells of the testes to produce testosterone, a main androgen for spermatogenesis, which affects sperm production and maturation. FSH exerts a direct effect on testicular Sertoli cells by playing a role in their functional development, maturation at puberty, and maintenance of their cytoskeleton and cell junctions (McLachlan, 2000; Figure 2). Anti-Müllerian hormone expression and secretion are controlled by testosterone and follicle stimulating hormone and are related to Sertoli cell function and maturation and spermatogenesis progression (Sinisi et al., 2008). In addition, inhibin concentrations are suggested to FSH-stimulated Sertoli cell

function (Andersen et al., 1997). Sertoli cells, also known as sustentacular cells of the testis, are commonly used as an indication of reproductive status in mammals (Setchell, 1978) and play a crucial role in the initiation and maintenance of spermatogenesis.

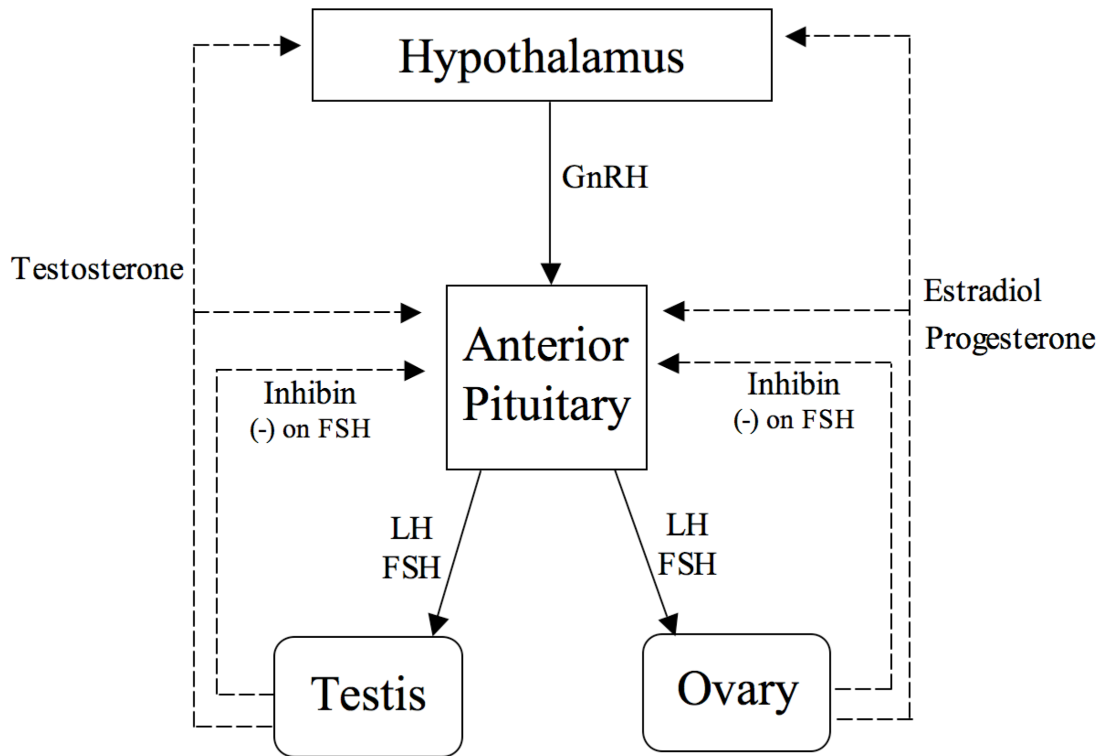

FIGURE 1. Regulation of the typical mammalian hypothalamic-pituitary-gonadal axis. Dashed lines indicate negative feedback and solid lines indicate positive influences. GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone.

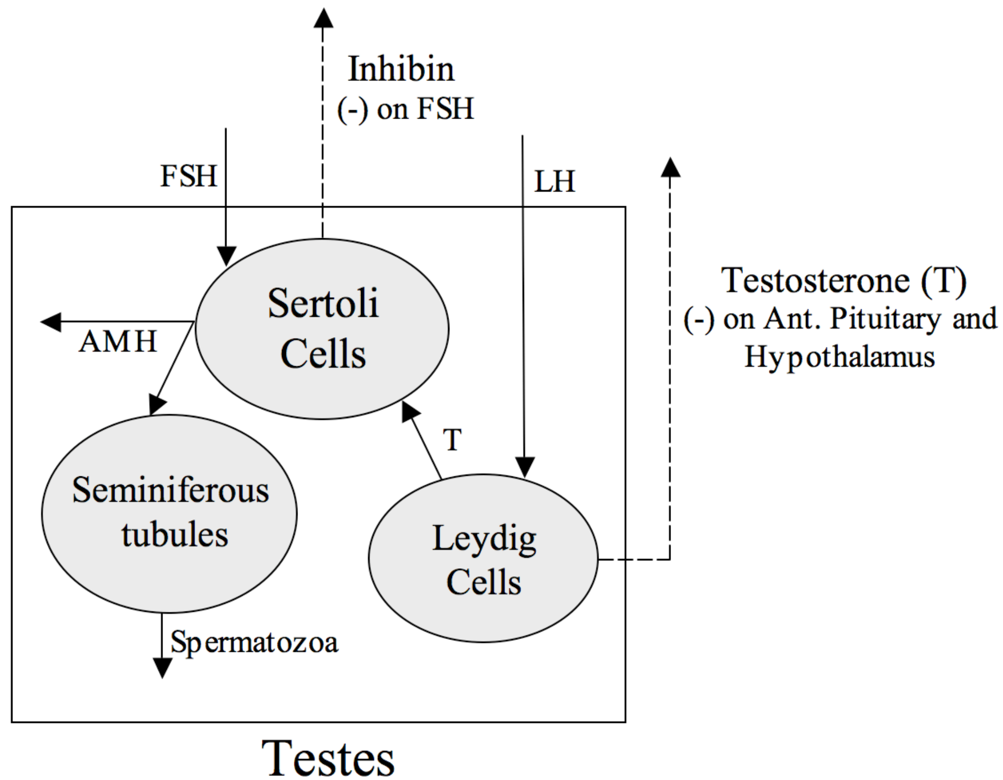

FIGURE 2. Reproductive endocrinology in the male. Dashed lines indicate negative feedback and solid lines indicate positive influences. LH, luteinizing hormone; FSH, follicle-stimulating hormone; T, testosterone; AMH, anti-Müllerian hormone.

In females, follicle stimulating hormone and luteinizing hormone govern the reproductive system and primarily target the granulosa cells within the ovary. Follicle stimulating hormone influences the development of the ovarian follicle, a sac-like aggregation of cells within the ovary that contains a single oocyte (an immature ovum or egg). Luteinizing hormone plays a role in final follicular and oocyte maturation, the induction of ovulation, and formation of the corpus luteum (CL) post-ovulation; the remaining part of the follicle becomes the CL, which secretes progesterone and helps to establish and maintain pregnancy. During folliculogenesis, follicle stimulating hormone converts estrogen from androgens within the granulosa cells, and luteinizing hormone stimulates androgen production from the ovarian theca cells, which are endocrine cells

associated with ovarian follicles. Under the influence of both gonadotropins, the granulosa cells produce several peptide hormones, including inhibins, activin, follistatin, and AMH (Figure 3). Inhibins regulate folliculogenesis through suppression of pituitary FSH secretion, whereas activin promotes granulosa cell proliferation, suppresses androgen production in the theca cells, enhances oocyte maturation, and increases inhibin and follistatin secretion. AMH modulates primordial follicle recruitment and inhibits cyclic follicle recruitment for folliculogenesis, primarily by inhibiting the follicle stimulating hormone effect on follicle growth (Roudebush et al., 2008).

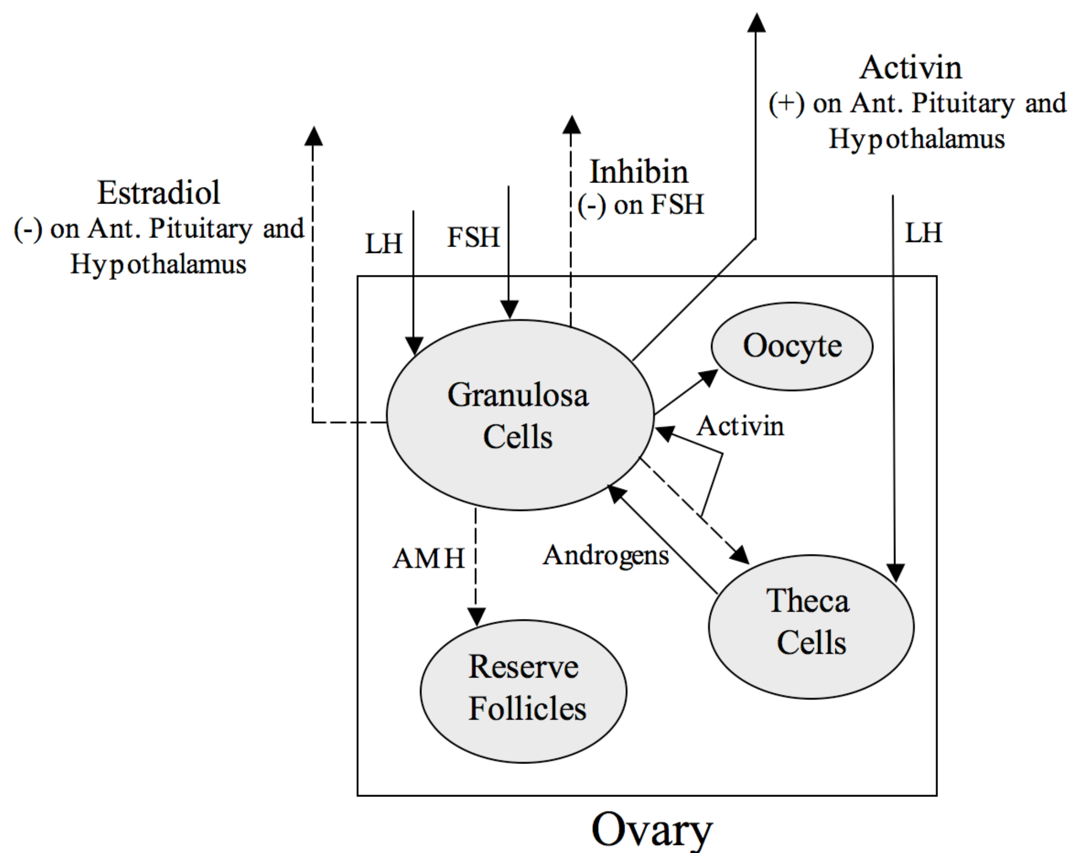

FIGURE 3. Reproductive endocrinology in the female. Dashed lines indicate negative feedback and solid lines indicate positive influences. LH, luteinizing hormone; FSH, follicle-stimulating hormone; AMH, anti-Müllerian hormone.

Cetacean Reproductive Endocrinology and Biology

The cetacean reproductive tract conforms to the general mammalian reproductive system centered on the hypothalamic-pituitary-gonadal axis. There are several similarities between the reproductive anatomy of terrestrial animals and cetaceans. Females possess paired ovaries, fallopian tubes, a diffuse-type placenta, a bicornuate uterus, and a vaginal opening. Individual males posses a fibroelastic penis, vasa deferentia, epididymides, and elongate testes, which perform similar functions to those of other mammals: the production of sperm and steroid hormones (Boyd et al., 1999; Pabst et al., 1999).

The earliest findings on cetacean hormone cycling were discovered through 0captive breeding programs, where reproductive state could be closely monitoring and sampling occurred frequently. Harrison and Ridgway (1971) first described gonadal hormone production and testicular maturity in captive bottlenose dolphins by measuring serum testosterone concentrations over a two-year period in immature and mature males. Their findings indicated higher testosterone levels with increasing testicular size and body length. Levels of progesterone, one of the most common steroids measured in female cetaceans, have shown a clear link between gonadal state and sexual maturity, where levels are generally very low in immature (or pre-pubertal) bottlenose dolphins, and then begin to rise at the onset of sexual maturity and ovulatory activity (Sawyer-Steffan et al., 1983). *T. truncatus* serum progesterone at sustained levels above 3,000 pg mL^{-1} is indicative of pregnancy, whereas ovulation seems to correlate with progesterone values > 3,000 pg mL^{-1} that return to < 1,000 pg mL^{-1} within one month (Sawyer-Steffan et al., 1983). These concentrations are significantly higher than baseline levels for non-

cycling females (Sawyer-Steffan and Kirby, 1980; Robeck et al., 1994). A more recent study used urinary luteinizing hormone and estrogen conjugate (EC) concentrations to correlate follicular growth and ovulation, which has helped define female *T. truncatus* reproductive endocrinology (Robeck et al., 2005).

Bottlenose dolphins are spontaneous ovulators (Kirby and Ridgway, 1984; Robeck et al., 1994) that breed seasonally in the Northwest Atlantic, with peak conception months during spring to June and peak calving between March and June (Mead and Potter, 1990). A second calving peak has been documented in late summer to early fall (Scott et al., 1990). The gestation period is ~12 months, lactation duration is ~1.6 years, and females typically produce a calf every 3-6 years (Reynolds et al., 2000). Reproduction can continue into the late 40s. Hormone analysis has demonstrated that estrous cycles last between 21-42 days (Kirby and Ridgway, 1984; Schroeder, 1990) and considerable variation has been observed in the number of estrous cycles per year (between 2-7 ovulations; Yoshioka et al., 1986). *T. truncatus* show evidence of seasonal reproductive activity, including polyestrus, seasonal polyestrus, and one to two year periods of anestrus (Cornell et al., 1977; Kirby and Ridgway, 1984; Schroeder, 1990).

T. truncatus generally exhibit a promiscuous mating system, where mating strategies and behavior can include the formation of male alliances and male-male competition for females (indicated by scarred bodies and rakes), which is characteristic of a polygynous system (Scott et al., 1990). In addition, the moderately large testicular size (relative to body size) of bottlenose dolphins, compared to some delphinids, likely indicates sperm competition, which occurs when multiple males compete for access to a single estrous female (Kenagy and Trombulak, 1986; Reynolds et al., 2000). Studies of

captive *T. truncatus* indicate seasonal variations in serum testosterone concentrations, total sperm per ejaculate, and sperm density, which reflect seasonal testicular activity (Schroeder and Keller, 1989). Sexually mature males produced testosterone levels between 2-5 ng mL^{-1} and increased up to 10 ng mL^{-1} during the breeding season. Pubescent individuals show fluctuating levels, between 1-10 ng mL^{-1}, whereas immature males had concentrations < 1 ng mL^{-1} (Kirby, 1990).

Endocrinology Techniques

The field of endocrinology has progressed as technological advancements have enabled accurate and consistent assessment of hormones in a variety of biological matrices (serum, saliva, urine, feces, and blubber; Atkinson and Yoshioka, 2007). Initially, a sensitive competitive protein-binding assay was used to measure serum testosterone levels (Harrison and Ridgway, 1971), but this was replaced by the more specific radioimmunoassay (RIA). In addition to RIA, enzyme immunoassays (EIA) are frequently used to measure reproductive hormones. Both are binding assays that depend on the progressive saturation of a specific antibody by a substance and the subsequent determination of bound and free phases using antibodies labeled with either a radioactive isotope (RIA) or an enzyme (EIA; Chard, 1990).

In some cases, RIAs can produce misleading results, as they measure not only dimeric forms of a hormone, but also the free subunits (Buzzard et al., 2004). Improvements in EIA techniques have resulted in highly specific and sensitive ELISAs (Enzyme-Linked ImmunoSorbent Assay) that utilize monoclonal antibodies and allow for quantitative measurement of biologically active peptide hormones, such as AMH, inhibin

A, and inhibin B in serum of individual mammals for both sexes. These assays demonstrate minimal cross-reactivity and enable the study of hormone secretion, without the limited specificity observed in other assays.

These methods are practical for assessing hormone levels in captive species, where animals are readily accessible and sampling can occur frequently. However, due to its invasive nature, blood collection is not generally performed on a daily basis, which limits detailed analyses of daily hormone fluctuations. Although assessment of reproductive hormone levels is possible in wild populations, it is logistically challenging and requires full restraint of the animal for blood collection.

Advantages of AMH and Inhibins

Testosterone, progesterone, estrogens (mainly estradiol-17ß [E2]) and, to some extent, gonadotropins have long been measured in captive cetaceans to assess reproductive condition, mostly for systematically planned breeding programs (Robeck et al., 1994). Hormone assays have been used to assess breeding condition, reproductive status, pregnancy rates, and to differentiate age groups. Testosterone levels have been correlated with testicular weight, spermatocyte density, germ cell density, and used to determine maturity status (Kirby and Ridgway, 1971; Kita et al., 1999). In females, progesterone levels have been monitored in captive delphinids to assess ovulation and pregnancy (Sawyer-Steffan et al., 1983) and also to establish the onset of sexual maturity in free-ranging animals (Atkinson and Yoshioka, 2007). Estrogens are generally measured to indicate the follicular phase of the estrous cycle, and gonadotropins (follicle stimulating hormone and luteinizing hormone) are typically measured to assist with

advanced reproductive technologies (ART) and the development of artificial reproductive management (Robeck et al., 1993).

Measurement of these reproductive hormones in cetaceans has increased our understanding of their reproductive physiology and endocrinology, and provided the capacity to assess reproductive state. However, many of the aforementioned hormones exhibit large variations during reproductive cycles and breeding seasons. Testosterone concentrations, for example, exhibit frequent fluctuations, vary among individual cetaceans with similar testicular size (Kita et al., 1999), and do not appear to correlate with peak sperm production and density in bottlenose dolphins (Schroeder and Keller, 1989). In addition, pre-ovulatory estrogen elevations may be variable, typically rising for days during the follicular phase, but may rise for only a few hours (Schroeder, 1990).

Three hormones, among many, yet to be measured in *T. truncatus* include AMH, inhibin A, and inhibin B, which are structurally-related dimeric glycoproteins that belong to the transforming growth factor-ß (TGF-ß) superfamily (Cate et al., 1986; Kingsley, 1994) and are chiefly expressed by the testicular Sertoli cells in males (Figure 2) and the ovarian granulosa cells in females (Figure 3). Initially studied to understand their role in reproduction and development in humans (Welt et al., 2002; Knight and Glister, 2006), these peptide hormones have also been investigated in several terrestrial mammalian taxa (such as rodents [Buzzard et al., 2004; Kevenaar et al., 2006], bovine [Ireland et al., 2008], ovine [Campbell and Baird, 2001], etc.) to further promote understanding of expression and function. The utility of measuring these hormones in a comprehensive evaluation of the conservation status of protected mammalian species has been considered (Marsh et al., 2011; Wilson et al., 2011), but seldom employed.

AMH expression is closely regulated developmentally in fetal, neonatal, prepubertal and adult Sertoli and granulosa cells, is conserved among mammalian species, and seems to be produced only in the reproductive organs (Teixeira et al., 2001). AMH is an appealing measure of gonadal function, since secretion is not dependent on gonadotropins, which are involved in follicular development and atresia, and AMH levels experience minor fluctuations during a normal menstrual cycle (Cook et al., 2000). In addition, AMH serum levels may be a more strongly related to ovarian follicular status and be a more discriminatory marker of ART outcome than FSH or estradiol (Fréour et al., 2007). Inhibins were first isolated from follicular fluid as a classical endocrine hormone by Robertson et al. (1985) and are known to regulate the reproductive system by acting on the anterior pituitary gland to block the synthesis of FSH via a negative feedback loop (de Kretser & Robertson, 1989; Figure 1). Therefore, inhibin decisively influences the regulation of gonadal function.

Anti-Müllerian Hormone

AMH is a homodimeric disulfide-linked molecule composed of two 72 kDa monomer subunits. Secretion of AMH from testicular Sertoli cells during embryonic development promotes involution of the Müllerian ducts, thus enabling normal development and differentiation of the male reproductive tract. Müllerian ducts are the primordium for the uterus, Fallopian tubes, and upper portions of the vagina (Lee and Donahoe, 1993).

The role of AMH secretion in male adults is not fully understood, but previous research indicates that seminal AMH concentrations may be a proper marker of the

function and maturation of Sertoli cells and, therefore, an indicator of spermatogenesis (Fujisawa et al., 2002; Sinisi et al., 2008). Alteration in Sertoli cell function can possibly lead to impaired spermatogenesis, germ cell losses, and male infertility (Monsees et al., 2000). AMH expression and secretion is regulated by inhibitory actions of testosterone, germ cells, and by FSH stimulation (Josso et al., 1990; Lee et al., 1996; Rajpert-De Meyts et al., 1999).

In males, AMH production commences during embryogenesis and continues well into adulthood. Levels are elevated in prepubertal individuals, and then slowly decline until puberty, when lower concentrations are maintained throughout adulthood (Lee et al., 1996). The high pre-puberty AMH levels correspond with the onset of testicular differentiation until puberty, at which point high testosterone levels take over (Josso et al., 1993; Rey et al., 1993). In several species, AMH expression decreases to trace amounts when Sertoli cells reach maturity (Kuroda et al., 1990; Lee et al. 1994), and an inverse relationship is observed between postneonatal serum AMH and testosterone levels (Josso et al., 1993; Rey et al., 1993; Lee et al., 1996).

AMH is produced by the ovarian granulosa cells of early developing follicles in females (Figure 3), can inhibit the initiation of primordial follicle growth, and is considered a negative regulator of follicular development (La Marca and Volpe, 2006). AMH is expressed throughout folliculogenesis, from primary to early antral stages; thus, serum levels may represent both the quantity and quality of the ovarian follicle pool (La Marca and Volpe, 2006). The mechanisms and precise function of ovarian AMH expression are still unclear, but circulating levels should reflect the volume of immature

granulosa cells in the ovaries and the combined number of growing follicles (Andersen et al., 2010).

Ovarian AMH expression has been detected as early as 36 weeks gestation in human fetal ovaries, though AMH serum levels in females can be nearly undetectable at birth (Rajpert-De Meyts et al., 1999). AMH is secreted in females from birth up to menopause, and a slight increase might occur from 2-4 years of age. Relatively low levels are maintained from puberty until menopause, when AMH decreases in correlation with a decline in the ovarian follicle pool (Hudson et al., 1990; Lee et al., 1996). Previous studies have utilized AMH as a tool for examining ovarian toxicity (Uzumcu et al., 2006) and ovarian reserve (Kwee et al., 2008).

Inhibins

Inhibins are heterodimeric protein hormones that consist of two covalently, disulfide linked α (18 kDa) and ß (14 kDa) subunits. The ß subunit exists in two forms, $ß_A$ and $ß_B$, where inhibin A consists of an α-subunit and $ß_A$ subunit, and inhibin B comprises an α-subunit and $ß_B$ subunit (Miyamoto et al., 1985; Robertson et al., 1985). Several inactive α subunits circulate as inert monomers, but only the dimeric forms of inhibin are biologically active (Burger, 1993). Primarily, inhibins regulate gametogenesis by inhibiting the production and/or secretion of follicle stimulating hormone from the anterior pituitary. An understanding of the mechanisms behind inhibin signaling is limited, but inhibin may act by suppressing activin action, which stimulates rather than inhibits FSH secretion (Ling et al., 1986).

Using specific assays that detect only dimeric inhibins, Anawalt et al. (1996) and Illingworth et al. (1996) demonstrated that inhibin B is the major circulating and most physiologically relevant inhibin in males; inhibin A has been undetectable in plasma of normal men. Previous research suggests inhibin B as a potential marker for spermatogenesis and testicular function (Anawalt et al., 1996; Klingmüller and Haidl, 1997; Kumanov et al., 2006), and inhibin B concentrations may reflect a degree of spermatogenic suppression or damage (Andersen et al., 1997). The negative feedback regulation by inhibin B on FSH appears to coincide with puberty and the maturation of the hypothalamic-pituitary-testis axis (Stewart and Turner, 2005), and an inverse relationship generally exists between circulating inhibin B and FSH levels in both normal men and those with varying degrees of testicular dysfunction (Anawalt et al., 1996; Illingworth et al., 1996). Other studies have shown that post-pubertal inhibin B production is directly proportional to the "amount" of spermatogenic activity and is correlated to sperm concentration (Pierik et al., 1998).

Profiles of circulating inhibin B levels in male humans are low but detectable throughout childhood, until the onset of puberty when inhibin B increases to relatively stable adult concentrations (Andersson et al., 1997; Byrd et al., 1998). Several studies have described a relationship between inhibin B peaks and the proliferation and number of Sertoli cells in both humans and rats (Cortes et al., 1987; Sharpe et al., 1999; Buzzard et al., 2004), and the stimulatory effect of FSH on inhibin B is the predominant control when Sertoli cells proliferate (Cortes et al., 1987).

Similar to males, inhibin in females is regulated by FSH stimulation, which induces proliferation and differentiation of granulosa cells from mammalian follicles, but

the role of inhibin secretion in FSH regulation and follicular development has yet to be clarified (Welt et al., 2002). Both dimeric inhibins are produced and detectable in female serum and exhibit unique patterns during the menstrual cycle. Inhibin B increases during the luteal-follicular transition, with peaks in the mid-follicular phase and again at ovulation, whereas inhibin A is synthesized during the late follicular phase, peaking during mid-luteal phase (Groome et al., 1994, 1996; Welt et al., 1997). The dominant follicle (during estrus) suppresses the development of neighboring small follicles by increased secretion of inhibin into blood vessels (Gibbons et al., 1997). The use of both inhibins as markers of follicular development is a well-accepted and established practice.

Toxicant Interactions and Biomarkers

Endocrine disrupting compounds (including PCBs and OCPs) may damage the health of wildlife by interacting with the endocrine system and are known to be estrogenic. Endocrine disruptors can mimic steroid sex hormones by binding to hormone receptors, influencing cell pathways, or by blocking hormonal binding to receptors, therefore affecting reproductive function (Fossi and Marsili, 2003). So, how might AMH and inhibin secretion by the Sertoli cells and ovarian granulosa cells be affected by a toxicant?

As previously discussed, Sertoli cells play a crucial role in spermatogenesis, and their proliferation is mostly stimulated by FSH. Exposure to xenoestrogens can lead to FSH suppression and, therefore, reduce the rate of Sertoli cell proliferation (Sharpe et al., 1995). Various toxicants are known to affect Sertoli cells, including chemotherapeutics (Wallace et al., 1989), plasticizers (Thomas et al., 1979), and pesticides, such as DDT

(Hodgson and Levi, 1996). Toxicants may cause Sertoli cell disruption by altering germ cell attachment or disturbing apical cytoskeletal transport, which can ultimately lead to germ cell loss and disruption of seminiferous epithelium (Monsees et al., 2000). It is also known that toxin exposure to antimetabolites can inflict morphological changes in Sertoli cells and cause direct cytotoxicity of germ cells during spermatogenesis (Takizawa and Horii, 2002). On the other hand, endocrine disruptors have been shown to affect adult ovarian morphology, female fertility, and inhibit folliculogenesis (Uzumcu et al., 2006). Exposure to chlorinated pesticides has caused irregular estrous cycle, inhibited ovarian development, and reduced fecundity in rats (Chapin et al., 1997).

The influences of anthropogenic stressors have been difficult to assess in marine mammals, given the complex logistics and legal limitations imposed on experimental studies for protected species. Diagnostic biomarkers, substances used to indicate normal biologic processes, can be used to suggest anthropogenic impacts and are proposed as a prospective and developing field of study to complement more conventional ecologically-based monitoring methods. Many biomarker and bioassay responses are readily quantifiable and can form a basis of long-term observational series to determine change over time (Langston et al., 2007). In addition, biomarkers strengthen our ability to detect the effects of various environmental stressors on the cellular and molecular processes that regulate organismal health (Reddy et al., 2001) and, ideally, allow for a correlation between causes and effects.

Baseline studies are becoming increasingly important in view of the potential effects that a number of environmental pollutants might have on the endocrine system of marine mammals. It is important to understand whether reproduction is being impaired,

especially for endangered and protected taxa. Policy makers and managers need convincing evidence regarding the possible subeffects of environmental and anthropogenic stressors on fertility, so effective steps can be taken towards mitigating the potentially harmful stressors (O'Hara and O'Shea, 2005). The utility of enhanced knowledge of reproductive capacity for assessing population status may lead to significant conservation applications.

OBJECTIVES AND STUDY DESIGN

The specific objectives for the research were to:

1. Determine whether AMH, inhibin A, and inhibin B are detectable in bottlenose dolphin serum using ELISA techniques,
2. Describe AMH and inhibin concentrations in free-ranging dolphins and establish baselines for individuals of different gender, age, and maturity status,
3. Investigate whether AMH and/or inhibins can be utilized as reproductive biomarkers in bottlenose dolphins,
4. Examine how hormone trends might align with current knowledge of bottlenose dolphin reproductive biology, and
5. Explore the possibility of utilizing these hormones as a biomarker for the impacts of toxicants or other stressors on reproduction in dolphins through a comparison of populations that experience different stress levels.

This research implemented a comparative study design, which included three bottlenose populations that may be affected by stressors, including contaminant burdens, associated with different geographic locations: Sarasota Bay, FL, Indian River Lagoon, FL, and southern Georgia. Variations in POP patterns have been observed in the populations assessed in this study (see detailed description in Approach and Methods). Dolphins in our estuarine study sites exhibit high site fidelity as indicated by long-term photo-identification data (Scott et al., 1990; Wells and Scott, 1990; Mazzoil et al., 2005;

Balmer et al., 2011) and, therefore, contaminant body burdens in these animals should reflect the local habitat. This research aimed to measure fertility hormone concentrations in serum from free-ranging bottlenose dolphins individuals of different gender, age, and maturity status during summer months (May-August) and peak breeding season, which presented a control for potential seasonal hormonal changes and even seasonal contaminant fluctuations (Kucklick et al., 2011). This study represents the first report of AMH, inhibin A, and inhibin B detection in cetaceans and, in particular, the bottlenose dolphin. The specific research questions for the study included:

1. What are the relationships between AMH, inhibin A, and inhibin B hormone levels and gender, age, and body size? Do hormone levels align with known reproductive biology and endocrinology of *T. truncatus*?
2. Are there population differences in AMH and inhibin concentrations?
3. Can any inferences be made about toxicant effects on hormone concentrations?

APPROACH AND METHODS

Study Design and Populations

Three bottlenose dolphin populations were targeted based on overall health condition and pollutant exposure: Sarasota Bay, FL (SRQ), Indian River Lagoon, FL (IRL), and the southern Georgia area (SGA). Blood, blubber, and other biological samples were collected from free-ranging dolphins during annual health assessment studies being conducted by the Chicago Zoological Society's Sarasota Dolphin Research Program (SDRP, NMFS Permit No. 522-1785), the Bottlenose Dolphin Health and Environmental Risk Assessment (HERA) Program (NMFS Permit No. 14352-01), and NOAA-sponsored dolphin health assessments (NMFS Permit No. 932-1905). These multi-disciplinary programs aim to assess the population status, health, and long-term viability of dolphins residing in the associated coastal areas, while investigating associations between dolphin health and stressors (Wells et al., 2004; Fair et al., 2006). Sampling was conducted during the summer months and corresponded with the previously mentioned peak breeding and calving seasons for bottlenose dolphins in the Northwest Atlantic and the Gulf of Mexico.

Sarasota Bay

Sarasota Bay (27˚ N, 82˚ W), a high salinity estuary, extends ~40 km along the west central coast of Florida and receives limited freshwater inflow. Stretches of barrier islands separate the estuary from the Gulf of Mexico (Figure 4). In 1989, the US

Congress named Sarasota Bay an estuary of national significance (Sarasota Bay National Estuary Program, 1995). The coastline in this region is densely populated, with medium to high suburban development and documented pollution stress (Sarasota Bay National Estuary Program, 1992; Sherblom et al., 1995; Lipp et al., 2001).

The Sarasota Bay bottlenose dolphin community, likely the most extensively studied cetacean population in the world (over 40 years), is composed of ~160 identifiable individuals from five generations of resident animals, many of known gender, age, reproductive status, and birth order (Scott et al., 1990; 2011, R. Wells, personal communication). The Sarasota Dolphin Research Program has performed physical examinations, obtained body condition measurements, and collected blood samples since the mid-1980s (Wells et al., 2004). The Sarasota Bay dolphins are considered to represent a healthy and stable population, with relatively low contaminant concentrations, in comparison to populations of the same species in other locations (Kucklick et al., 2011), but the animals do experience occasional threats from disease and red tides (Scott et al., 1990; Wells et al., 2005).

All contaminant concentrations referenced in this section were made on the basis of extractable lipids, i.e., lipid weight. PCB concentrations, the predominant contaminant class measured in dolphin blubber samples, in Sarasota Bay dolphins fall toward the lower end of the documented range, compared to other populations in the U.S. Atlantic and Gulf of Mexico (Kucklick et al., 2011). Yet, many individuals still exceeded the established threshold PCB level known to cause adverse health effects in some mammalian species (17,000 ng g^{-1}; Kannan et al., 2000). Yordy et al. (2010) documented $\sum_{61}$PCB concentrations (sum of 61 PCB congeners or congener pairs) in Sarasota Bay

adult males, adult females and juveniles at 61,300, 3,420 and 33,900 ng g^{-1}, respectively. Chlordane ($\sum_5$CHL; sum of five chlordanes) levels were 21,200, 896, and 11,400 ng g^{-1} in adult males, adult females and juveniles, respectively. DDT ($\sum_6$DDT; sum of six DDTs) concentrations were 29,600, 952, and 16,600 ng g^{-1} in adult males, adult females, and in juveniles, respectively (Yordy et al., 2010).

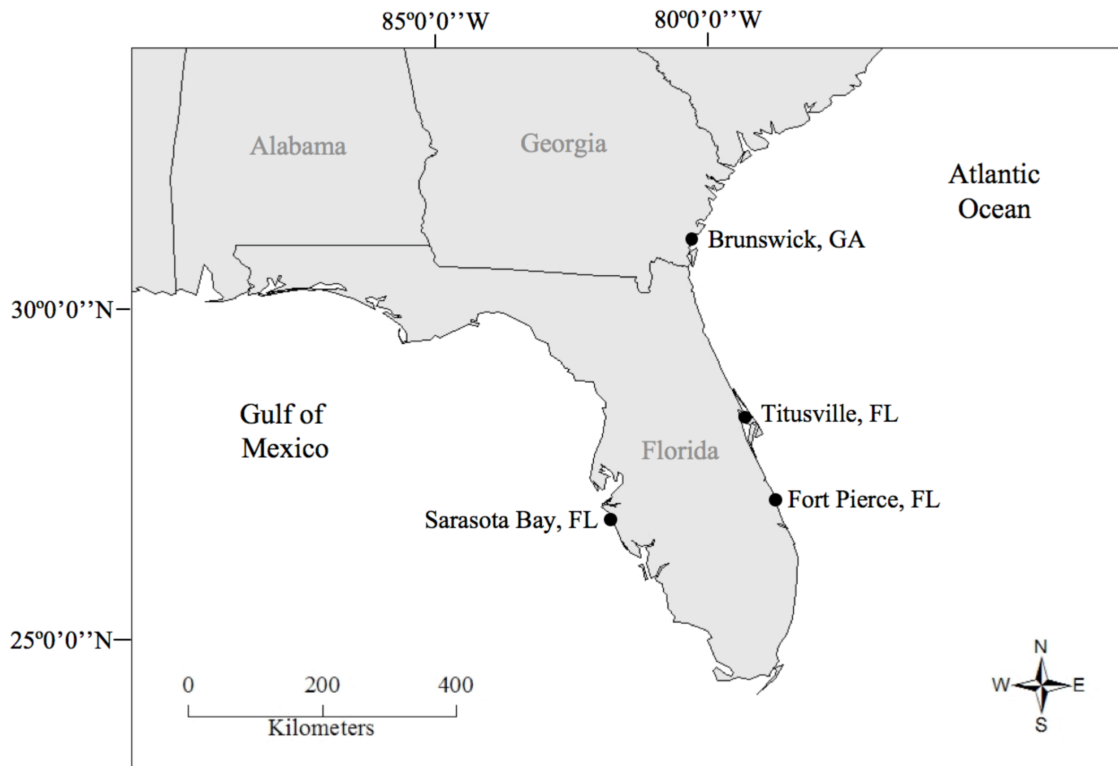

FIGURE 4. Study sites in Sarasota Bay, FL, Indian River Lagoon, FL (between Titusville and Fort Pierce, FL), and southern Georgia (near Brunswick, GA).

Indian River Lagoon

The Indian River Lagoon system is a 250 km linear estuary located on the east central coast of Florida (Figure 4) and is composed of four distinct water bodies: Mosquito Lagoon, Indian River, Banana River, and the St. Lucie Estuary. The shallow lagoon, connected to the Atlantic Ocean through five inlets and the Canaveral Lock, is

located near a rapidly growing urban and agricultural area. The Indian River Lagoon was designated an estuary of national significance in 1990 and is the most biodiverse estuary in North America (Indian River Lagoon National Estuary Program, 2007). Limited tidal exchange and slow flushing rates in the northern/central section create habitats that are susceptible to pollutant loading (Sigua et al., 2000). Freshwater releases from Lake Okeechobee and runoff from agriculture watersheds affect water quality in the southern section (Sime, 2005) and introduce nutrients, metals, and pesticides (Woodward-Cycle Consultants, 1994).

The Health and Environmental Risk Assessment Program initiated in 2003, and the dolphin health assessments operate from two field sites: Titusville, FL (28˚ N, 80˚ W) in the northern Indian River Lagoon and Fort Pierce, FL (27˚ N, 80˚ W) in the south. Dolphins in the Indian River are experiencing health problems including, but not limited to, morbillivirus infection (Bossart et al., 2010), lobomycosis, biotoxins, and acute gastric inflammation (Bossart, 2006). The population size is approximately 1000 (Bossart et al., 2010).

Previous research by Fair et al. (2010) reported that many IRL adult male dolphins generally exceeded the established threshold PCB level known to cause adverse health effects (17,000 ng g^{-1}; Kannan et al., 2000) by a 5-fold order of magnitude. $\sum_{73}$PCB concentrations were reported at 79,800 ng g^{-1}, and the highest adult male concentration was 227,000 ng g^{-1} (Fair et al., 2010). As expected, adult females and juveniles harbored lower PCB levels than adult males: 25,500 and 48,400 ng g^{-1}, respectively. Chlordane ($\sum_{6}$CHL) and DDT ($\sum_{6}$DDT) levels were 7,660 and 18,600 ng

g^{-1} in adult males, 1,490 and 4,600 ng g^{-1} in adult females, and 3,800 and 10,900 ng g^{-1} in juveniles (Fair et al., 2010).

Southern Georgia

The southern Georgia study area, comprising ~60 km of estuarine shoreline, ranges from Sapelo Sound southward to St. Simons Sound (31˚ N, 81˚ W; Figure 4). The Georgia study area was divided into two sampling sites; (1) the Brunswick site, which included the Turtle/Brunswick River Estuary (TBRE) and all estuarine waters from St. Simons Sound north to Altamaha Sound, and (2) the Sapelo field site which encompassed waters near the Sapelo Island National Estuarine Research Reserve (NERR).

The Turtle/Brunswick River Estuary is known for contamination by highly chlorinated (octa- through deca-chlorobiphenyl) PCBs associated with a rare mixture known as Aroclor 1268 (Kannan et al., 1997; Maruya and Lee, 1998). The contamination in this estuary resulted from the use of Aroclor 1268 by a chlor-alkali plant in Brunswick, GA (USA) from 1955 to 1994, which led to its designation of a National Priority List site (i.e., Superfund) in 1996 (Kannan et al., 1997). Extremely high PCB concentrations, in some cases 2,900 ppm (mg kg^{-1} lipid weight), have been documented in bottlenose dolphins along Georgia's southern coast (Pulster et al., 2009; Balmer et al., 2011).

A recent study by Balmer et al. (2011) measured POP concentrations for the same Georgia dolphins captured and sampled for our research. The study compared concentrations in Brunswick and Sapelo dolphins separately, as the Turtle/Brunswick River Estuary area is known for specific contamination of Aroclor 1268. $\sum_{54}$PCB concentrations in Brunswick males (509,960 ng g^{-1}) were significantly higher than Sapelo

males (115,730 ng g^{-1}), and the proportion of Aroclor 1268 was relatively high at both sites (77%, Brunswick; 60%, Sapelo). Female body burdens were generally lower, with $\sum_{54}$PCB levels of 116,470 ng g^{-1} in Brunswick and 48,270 ng g^{-1} in Sapelo. $\sum_{6}$CHL in males was 6,300 and 3,830 ng g^{-1}, and $\sum_{5}$DDT was 36,770 and 20,490 ng g^{-1} in Brunswick and Sapelo, respectively. $\sum_{6}$CHL in females was 630 and 1,310 ng g^{-1}, and $\sum_{5}$DDT was 52,450 and 10,030 ng g^{-1} in Brunswick and Sapelo, respectively. In addition to contaminant exposure, the southern Georgia dolphins suffer from anemia, reduced thyroid hormone levels, and increased susceptibility to infectious disease (demonstrated by decreased T-lymphocyte indices of innate immunity; Schwacke et al., 2011).

Sample Collection

Techniques used for the dolphin health assessments are described by Wells et al. (2004), Fair et al. (2006), and Schwacke et al. (2011). Briefly, small groups of dolphins were encircled with a seine net in shallow water. Within the first 10 min of initial restraint in the water, blood was collected by venipuncture of the ventral fluke vessels with a 19-gauge, 1.9 cm, butterfly catheter into either Vacutainer Serum Separator Tubes (Sarasota Bay and Georgia) or green-top lithium heparin Vacutainer tubes (Indian River). The dolphins were then transported to a processing vessel for veterinary examination and further sample collection. Either serum or lithium heparin plasma are recommended for the assays performed (Beckman Coulter, 2008). In the field, blood samples were placed on ice immediately after collection. Upon return to the laboratory, blood tubes were centrifuged (5 min at 3,000 rpm), and serum was transferred to sterile cryogenic vials and

frozen at -80º C until analyzed. Prior to analysis, samples were vortexed and divided into aliquots (~300 µL) to avoid repeated freezing and thawing of serum.

Sterile, solvent-rinsed instruments were used to surgically remove a full depth blubber biopsy (3 cm x 5 cm x 2 cm) from a site approximately 10 cm caudal to the dorsal fin and 10 cm ventral to the dorsal ridge. Lidocaine hydrochloride and epinephrine were administered for local anesthesia, and the site was pre-cleaned with a chlorhexiderm then methanol scrub, followed by a methanol rinse. The blubber wedge was sub-sampled for various studies, then placed in Teflon vials and stored in liquid nitrogen until frozen at -80º C.

Age Class Determination

For selected individuals of unknown age, a single tooth was extracted under local anesthesia, and Dolphin Health Assessment Programs determined age through examination of growth layer groups in teeth (Hohn et al., 1989). Generally, bottlenose dolphins are considered sexually mature between 5-12 years or >220 cm for females and 10-13 years for males or >240 cm (Mead and Potter, 1990; Wells and Scott, 1999; McFee and Hopkins-Murphy, 2002).

When available, age was used primarily to categorize juveniles and adults; body length was utilized as a secondary index of sexual maturity. It is important to note that onset of sexual maturity in bottlenose dolphins can vary among populations in different geographic locations, but for the purposes of this study, females >8 years or ≥220 cm and males >10 years or ≥240 cm were classified as adults.

ELISA Analysis

AMH, inhibin A, and inhibin B concentrations were measured in serum and plasma with an automated ELISA instrument (Dynex DS2, Dynex Technologies, Chantilly, VA). The ELISAs performed were enzymatically amplified multi-site sandwich assays (Beckman Coulter, Inc., Brea, CA) and were programmed individually in the DS-Matrix software (Dynex Technologies) according to the manufacturer's instructions. In all assays, calibrators, controls, and samples were run in duplicate and accepted precision (expressed as coefficient of variation [%CV]) was <15%. All incubations were performed with linear microplate shaking (600 - 800 rpm) at ambient temperature (~25˚ C).

For the AMH assay, 20 µL of calibrators, low and high controls, and serum samples were incubated with 100 µL of the AMH Gen II assay buffer in microtitration wells coated with anti-AMH IgG antibody. Seven calibrators with AMH concentrations of approximately 0, 0.16, 0.4, 1.2, 4.0, 10, and 22.5 ng mL^{-1} were utilized. Following the first incubation (60 min), a five-cycle washing step was used. Each cycle comprised an aspiration and wash with 400 µL of a wash solution, followed by a final aspiration. Next, the wells were treated with an anti-AMH detection antibody (100 µL) labeled with biotin. After a second incubation (60 min) and washing cycle (as described above), the wells were incubated with 100 µL of streptavidin-horseradish peroxidase (HRP). A third incubation (30 min) and washing step were employed, and then 100 µL of the substrate tetramethylbenzidine (TMB) was added to the wells. A short incubation (8 - 12 min) occurred, during which the colorimetric change was visually observed to optimize the

incubation time. Finally, an acidic stopping solution (100 µL; 0.2 M H_2SO_4) stopped the color formation.

The highly characterized dual monoclonal antibody pair used in the AMH assay bind only to the mature region of AMH and do not detect inhibin A or B, activin A, follicle stimulating hormone, or luteinizing hormone. The assay sensitivity, or limit of detection (LOD), for the AMH Gen II assay was 0.8 ng mL^{-1} (Beckman Coulter Inc., 2009).

Variations in the inhibin A method were as follows: 50 µL of calibrators, low and high controls, and serum samples were incubated with 50 µL of the inhibin A sample buffer A and 50 µL of the sample buffer B in microtitration wells coated with anti-inhibin $ß_A$ subunit antibody. Seven calibrators with inhibin A concentrations of 0, 9.5, 26, 92, 211, 423, and 781 pg mL^{-1} were utilized. Following an incubation (3 hr) and six-cycle washing step (with 350 µL of wash solution), the wells were treated with an anti-inhibin α-subunit detection antibody (100 µL) labeled with the enzyme HRP that was freshly diluted 10 min prior to use. After the second incubation (60 min) and washing, 100 µL of tetramethylbenzidine was dispensed into each well, followed by a third incubation (15 min) and washing cycle. Lastly, the stopping solution ended the colorimetric change.

The ACTIVE Inhibin A ELISA kit is highly specific for inhibin A. Minimal cross-reactivity was measured against other hormones: 1) Inhibin B at 1 µg mL^{-1} = 0.012% cross-reactivity; 2) Activin A at 1 µg mL^{-1} = 0.002%; and 3) Activin B at 1 µg mL^{-1} = 0.001%. The assay sensitivity was 1.0 pg mL^{-1} (Beckman Coulter Inc., 2008).

For the inhibin B assay, 50 µL of calibrators, low and high controls, and serum samples were incubated with 50 µL of the inhibin B Gen II assay buffer in microtitration

wells coated with anti-Activin B antibody. Six calibrators with inhibin B concentrations of approximately 0, 5, 10, 30, 100, and 250 pg mL^{-1} were utilized. Following a shaker-incubation (2 hr) and five-cycle washing step, the wells were incubated (60 min) with a biotinylated anti-inhibin α-subunit detection antibody (100 μL), which was freshly diluted 10 min prior to use. After the washing cycle, 100 μL of the streptavidin-enzyme conjugate was added to each well, followed by incubation (30 min) and washing. TMB (100 μL) was dispensed into each well, followed by a third incubation (8 - 12 min) and washing cycle. The colorimetric change was visually observed, and the stopping solution ended the reaction.

The Inhibin B Gen II ELISA kit used a highly characterized antibody pair that measured 100% inhibin B in human, monkey, and rat. Inhibin A, activin A, activin B, AMH, follicle stimulating hormone, and luteinizing hormone were tested as potential cross reactants and found non-detectable. The limit of detection was 2.6 pg mL^{-1} (Beckman Coulter Inc., 2010).

In all assays, the absorbance in each well was measured within five minutes of the reaction termination. The degree of enzymatic turnover of the substrate was determined on a plate reader by dual wavelength absorbance measurement at 450 nm (primary test filter) and 620 nm (primary reference filter). The absorbance measured was directly proportional to the concentration of hormone present. Calibrators were used to plot a log-log linear regression calibration curve of optical density versus hormone concentration. The absorbance measured was directly proportional to the hormone concentration present in the sera.

Upon arrival, calibrators and controls were divided into appropriate aliquots (~200 μL) and frozen at -20˚ C. To preserve stability, individual aliquots were thawed for use only once. Prior to assay initiation, all calibrators, controls, reagents, and samples were mixed by either gentle inversion or vortexed, then brought to room temperature. Due to high AMH concentrations in male *Tursiops*, samples were typically diluted 1:80, but in some cases, 1:100 or 1:120 dilutions were necessary. Trial dilutions performed at 1:20, 1:40, and 1:60 over-saturated the plate reader. In all assays, any sample reading above the highest calibrator was appropriately diluted and reassayed.

Reproducibility was determined for all three ELISAs using five *Tursiops* serum pools. These samples were run in duplicate in two assay runs on different days, using two reagent lots, and the precision was measured for between-run variability. The AMH inter-assay variability calculated on the five samples was 1.1, 7.4, 1.1, 1.3, and 2.9%. For inhibin A, the imprecision was 5.0, 6.6, 0.1, 2.4, and 1.8%, and for inhibin B, 5.7, 6.9, 4.2, 4.3, and 1.5%. The percent coefficient of variation was calculated as follows:

$$\%\text{Coefficient of Variation} = \frac{\text{Standard deviation of means of duplicates}}{\text{Grand mean of duplicates}} * 100$$

Contaminant Analysis

Organic pollutant analysis was performed at Mote Marine Laboratory in Sarasota, FL. Blubber samples were collected from 10 Sarasota Bay dolphins. Individual blubber samples (~1 g) were weighed, macerated, then homogenized with diatomaceous earth and added to a 66-mL stainless steel accelerated solvent extraction (ASE) cell along with the appropriate internal standards. Using pressurized fluid extraction (PFE), the samples were extracted with methylene chloride for three sequential cycles at 100° C and 2000 psi

on an ASE 300 (Dionex Corporation, Sunnyvale, CA, USA). The extracts (~80 mL) were reduced to ~1-2 mL using a RapidVap (Labconco Corp., Kansas City, MO, USA), then reduced to dryness under purified N_2 in pre-cleaned and pre-weighed scintillation vials. Lipid content was determined gravimetrically, and sample extracts were reconstituted in 5 mL of hexane. Lipid removal and sample clean up was performed on a multi-column clean up system (Automated Power-Prep System, Fluid Management Systems, Watertown, MA, USA). Targeted analytes were eluted with 50% CH_2Cl_2 / hexane (v/v) through a high capacity acidic silica column (30 g), an ABN (acidic basic neutral) silica column (11 g), and an alumina column (11 g) in tandem. The eluted fraction containing compounds of interest was collected and reduced to dryness under nitrogen; samples were reconstituted in 200 µL of hexane.

From this extract (1 µL injection volume), polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) were analyzed using gas chromatography/mass spectrometry (GC/MS; Agilent 7890A/5975C; Agilent Technologies, Inc., Andover, MA, USA). Analyte separation was achieved on a ZB-5MS capillary column (30 m x 0.25 µm film thickness x 0.25 mm i.d.; Phenomenex, Torrance, CA, USA) with ultrahigh-purity helium as the carrier gas (1 mL min^{-1}). PCBs were quantified in electron impact selective ion-monitoring mode (EI-SIM), and the injector (splitless mode) and transfer line temperatures were 280° C. The oven temperature program was as follows: 60° C (0.55 min hold), then 72.98° C/min increase to 110° C (1.1 min hold), and then 9.12°C min^{-1} increase to 325° C (9.87 min hold) for a total run time of 35.78 min. The source and quadrapole temperatures were 230° C and 150° C, respectively. PBDEs were identified in negative chemical ionization (NCI-SIM) mode with methane as the moderator gas.

The GC oven was programmed for PBDE detection as follows: 125 º C (1 min hold) and increased to 325º C at 17 ºC min^{-1} (10 min hold) for a total run time of 22.8 min. The source and quadrapole temperatures were 150° C.

Concentrations were calculated from the slope and y-intercept generated by a seven-point linear calibration curve. Since only 30 of the 60 PCB congeners were detected and quantified in the dolphin blubber, total PCB concentration (∑PCB) in blubber was represented as the sum of the 30 detectable congeners, which included: 28/31, 52, 66, 74, 85, 95, 99, 101, 105/152, 118, 128/167, 137, 138, 146, 149, 151, 153, 170, 174, 177, 178, 180, 183, 187, 194, 195, 196/203, 200, 206, and 209. Only five of the seven PBDE congeners were detected, therefore total PBDE concentration (∑PBDE) was represented as the sum of the five congeners: 47, 99, 100, 153, and 154.

A performance-based quality-assurance and quality control program, which included the parallel analysis of procedural blanks, matrix spikes, and Standard Reference Materials (SRM 1945; organics in whale blubber from the National Institute of Standards and Technology [NIST]), was implemented to ensure data of the highest quality. The GC response was monitored every 10 to 12 samples with product check standards. Procedural blanks were clear of targeted analytes. Individual and standard mixtures of PCBs and PBDEs were purchased from AccuStandard (New Haven, CT, USA). PBDE concentrations determined in SRM 1945 averaged within 10% (92 ± 12%) of the certified values. Mean recovery of dibromooctofluorobiphenyl (DBOFB) was 90 ± 7%, and mean recoveries for dolphin blubber spiked with 24 PCB congeners (PCB B) and 7 PBDE congeners (PDBE mix) were 97 ± 15% and 93 ± 7, respectively. Mean

recovery of BDE (brominated diphenyl ether) 71 and BDE 172 were 81 ± 11% and 62 ± 14%, respectively.

The method of detection limit (MDL) is defined as the mass of the analyte in the lowest detectable calibration solution multiplied by the extract volume and divided by the sample mass. All mass spectral data were compared to spectra produced by authentic standards and to previously published library spectra.

Statistical Analysis

Concentrations were expressed as ng mL^{-1} for AMH, pg mL^{-1} for inhibin A and inhibin B, and ng g^{-1} for contaminants. Shapiro-Wilk normality tests were performed, and data were log transformed when appropriate to meet assumptions of normality. For all tests, $\alpha = 0.05$. Statistical differences between hormone concentrations and gender, age class, and location were assessed by a series of Student's *t*-test or Mann-Whitney-Wilcoxon (for two categories) and by analysis of variance (ANOVA), Kruskall Wallis analysis of variance, or analysis of covariance (ANCOVA) for greater than two categories within and among populations. If significant, pairwise comparisons were performed using Tukey's Honestly Significant Difference (HSD) test. Linear regression analyses examined functional relationships between hormone levels and age and various morphometrics (i.e., body length, body weight, and maximum girth), and regression plots utilized least square regression equations. Given the error in x and y variables in this biological data set, the geometric mean and 95% confidence limits (CL) were calculated and reported for geometric mean regression equations (Ricker, 1973; Laws and Archie, 1981; McArdle, 1987). In addition, the predictive relationship between age and body

length were assessed. Data were analyzed using IBM SPSS software (version 19.0; SPSS, Inc., Chicago, IL).

RESULTS

Comparison of Sample Size, Age and Morphometrics

Serum samples were collected from 129 individual dolphins during spring and summer (May, June, and August) of 2009-2011 (41 SRQ; 52 IRL; 26 SGA). Ages were available for only 57% of the individuals sampled, and dolphin demographic and morphometric data are provided in Table 1. In the absence of age data, body length was used to infer individual maturity status (juvenile or adult). Student's *t*-tests indicated that there was a significant decreasing trend in adult body lengths of both sexes across populations, where Sarasota Bay > Indian River Lagoon > Georgia (Table 1).

TABLE 1. Sample demographics in bottlenose dolphins (*Tursiops truncatus*) from Sarasota Bay, Indian River Lagoon, and southern Georgia. Age, length, and weight values are means for each category (mean ± SD). Significant *p*-values ($p \leq 0.05$) of comparison of serum concentrations between locations are indicated in bold

Location	Category	*n*	Age (years)	Length (cm)	Weight (kg)
Sarasota Bay	Juvenile male	10	3.6 ± 1.8	208.9 ± 14.3	105.4 ± 25.5
	Adult male	9	18.9 ± 5.9	**261.2**[a] ± 8.1	231.6 ± 33.1
	Juvenile female	6	3.7 ± 1.9	207.2 ± 25.3	104.0 ± 35.7
	Adult female	16	18.0 ± 10.7	**242.9**[b] ± 12.0	167.7 ± 24.7
Indian River	Juvenile male	17	8.4 ± 2.0	212.3 ± 25.9	118.9 ± 21.2
	Adult male	19	19.0 ± 2.6	**257.4**[c] ± 14.4	183.9 ± 28.0
	Juvenile female	0	-	-	-
	Adult female	9	10.8 ± 0.4	239.0 ± 11.6	137.2 ± 21.0
Southern Georgia	Juvenile male	0	-	-	-
	Adult male	13	19.3 ± 6.6	**244.0**[a,c] ± 9.7	-
	Juvenile female	2	-	203.0 ± 21.2	-
	Adult female	11	24.3 ± 11.1	**230.4**[b] ± 11.6	-

[a] Student's *t*-test comparing Sarasota and Georgia adult male body lengths ($p < 0.001$)
[b] Student's *t*-test comparing Sarasota and Georgia adult female body lengths ($p = 0.01$)
[c] Student's *t*-test comparing Indian River and Georgia adult male body lengths ($p = 0.01$)

Linear regression analyses were performed to assess the predictive relationship between age and body length in males in all populations (Table 2). For females, regressions were performed only in Sarasota Bay, as the minimal age data available for females in Indian River Lagoon and Georgia restricted the sample size. All regression analyses resulted in statistically significant relationships between age and body length.

TABLE 2. Model II linear regression analyses of predictive relationships between age and body length in both sexes in Sarasota Bay, Indian River Lagoon, and southern Georgia. All relationships were statistically significant ($p \leq 0.002$)

Males	**Sarasota Bay**	**Indian River**	**Southern Georgia**
Regression Equation	y = 0.304x – 59.9	y = 0.237x – 42.9	y = 0.676x – 145.1
n	17	12	9
r^2	0.737	0.650	0.613
95% Confidence Limit	0.236 – 0.406	0.139 – 0.335	0.3 – 1.05
Females			
Regression Equation	y = 0.486x – 98.6	-	-
n	19	2	4
r^2	0.436	-	-
95% Confidence Limit	0.3 – 0.672	-	-

Anti-Müllerian Hormone

AMH levels were significantly different ($p < 0.001$) between males and females in all locations. In Sarasota Bay, AMH ranged from 513 - 2,080 ng mL^{-1} (1,341 ± 409 ng mL^{-1}; mean ± SD) in males (Table 3) and from 0 - 5.4 ng mL^{-1} (1.6 ± 1.5 ng mL^{-1}) in females (Table 5). The Indian River Lagoon ranges were 359 - 1,851 ng mL^{-1} (1,128 ± 370 ng mL^{-1}) and 0.18 - 2.64 (0.97 ± 0.86 ng mL^{-1}) in males and females, respectively, and the Georgia AMH concentrations ranged from 65.2 - 1,185 (805 ± 397 ng mL^{-1}) in males and from 0.9 - 1.35 (0.45 ± 0.33 ng mL^{-1}) in females.

Male AMH levels showed a decrease with age, and comparisons of juvenile and adult AMH concentrations were significantly different in Sarasota Bay ($p = 0.01$) and

Indian River ($p < 0.01$, Table 3). Linear regression analyses were performed to identify predictive relationships between AMH concentrations and age, body length, body weight, and maximum girth. In all populations, the regressions showed a significant relationship between male AMH levels and the selected morphometrics, whereby AMH exhibited a negative relationship with age (Figure 5), body length (Figure 6), body weight, and maximum girth (Table 4).

The coefficients of determination (r^2) were greatest for the relationships between AMH levels and age in all populations (Table 4). When comparing relationships between male AMH levels and age across populations, a separation of regression lines was not apparent (Figure 5), and the geometric mean regression slopes varied (Table 4). An ANCOVA was performed to look at differences in male AMH concentrations among populations after removing the variance of age, and no statistical difference was observed in the unbiased means among populations. Since body length is commonly used to infer maturity status, relationships between male AMH levels and body length were assessed across populations (Figure 6). A separation of Sarasota Bay and Indian River Lagoon regression lines was observed, and the slopes of these two populations differed greatly from Georgia (Table 4). ANCOVA results showed a significant difference in male AMH levels across populations while controlling for body length ($p < 0.01$). However, the estimated mean for the Georgia population did not include juveniles.

Since samples from Georgia included only adult males, an additional set of regressions was performed to assess the relationships between adult male AMH levels and either age or body length in all three populations. For age, the functional relationship was not significant in Sarasota Bay ($r^2 = 0.417$, $p > 0.05$, $n = 7$), but was significant in

Indian River ($r^2 = 0.756$, $p = 0.01$, $n = 8$) and Georgia ($r^2 = 0.926$, $p < 0.01$, $n = 7$, Figure 7). The regressions between adult male AMH and body length showed no significant relationships in Sarasota Bay and Indian River ($r^2 = 0.030$, $p > 0.05$ and $r^2 = 0.066$, $p > 0.05$, respectively), whereas the relationship in Georgia was significant and explained a greater percentage of the variation ($r^2 = 0.427$, $p < 0.01$, Figure 8). ANCOVA results showed no significant difference in male adult AMH levels across populations while controlling for body length ($p > 0.05$).

TABLE 3. Mean (range) serum anti-Müllerian hormone (AMH), inhibin B, and inhibin A concentrations in male bottlenose dolphins from Sarasota Bay, Indian River Lagoon, and southern Georgia. Significant *p*-values ($p \leq 0.05$) of comparison of serum concentrations between locations are indicated in bold

Location	**Category (Males)**	**AMH ($ng\ mL^{-1}$)**	**Inhibin B ($pg\ mL^{-1}$)**	**Inhibin A ($pg\ mL^{-1}$)**
Sarasota Bay	Juveniles	**1589**[a] (1122-2080)	7.1 (2.9-13.7)	**30**[c] (4.1-113)
	Adults	**1030**[a] (513-1397)	5.7 (4.0-6.9)	45 (7.7-84.1)
Indian River	Juveniles	**1399**[b] (875-1851)	10.7 (3.9-46.6)	**52.8**[c] (15.4-184)
	Adults	**850**[b] (359-1198)	8.6 (2.7-25.0)	**34.0**[d] (16.5-72.6)
Southern Georgia	Juveniles	-	-	-
	Adults	805 (65-1184)	17.4 (4.0-74.4)	**21.2**[d] (7.4-60)

[a] Student's *t*-test comparing Sarasota Bay juveniles and adults ($p = 0.01$)
[b] Student's *t*-test comparing Indian River juveniles and adults ($p = 0.00$)
[c] Mann-Whitney comparing Sarasota Bay and Indian River juveniles ($p = 0.02$)
[d] Mann-Whitney comparing Indian River and Georgia adult ($p = 0.02$)

TABLE 4. Model II linear regression analyses relating anti-Müllerian hormone (AMH) concentrations and selected morphometrics in male bottlenose dolphins. All relationships were statistically significant ($p \leq 0.02$)

AMH vs. Age	**Sarasota Bay**	**Indian River**	**Southern Georgia**
Regression Equation	y = -46.2x – 1827.6	y = -57.8x – 2034.9	y = -74.3x – 2393.5
n	17	12	7
r^2	0.604	0.683	0.926
95% Confidence Limit	-62.2 – -30.2	-80.7 – -34.9	-97.5 – -51.1
AMH vs. Body length			
Regression Equation	y = -14.1x – 4617.2	y = -13.6x – 4377.7	y = -49.9x – 13089.1
n	18	33	11
r^2	0.485	0.414	0.427
95% Confidence Limit	-19.5 – -8.76	-17.4 – -9.8	-78.4 – -21.4
AMH vs. Body weight			
Regression Equation	y = -5.86x – 2281.5	y = -8.81x –2471.5	-
n	18	29	-
r^2	0.517	0.51	-
95% Confidence Limit	-8.0 – -3.7	-11.2 – -6.4	-
AMH vs. Max girth			
Regression Equation	y = -19.9x – 3911.1	y = -33.7x – 5090.0	-
n	18	29	-
r^2	0.530	0.414	-
95% Confidence Limit	-27.1 – -12.66	-43.9 – -23.5	-

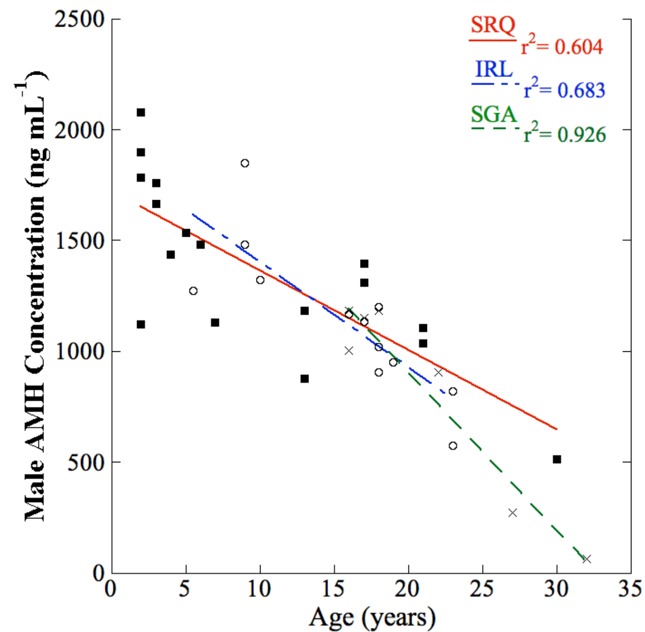

FIGURE 5. Relationship between anti-Müllerian hormone (AMH) and age in free-ranging male bottlenose dolphins (*Tursiops truncatus*) from all study populations. ■ = Sarasota Bay (SRQ), O = Indian River Lagoon (IRL), and X = Georgia (SGA).

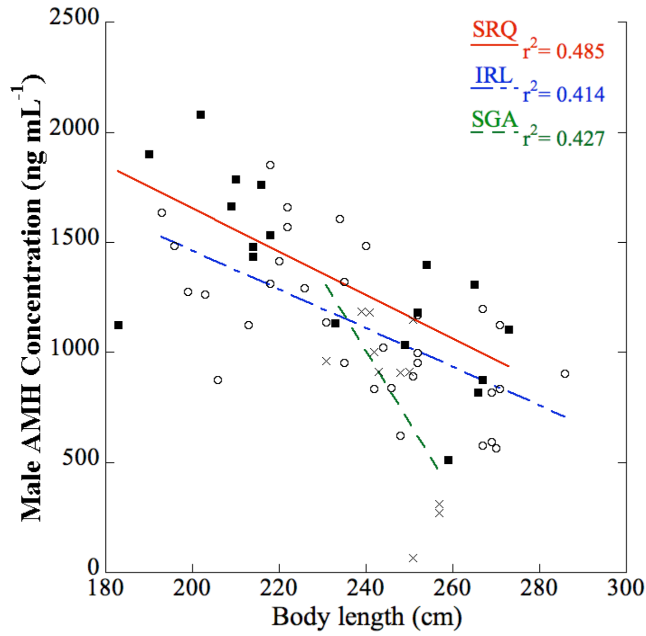

FIGURE 6. Relationships between anti-Mullerian hormone (AMH) and body length in male bottlenose dolphins from all study populations. ■ = Sarasota Bay (SRQ), ○ = Indian River Lagoon (IRL), and X = Georgia (SGA).

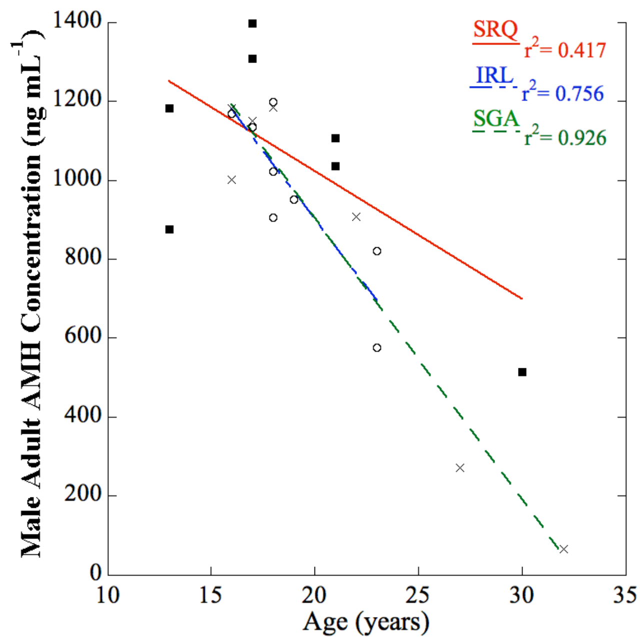

FIGURE 7. Relationships between anti-Müllerian hormone (AMH) and age in adult male bottlenose dolphins from all study populations. ■ = Sarasota Bay (SRQ), ○ = Indian River Lagoon (IRL), and X = Georgia (SGA).

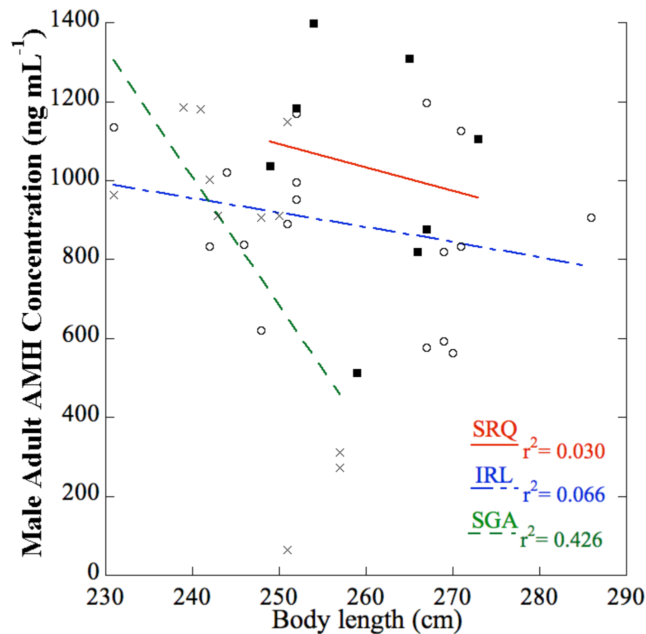

FIGURE 8. Relationships between anti-Müllerian hormone (AMH) and body length in adult male bottlenose dolphins in all study populations. ■ = Sarasota Bay (SRQ), O = Indian River Lagoon (IRL), and X = Georgia (SGA).

Mean AMH concentrations were compared among populations for both male juveniles and adults. The comparison between Sarasota Bay and Indian River juveniles showed no statistical difference ($p > 0.05$); no juveniles were available for comparison in Georgia. No statistically significant differences in the mean adult AMH concentrations were found among or between populations ($p > 0.05$).

The relationships between female AMH levels and certain morphometrics were not as clear. In Sarasota Bay, a significant relationship was observed between AMH and maximum girth ($p = 0.05$), but no significant relationships were observed between AMH and age, body length or body weight. In the other two populations, no significant relationship was found between AMH levels and any selected morphometric.

Unlike in males, female AMH concentrations were not significantly different between juveniles and adults in any population (Table 5). However, comparisons of the

different age classes among locations resulted in significant findings. Juvenile mean AMH concentrations in females were significantly different between Sarasota Bay and Georgia (p = 0.04); no juveniles were available for comparison in Indian River. In addition, a statistically significant difference was found in the mean adult female AMH concentrations among all populations (p = 0.04), and AMH was significantly different between Sarasota Bay and Georgia (p = 0.04, Figure 9).

TABLE 5. Mean (range) serum anti-Müllerian hormone (AMH), inhibin B, and inhibin A concentrations in female bottlenose dolphins from Sarasota Bay, Indian River Lagoon, and southern Georgia. Significant p-values ($p < 0.05$) are indicated in bold

Location	Category (Females)	AMH (ng mL^{-1})	Inhibin B (pg mL^{-1})	Inhibin A (pg mL^{-1})
Sarasota Bay	Juveniles	**2.01**[a] (0.6-2.9)	10.8 (5.5-19.2)	35.3 (8.5-128)
	Adults	**1.5**[b] (0-5.4)	**10.7**[c] (2.7-60)	27.8 (3.7-102)
Indian River	Juveniles	-	-	-
	Adults	1.04 (0.18-2.6)	**18.6**[c] (3.6-60.9)	26.5 (9.9-72.5)
Southern Georgia	Juveniles	**0.38**[a] (0.33-0.42)	8.3 (6.0-10.6)	34.1 (22.9-45.3)
	Adults	**0.46**[b] (0.09-1.35)	9.8 (4.7-26.0)	24.3 (7.9-44.9)

[a] Student's t-test comparing Sarasota Bay and Georgia juveniles (p = 0.04)
[b] Tukey's HSD pairwise comparison of Sarasota Bay and Georgia adults (p = 0.04)
[c] Mann-Whitney comparing Sarasota Bay and Indian River Lagoon adults (p = 0.03)

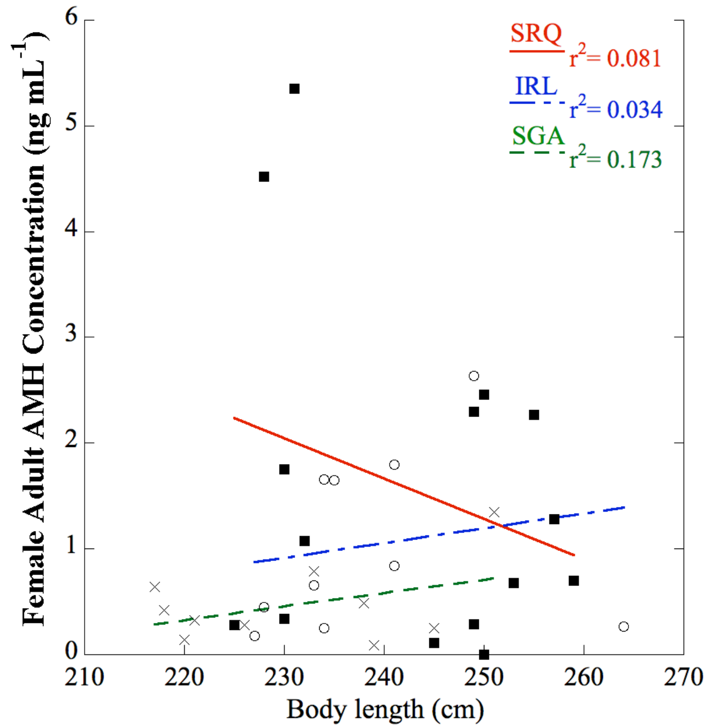

FIGURE 9. Plot of anti-Müllerian hormone (AMH) and body length in adult female bottlenose dolphins in all study populations. ■ = Sarasota Bay (SRQ), O = Indian River Lagoon (IRL), and X = Georgia (SGA). No relationships were statistically significant (*p* > 0.05).

Inhibins

Inhibin B levels between juveniles and adults were not significantly different within sexes in any location. In Sarasota Bay, inhibin B ranged from 2.9 -13.7 pg mL^{-1} (6.4 ± 3.0 pg mL^{-1}) in males and from 2.7 - 59.9 pg mL^{-1} (10.7 ± 12.7 pg mL^{-1}) in females. The Indian River ranges were 2.7 - 46.6 pg mL^{-1} (9.9 ± 8.9 pg mL^{-1}) and 2.6 - 60.9 pg mL^{-1} (15.6 ± 17.9 pg mL^{-1}) in males and females, respectively, and the Georgia inhibin B concentrations ranged from 4.0 - 74.4 pg mL^{-1} (17.4 ± 20.7 pg mL^{-1}) in males and from 4.7 - 26.0 pg mL^{-1} (9.6 ± 7.2 pg mL^{-1}) in females. In order to minimize skewing, outliers were not included in means or comparisons, as discussed in detail in the Discussion.

Comparison of the different age classes among sexes showed no significant difference in inhibin B in Sarasota Bay or Georgia, but adult male (8.6 pg mL^{-1}) and female (15.6 pg mL^{-1}) inhibin B concentrations were significantly different in Indian River (p = 0.03). Inhibin B means were then compared among populations for both sexes and age classes. No significant differences were observed among juveniles for either sex, and inhibin B levels in male adults were not statistically different among or between populations ($p > 0.05$). However, a Mann-Whitney-Wilcoxon test comparing females found significantly different mean inhibin B levels between Sarasota Bay and Indian River (p = 0.03, Table 5).

Inconsistent trends were observed from linear regression analysis used to define functional or predictive relationships between inhibin B concentrations and age, body length, body weight, and maximum girth. In males, no significant relationships were found between inhibin B levels and the selected morphometrics in any population (Figure 10, showing body length). Opposite, but non-significant, relationships were observed in females between inhibin B and body length, where the relationship was positive in Indian River and negative in Georgia (IRL: $r^2 = 0.378$, $p > 0.05$, $n = 9$; SGA: $r^2 = 0.216$, $p > 0.05$, $n = 13$).

Inhibin A means were higher than those for inhibin B when comparing similar sex and age classes (Tables 3 and 5). In Sarasota Bay, inhibin A ranged from 4.1 - 113.7 pg mL^{-1} (37.5 ± 34.9 pg mL^{-1}) in males and from 3.7 - 128.4 pg mL^{-1} (30.0 ± 34.3 pg mL^{-1}) in females. The Indian River ranges were 15.4 - 183.7 pg mL^{-1} (42.9 ± 38.5 pg mL^{-1}) and 9.9 -72.5 pg mL^{-1} (26.5 ± 19.1 pg mL^{-1}) in males and females, respectively, and the

Georgia inhibin A concentrations ranged from 7.4 - 60.0 pg mL^{-1} (21.2 ± 16.5 pg mL^{-1}) in males and from 7.9 - 45.3 pg mL^{-1} (26.5 ± 15.2 pg mL^{-1}) in females (Figure 11).

Two-sample test of means (Student's *t*-tests or Mann-Whitney-Wilcoxon tests) did not show a significant difference for inhibin A levels between age classes or between sexes in any population. Comparisons of mean inhibin A levels among populations for both sexes and age classes showed no significant difference in female juveniles, but inhibin A levels in male juveniles were significantly different between Sarasota and Indian River ($p = 0.02$, Table 3). In adult males, a Kruskall Wallis analysis of variance found a significant difference in inhibin A among populations ($p = 0.03$). No significant differences were observed in female adults across populations.

No significant relationships were observed using linear regression analysis to assess relationships between inhibin A concentrations and morphometrics. However, two marginally significant, positive correlations were found in Sarasota Bay males between inhibin A and body length ($r^2 = 0.206$, $p > 0.05$) and also body weight ($r^2 = 0.177$, $p > 0.05$).

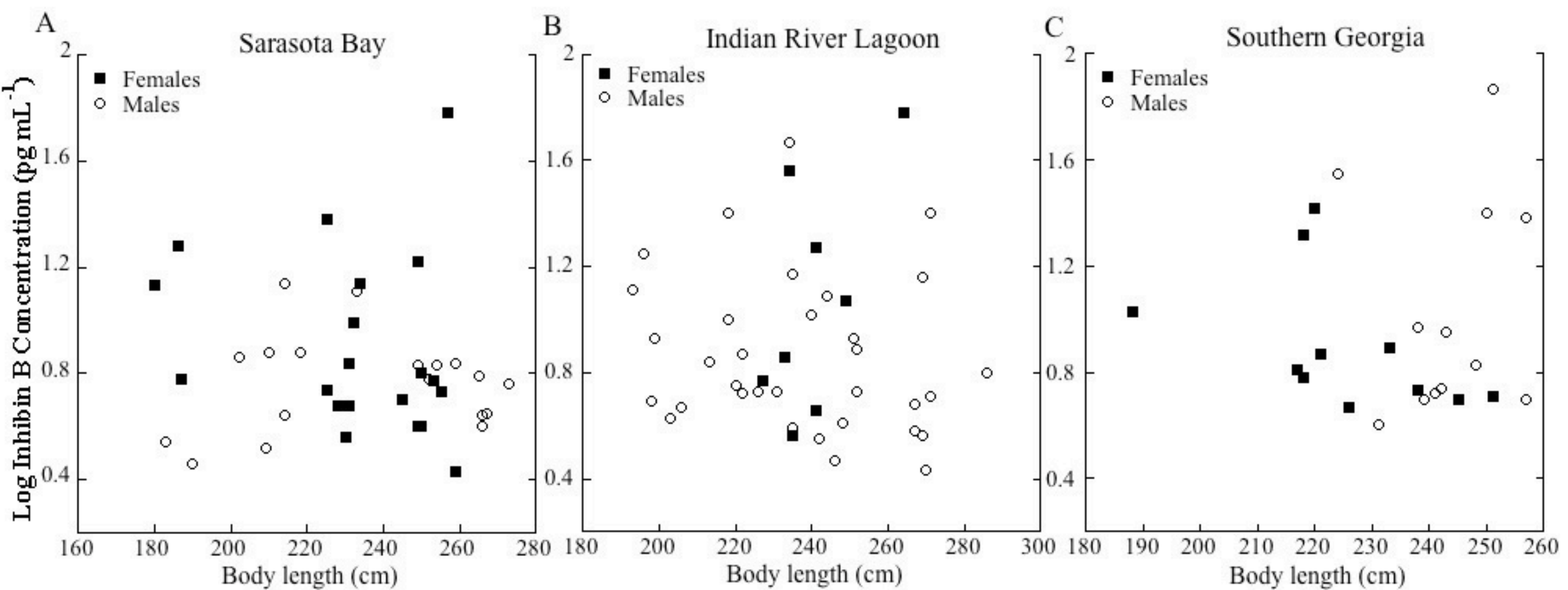

Figure 10. Plot of female and male log transformed inhibin B concentrations against body length in (A) Sarasota Bay, (B) Indian River Lagoon, and (C) southern Georgia. ■ = females and O = males.

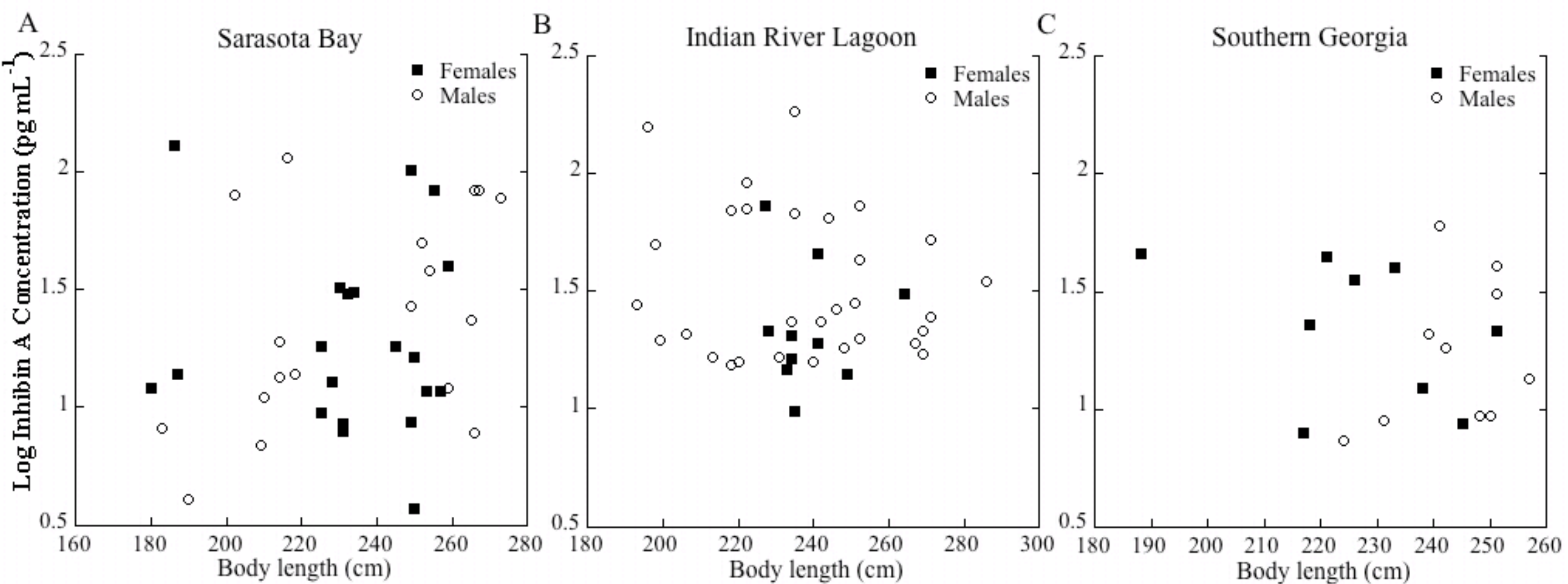

Figure 11. Plot of female and male log transformed inhibin A concentrations against body length in (A) Sarasota Bay, (B) Indian River Lagoon, and (C) southern Georgia. ■ = females and ○ = males.

Contaminants

Blubber samples from the Sarasota Bay dolphins (n = 10) were analyzed for PCBs and PBDEs (Table 6). Total lipid content averaged 32% wet weight (SD ± 7), with high variability between blubber samples (23 to 41%), and contaminant concentrations were made on the basis of extractable lipids, i.e., lipid weight. PCBs found in female dolphins ranged from 2,930 to 44,340 ng g^{-1}. Body burdens found in the males were within the same order of magnitude as the females, ranging from 19,820 to 24,270 ng g^{-1}. PBDE levels were up to three orders of magnitude lower than PCBs, and PBDEs ranged from 55 to 1,000 ng g^{-1} and 370 to 410 ng g^{-1} in females and males, respectively (Table 6). No significant correlations were found between male hormone and contaminant concentrations, and due to the small sample size and distribution, statistical analyses were limited for females. PCBs in the two females with documented calves (between 2 – 7 offspring) were significantly different and an order of magnitude lower than PCB levels in females with no recorded calves ($p = 0.045$). PCB and PBDE levels in males and those females without recorded calves were no significantly different ($p > 0.05$).

TABLE 6. Persistent organic pollutant concentrations (ng g^{-1} lipid weight), biometrics, and percent lipid content for blubber in Sarasota Bay juvenile, adult male, and adult female bottlenose dolphins

Individual	**Gender**	**Age**	**Recorded Calves**	**% Lipid**	**∑PCB[a]**	**∑PBDE[b]**
FB 54	F	39	7	23	2930	55
FB 151	F	10	0	28	44340	480
FB 211	F	5	0	36	44020	1000
FB 223	F	8.5	0	25	34230	660
FB 227	F	5	0	41	11680	240
FB 229	F	2	0	31	22080	520
FB 231	F	26	2	36	3110	65
FB 20	M	21	-	32	24270	370
FB 258	M	17	-	24	20720	390
FB 260	M	2	-	41	19820	410

[a] Sum of IUPAC congeners 28/31, 52, 66, 74, 85, 95, 99, 101, 105/152, 118, 128/167, 137, 138, 146, 149, 151, 153, 170, 174, 177, 178, 180, 183, 187, 194, 195, 196/203, 200, 206, 209

[b] Sum of IUPAC congeners 47, 99, 100, 153, 154

DISCUSSION

Previous efforts to define serum hormonal profiles in free-ranging bottlenose dolphins have been only moderately successful, as monitoring hormonal levels and changes over time is difficult given the challenges involved with sampling wild dolphins (i.e., logistical constraints, permitting, access, cost, etc.). Establishing baseline hormone concentrations in wild populations and understanding the relationships between hormone trends and reproductive biology will advance our knowledge of cetacean endocrinology and provide a basis for investigating potential effects of stressors on population level reproductive potential. Through the use of ELISA techniques, we successfully detected AMH, inhibin A, and inhibin B in bottlenose dolphins with reproducible results. This research reports hormone concentrations in free-ranging bottlenose dolphins from three locations and describes observed trends and influences of gender, age, location, and known *Tursiops* reproductive biology.

Relationships between Gender, Age, Body Size, and Reproductive Biology

This study assessed the relationships between age and body length in both sexes and all populations (when data were available) and found statistically significant predictive relationships between these parameters in all analyses ($p \leq 0.002$, Table 2). However, considerable variation in size-at-age has been observed in bottlenose dolphin populations, which should provide caution against using length as a precise indicator of age (Read at el., 1993).

Anti-Müllerian Hormone in Males

For many mammalian species, AMH production is largely involved in the normal development of the male reproductive tract and sex differentiation *in utero*. In this study, high pre-pubertal circulating AMH levels in male dolphins were significantly greater than in adults (Table 3) and can be expressed as a linear decrease with age and body size (Figures 5 and 6). Comparable trends have also been observed in African and Asian elephants (Dow et al., 2011), cattle (Rota et al., 2002), and mice (Al-Attar et al., 1997). Juvenile dolphin AMH levels (up to 2,080 ng mL^{-1}) are among the highest currently reported in any mammalian species. The elevated concentrations seen in male juvenile dolphins are likely related to pre-pubertal testicular development, as high AMH levels are secreted in humans by immature Sertoli cells in correspondence with testicular differentiation and development (Josso et al., 1993; Rey et al., 1993). Therefore, AMH appears to be a measure of gonadal function and is related to maturity status in male bottlenose dolphins.

Dolphins exhibited an AMH decline with age (Figure 5), where lower AMH levels were measured in adult males, compared to juveniles. Yet, the decline was much less abrupt, than that observed in other species, and did not appear to be directly related to the onset of sexual maturity. Gonadal observations document that sexual maturity is attained between 10-13 years of age in most male bottlenose dolphins (Perrin and Reilly, 1984; Cockcroft and Ross, 1990). Yet, AMH concentrations for dolphins in this age category did not differ greatly from those in dolphins a few years younger or older, which indicated a lack of a rapid post-pubertal decline.

In humans, AMH levels decrease in association with Sertoli cell maturation, as AMH is expressed in prepubertal (or immature), but not mature, Sertoli cells (Josso et al., 1993; Steger et al., 1996). At puberty, luteinizing hormone stimulates Leydig cell maturation resulting in increased testicular volume and testosterone production, which induces Sertoli cell maturation and down regulates AMH expression in the seminiferous tubules (Rey et al., 1993; Rey et al., 2009). Post-puberty, AMH levels in adult men decline quite rapidly to the low levels observed in females; similar trends are seen in mice and cows (Al-Attar et al., 1997; Rota et al., 2002). However, we measured relatively high circulating serum AMH concentrations in post-pubertal male dolphins, when mature Sertoli cells should have been present and active.

In the testes, AMH is secreted bi-directionally: apically into seminiferous tubules and basally towards the interstitium and circulation (Josso et al., 1979; Vigier et al., 1983). After puberty, the apical pole of the Sertoli cell preferentially releases AMH, resulting in higher seminal fluid concentrations than in serum (Fallat et al., 1996; Fenichel et al., 1999). Previous research that measured higher seminal AMH concentrations suggests that AMH secretion from the apical aspect of Sertoli cells may be involved in sperm production and germ cell proliferation (Fujisawa et al., 2002).

There is little known about the role of immature or mature Sertoli cells in bottlenose dolphin spermatogenesis, and the clinical significance of AMH production in adult males is not entirely clear. Perhaps the elevated serum AMH concentrations measured in adult male dolphins during the breeding season, when normal spermatogenesis was likely occurring, imply that mature Sertoli cells are more actively involved in AMH production during spermatogenesis. This trend possibly suggests that

the relative roles of AMH, Sertoli cell function and/or directional secretion of AMH by Sertoli cells may differ with sexual maturity among species. However, a comparison of AMH levels in dolphin seminal plasma and serum warrants further investigation.

Dolphins exhibit seasonal breeding behavior, with known peaks in sperm production and density, along with testes enlargement during the breeding season (Schroeder and Keller, 1989). Harrison and Ridgway (1971) found seasonal variation in testosterone concentrations in captive bottlenose dolphins, and a more recent study discovered that sperm production peaks occurred when testosterone concentrations were lowest (Schroeder and Keller, 1989). This phenomenon has been documented in other seasonally reproducing species, such as deer (Asher et al., 1987), stallions (Byers et al., 1983) and monkeys (Matsubayashi et al., 1991). The delayed peak in sperm production may be related the inhibitory effects of high serum testosterone levels on spermatogenesis (Matsumoto, 1990).

Given the seasonal elevations of dolphin testosterone production and the fairly high serum AMH levels present in adult males in this study, it is possible that testosterone effects on AMH expression during spermatogenesis are not as strong as those observed in other species. Contrary to previous research findings, this may indicate that AMH plays a larger role in dolphin spermatogenesis and may also be affected by social maturity and status. In humans, AMH expression is down regulated and germ cells undergo meiosis and attain sperm production when testosterone levels increase during pubertal development (Rey et al., 2009), where testosterone and serum AMH concentrations are inversely correlated (Rey et al., 1993). Interestingly, a recent study on elephants showed no change in AMH concentrations, despite a dramatic increase in

testosterone during periods of heightened sexual behavior, and there was no significant relationship found between AMH and testosterone levels (Dow et al., 2011). Testosterone measurements were beyond the scope of this study; therefore, it is difficult to interpret the relationship between testosterone and AMH concentrations in dolphins. Also, due to our sampling design, it is unclear how AMH levels might change seasonally, as testosterone does, and warrants further examination.

In addition to the relationships assessed between dolphin AMH levels and age, linear regressions indicated a significant negative relationship between AMH and body length, body weight, and maximum girth (Table 4). Compared to the selected parameters, the functional relationship between AMH and age explained the highest percentage of variability in AMH values (60-93%), which suggests that age is the best predictor of circulating AMH levels in male dolphins. This was expected, given the previously mentioned patterns observed in humans, cattle, elephants, and mice. The relationships between AMH and body length explained less variation (41-49%) in AMH levels than age, but in the absence of age data, which is likely for marine mammal populations, body length might be a good alternative as a predictor of AMH levels in male dolphins.

Read et al. (1993) found that male dolphins generally grow in length, girth, and mass well beyond the first 10 years of life and attain only 70% of asymptotic mass when approaching sexual maturity at 10 years of age. Growth continues into the second decade, particularly in girth and mass, which suggests that body size may be an important factor in mating (Read et al., 1993). The relationships observed between AMH and these

growth parameters might relate to the comparable timing that male dolphins reach body length, body weight, and maximum girth asymptotes.

Interestingly, the AMH trend observed in adult male dolphins is opposite that seen in the Florida manatee (*Trichechus manatus latirostris*). Wilson et al. (2011) documented a significant, positive relationship between AMH and both total length and body weight in males. A study by Reynolds et al. (2004) documented the testicular mass:body mass ratio in manatees as 0.19% (on average) during non-winter months when spermatogenic activity is most evident. Given the highly promiscuous behavior of manatees, their testicular mass is relatively low. Bottlenose dolphins, on the other hand, have large testes, compared to body size (ratio of 1%), which provides further confirmation of their promiscuous mating system (Kenagy and Trombulak, 1986). Even though manatees have a lower body size to testicular size ratio, the increased AMH production of manatees during non-winter months may demonstrate that testicular size does not necessarily correlate with the amount of AMH production and that the model of AMH concentrations varies among marine mammal species.

Anti-Müllerian Hormone in Females

Female AMH levels in dolphins remained relatively low, regardless of age, and did not vary significantly between juveniles and adults. In addition, there were no consistent relationships observed between AMH and age or the selected morphometrics. These were expected trends, as minor fluctuations are observed during adulthood or the menstrual cycle in humans, consistent with continuous noncyclic growth of small follicles (Cook et al., 2000). Similarly, AMH levels measured in elephants did not differ

between phases of the ovarian cycle or between cycling and non-cycling females (Dow et al., 2011). AMH likely plays a role in regulating folliculogenesis in female dolphins, but does not appear to be the best gauge of reproductive potential in this species.

Lower AMH levels were measured in a few older female dolphins, and two individuals, a 36- and 39-year old, had AMH levels of 0.25 and 0.11 ng mL^{-1}, respectively. The oldest dolphin sampled (0.70 ng mL^{-1} AMH) was still of reproductive age (41 years old), had eight recorded calves and was potentially nearing senescence, as other odontocete species have shown reduced fertility with age (Kasuya and Marsh, 1986). Yet, her AMH level was higher than younger individuals. One dolphin population showed a negative relationship between AMH concentration and age ($p >$ 0.05), but the trends among age classes and populations were inconsistent.

Typically correlated with antral follicle number, female AMH serum concentrations decrease as the follicle pool is depleted with advancing age (de Vet et al., 2002). Similar AMH declines have been shown to reflect the size of the primordial follicle pool in aging mice and, therefore, were recommended as a marker to assess the quantitative aspect of ovarian reserve (Kevenaar et al. 2006), which is essentially the number and quality of oocytes available to produce a dominant follicle late in the follicular phase. A study on human ovarian activity suggested a correlation between the reduced primordial follicle stock and the declining number of small growing follicles (Gougeon et al., 1994). There may be a natural decline in AMH concentrations over time in long-lived dolphins. However, due to the small sample size of older females in this study, evidence for this trend was not clear. If applied to a greater number of older

dolphins, AMH might prove to be a promising marker of declining fertility in female cetaceans.

As seen in other species, this study observed a significant gender difference in AMH secretion in dolphins, where concentrations were approximately 1,000-fold higher in males than females. A similar trend was reported in cattle (Rota et al., 2002), manatees (Wilson et al., 2011), and elephants (Dow et al., 2011). The magnitude of gender difference is most similar to that found in the Florida manatee, where mean male AMH levels were 1,270 ng mL^{-1} in non-winter months, compared to 0.03 ng mL^{-1} in females.

Inhibins in Males

The cyclical and diurnal changes observed in inhibins have made interpretation of hormone levels challenging, and given the annual sampling of this study design, trends have been inferred under these constraints. Clear diurnal inhibin B rhythms have been observed in normal men, with the lowest concentrations expressed during evening or night and were similar to that of testosterone (Carlsen et al., 1999). The regulation of inhibin B is complex, and both stimulatory and inhibitory effects of testosterone influence its secretion.

In dolphins, no significant differences were observed in inhibin B concentrations between male juveniles and adults, but juveniles had slightly higher levels (Table 3). Inhibin B was elevated in several juvenile (between 12-47 pg mL^{-1}) and a few adult (ranging between 17-74 pg mL^{-1}) dolphins, compared to other individuals in the respective age classes, but no consistent patterns emerged with the elevated

concentrations and sexual maturity. In humans, Sertoli cell proliferation and FSH govern inhibin activity during childhood, whereas germ cells are the chief determinant of inhibin B synthesis in adults (Meachem et al., 2001). It is likely that the regulation of inhibin B differs in immature dolphins and adults, but given the lack of differences between hormone levels and age class or correlations with the selected morphometrics, the function of inhibin B at different stages of sexual maturity in male dolphins is not apparent.

With the exception of the few individuals with elevated inhibin B levels, a majority of both juveniles and adults had inhibin B levels < 10 pg mL^{-1}, which are among the lowest reported in any mammalian species. Although, two particular dolphins had atypical values: a juvenile at 112 pg mL^{-1} and an adult at 611 pg mL^{-1}. The testicular Sertoli cells are known to be the primary source of inhibins in male circulation (Robertson et al., 1988). In healthy adult men, mean serum inhibin B concentrations were ~200 pg mL^{-1} (Klingmüller and Haidl, 1997; Kumanov et al., 2006), and similar levels were observed in mature male rats (~180 pg mL^{-1}; Buzzard et al., 2004). The vast differences among species in male inhibin B secretion suggest that Sertoli cell function may differ across species.

Serum inhibin B levels reflect the functional state of the seminiferous epithelium, and production is regulated primarily by spermatogenic status, which has been shown by a direct correlation between serum inhibin B levels and sperm count (Pierik et al., 1998). When spermatogenesis is damaged, inhibin B decreases while follicle stimulating hormone increases (Anawalt et al., 1996), whereas follicle stimulating hormone stimulates inhibin B secretion in normal men and is mediated by the presence of germ

cells. A study performed on both normal men (with sperm counts > 20 x 10^6 mL^{-1}) and those with oligozoospermia (with average sperm counts of ~ 4 x 10^6 mL^{-1}) found that inhibin B averaged 223 pg mL^{-1} in normal men compared to 107 pg mL^{-1} in those with low sperm count (Klingmüller and Haidl, 1997).

Therefore, the elevated levels observed in mature dolphins are not likely related to impaired spermatogenesis, but possibly related to increased sperm count or Sertoli cell activity during the breeding season. Seasonal changes in inhibin were documented in Japanese monkeys (*Macaca fuscata fuscata*), where plasma immunoreactive-inhibin levels increased during the breeding season, likely in association with increased Sertoli cell activity (Matsubayashi et al., 1991). Given the timing of our sampling, the adult male dolphins were likely near or at sperm concentration peaks and in the midst of spermatogenesis. Since inhibin B in adults is regulated, to some degree, by spermatogenic activity, then perhaps the lower inhibin B concentrations in the majority of dolphins, compared to those seen in other species, reflect a level of spermatogenesis. However, a temporal inhibin profile is necessary to fully understand possible diurnal and seasonal fluctuations, which might increase our ability to use inhibin B as a marker of reproductive status.

Interestingly, this study successfully detected inhibin A in dolphins. Several human studies have discovered that inhibin A is undetectable in serum of normal men; thus, it has been accepted that inhibin B is the predominant form produced by the testes (Anawalt et al., 1996; Illingworth et al., 1996). The specific assays utilized for inhibin A measurement in this study were optimized for humans, rats, and monkeys, with minimal

cross-reactivity against inhibin B or activins, and these ELISAs were adapted for dolphins with reproducible results.

Dolphin inhibin A concentrations were higher than inhibin B (Table 3), which differs from the trend observed in rats. Testicular inhibin A concentrations are detectable in the rat, but levels are 100-fold lower than those of inhibin B. Even so, a postnatal increase in both inhibin A and inhibin B was observed in these rats, which paralleled Sertoli cell activity (Buzzard et al., 2004). Additionally, a few studies have shown that administration of inhibin A suppressed follicle stimulating hormone concentrations in both male sheep (Tilbrook et al., 1993) and primates (Ramaswamy et al., 1998), which confirm the inhibitory action of inhibins on FSH secretion.

Similar to inhibin B, no significant differences were observed in inhibin A concentrations between male juvenile and adult dolphins (Table 3), and the trends across age classes were not consistent among populations. Inhibin A means were higher in Indian River juveniles than adults, but the opposite was observed in Sarasota Bay (Table 3). Given the lack of consistent relationships between inhibin A and age class or the selected morphometrics, the function of inhibin A at different stages of sexual maturity in dolphins is not clear. The specific role of inhibin A in males has yet to be established and limited data are available on the topic; thus, it is difficult to interpret trends in dolphins. However, as with inhibin B, more frequent measurements and additional investigation into seasonal changes might improve our understanding of inhibin A synthesis and function in bottlenose dolphins.

Inhibins in Females

Inhibins exhibit distinct peaks during the follicular and luteal phases of the menstrual cycle (Groome et al., 1994, 1996; Welt et al., 1997), and in some mature dolphins, similar peaks were observed. Most of the inhibin levels in adult females were < 8 pg mL^{-1} and < 20 pg mL^{-1} for inhibin B and inhibin A, respectively. However, peaking hormone levels were significantly different in many cases and ranged from 20 - 550 pg mL^{-1} (for inhibin B) and 30 - 580 pg mL^{-1} (for inhibin A). With the outliers removed, juvenile and adult inhibin B concentrations were quite similar, but inhibin A was slightly higher in juveniles compared to adults (Table 5).

The estrous cycle in bottlenose dolphins is split evenly between the follicular and luteal phases (Yoshioka et al., 1986). Information about fertility variations that may occur during transitional periods in dolphins (i.e., puberty, entering or coming out of estrous, or anestrous) is lacking. Anestrous periods, characterized by little to no measurable levels of sex steroids have been documented in captive *T. truncatus*, lasting up to two years (Kirby and Ridgway, 1984; Yoshioka et al., 1986). Bottlenose dolphins are known to enter periods of anestrus, especially after giving birth and during rearing, which supports the hypothesis that lower inhibin levels correspond with periods of anestrus. It is likely that the lowest measured inhibin concentrations represent non-cycling or anestrous females. With the exception of one individual, lactating females also had lower inhibin B levels. In addition, immature females had similar inhibin levels to those individuals assumed to be non-cycling or in anestrus.

In most cases, the inhibin A peaks in dolphins did not occur simultaneously with those of inhibin B. In humans, inhibin A concentrations are low during the early

follicular phase when inhibin B begins to rise, and in contrast, inhibin B declines during the luteal phase as inhibin A increases. The different patterns of circulating inhibin A and inhibin B levels during the ovarian cycle indicate different physiological roles in follicle recruitment, maturation, and ovulation (Groome et al., 1996). Even without knowing the exact reproductive status of the female dolphins in this study, it appears that estrous individuals might exhibit similar inhibin expression patterns as seen in human menstrual cycles. The duration of inhibin peaks in dolphins is unclear, but these hormones appear to be an indicator of estrous cycle phases and may be a promising marker of follicular development in dolphins. In addition, inhibins may be a useful complement to serum estrogen concentrations, which remain elevated for 5 - 7 days during the follicular phase in dolphins (Schroeder and Keller, 1989).

Certain calves (~2 years of age) observed with their mothers had slightly elevated inhibin levels compared to other juveniles, not known to be nursing. Sex steroids are lipophilic, can concentrate in blubber and have been measured in breast milk (West et al., 2000). Though primarily secreted by the ovarian granulosa cells, inhibins and activins are also produced to a lesser degree by the mammary glands. Previous research has identified activin A in human breast milk (Alvarado et al., 1993; Di Loreto et al., 1999). Female cetaceans are known to depurate contaminants through lactation (Cockcroft et al., 1990; Borrell et al., 1995; Aguilar et al., 1999), so it may be possible that inhibins are transferred through milk. This might explain the elevated inhibin levels seen in some nursing juveniles.

Population Comparisons

Anti-Müllerian Hormone

No significant differences were found in male AMH concentrations across or between populations, but mean adult AMH levels expressed a decreasing trend, where Sarasota Bay > Indian River > Georgia (Table 3). Strong population differences were not evident based on predictive relationships between AMH concentrations and age. The comparison of linear regressions showed little separation in regression lines (Figure 5), and an ANCOVA showed no significant difference in AMH across populations when controlling for age effects. Age appeared to be the strongest predictor of male AMH levels over time, compared to other selected morphometrics.

On the other hand, comparisons of linear regressions for AMH and body length revealed a clear separation of regression lines between Sarasota Bay and Indian River, even though body lengths were not statistically different. Still, the difference in AMH concentrations between could possibly be an affect of body size. The regression line slope for the Georgia population was considerably steeper than the other two populations (Figure 6), which is likely an effect of the smaller body lengths observed in this population. Bottlenose dolphin populations can demonstrate substantial variation in body size (Perrin and Reilly, 1984). When comparing adult male AMH levels among populations, an ANCOVA analysis controlling for body length effects showed no significant difference. It is possible that the steep decline in the Georgia population resulted from a body length affect, but these dynamics merit further investigation.

Body lengths were significantly smaller in Georgia dolphins than in Sarasota Bay individuals. The largest Georgia male measured 257 cm, compared to larger dolphins in

Sarasota Bay (273 cm) and Indian River (271 cm). One 11-year old Georgia male measured 224 cm, while dolphins of a similar age in Indian River measured 235 - 240 cm. No dolphins > 10 years of age in Sarasota Bay measured below 249 cm. From this data set, one might infer that Georgia males reach the onset of sexual maturity at smaller body lengths, but given the small sample size, this is not conclusive.

Comparisons of AMH levels in dolphins of a similar age between locations were difficult to perform given the limited age data, but some variations appeared. For example, male dolphins between 15 - 17 years of age averaged 1,350, 1,150, and 1,143 ng mL^{-1} in Sarasota Bay, Indian River, and Georgia, respectively. In addition, two older male dolphins expressed considerably different AMH levels, one 30-year old in Sarasota Bay (513 ng mL^{-1}) and one 32-year old in Georgia (62 ng mL^{-1}). Variations in AMH levels were evident across populations, but discerning clear trends and population differences, while controlling for certain artifacts, such as age or body length, was challenging. The lowest AMH concentrations were observed in adult males in Indian River and Georgia, both populations that experience higher evidence of disease and contaminant body burdens (especially for PCBs), which might suggest that Sertoli cell function is possibly slightly reduced or altered.

On the other hand, females did show statistically significant differences in AMH concentrations among all three populations ($p = 0.04$), and the Georgia females had, on average, lower AMH levels than either Sarasota Bay or Indian River. The differences in AMH levels among populations were minor, but previous research has shown that slight, yet significant, decreases in AMH (from 2.1 to 1.3 μg L^{-1}) occur over time in normo-ovulatory women (de Vet et al., 2002). The observed variations in AMH between

Sarasota Bay and Georgia might indicate a decline in the number of early developing follicles that produce AMH and could support the hypothesis that AMH can be used as a marker to assess the quantitative aspect of ovarian reserve (Kevenaar et al., 2006).

Inhibins

It is still unclear how inhibin secretion relates to reproductive status in male bottlenose dolphins, so it is difficult to interpret population differences. An increasing trend was observed in mean adult male inhibin B concentrations across populations, where levels in Sarasota Bay < Indian River < Georgia. Interestingly, inhibin A levels showed the opposite trend, where concentrations in Sarasota Bay > Indian River > Georgia (Table 3). Inhibin B levels were not significantly different among or between populations, but inhibin A levels were significantly different. Inhibin B levels in Sarasota Bay males were relatively stable, and the observed elevations in inhibin B means resulted from several individuals with higher inhibin concentrations in the populations with higher contaminant body burdens. However, the majority of adult males in all populations had circulating levels < 10 pg mL^{-1}. Inhibin B levels have been shown to decrease with impaired spermatogenic activity, but the majority of dolphins in all populations expressed similar inhibin B levels. Again, it is unclear how inhibins are associated with spermatogenesis in dolphins. So, perhaps with a greater understanding of this relationship and an increased sample size, population differences might be clearer.

All study populations included females that expressed peaking inhibin A and inhibin B concentrations, which were likely indicative of the follicular and luteal phases of the estrous cycle. The slight differences observed across populations in mean adult

female inhibin B levels, alongside the nearly identical mean inhibin A concentrations indicated no strong population differences in female inhibins levels (Table 5).

Toxicant Effects

Based on the observed hormone trends across populations, what inferences can be made regarding possible toxic effects on AMH and inhibin production? Many research efforts have focused on links between contaminant exposure and adverse effects on reproduction in marine mammals, but only a few have demonstrated effects on sex hormones. Reduced oestradiol-17ß and testosterone levels have been observed in marine mammals in association with high levels of organochlorines, demonstrating that organochlorine contamination could cause an imbalance of sex hormones (Reijnders, 1986; Subramanian et al., 1987).

Adverse changes in male reproductive health have piqued an interest in detecting negative effects on testicular spermatogenesis and identifying testicular toxicity biomarkers. Proteins from the Sertoli cells are of particular interest to toxicologists because Sertoli cells are one of the three main target cells of toxicants that disrupt spermatogenesis (Boekelheide et al., 2005). Several studies have investigated the effects of various toxicants on Sertoli cells. Phthalates, such as mono-(2-ethlyhexyl) phthalate, can render Sertoli cells dysfunctional and induce testicular germ cell apoptosis (Lee et al., 1997). Monsees et al. (2000) assessed the effects of different toxicants, including pesticides, heavy metals, and oestrogens on cultured rat Sertoli cells. Exposure to the pesticide lindane and the oestrogens (ethinyloestradiol and bisphenol A) increased levels of inhibin B secreted into the cell culture. In contrast, the heavy metals (mercury [II] and

platinum [II]) decreased inhibin B levels, and the metal ions likely had a direct effect on inhibin secretion (Monsees et al., 2000). Human studies have shown that men exposed to lead demonstrated significantly lower sperm concentrations and higher serum inhibin B levels than normal men with low lead blood levels (Mahmoud et al., 2005).

These previous findings provide evidence of toxic effects on Sertoli cells and spermatogenesis. The increase of Sertoli cell inhibin B production in rats and humans exposed to certain toxicants aligns with our research findings, where elevated inhibin B levels were documented in dolphin populations with elevated contaminant loads (especially PCBs). Individuals in both Indian River and Georgia exhibited higher inhibin B levels than the stable concentrations observed in Sarasota Bay. Prior research suggests that Sertoli cells may respond to various stressors by increasing inhibin B production (Comhaire and Mahmoud, 2003), and increased inhibin B levels may directly suppress spermatogenesis (Bame et al., 1999). Monsees et al. (2000) suggested that at high enough doses, chemicals could have a direct effect on Sertoli cells or may induce alteration in enzyme activity involved in the synthesis of AMH or inhibins.

Conversely, Georgia dolphins had the smallest body sizes and the lowest AMH concentrations in males, where ∑PCB concentrations in adult males were significantly higher than Sarasota Bay or Indian River. It may be that endocrine disrupting compounds, such as PCBs and pesticides, acting as estrogen mimics can influence the expression of the AMH gene in the Sertoli cells, therefore decreasing AMH secretion (Toppari et al., 1996). PCBs are known estrogenic chemicals that elicit estrogenic responses, and exposure to xenobiotic estrogens may induce reductions in fertility and are correlated with disorders in the male reproductive system (Sharpe et al., 1993).

Previous research has examined the toxic effects of methoxychlor (MXC), a chlorinated hydrocarbon pesticide, and the effect of HPTE, an active, major metabolite of MXC, on the rat ovary. MXC is a weak estrogenic compound and a known endocrine disruptor that has been shown to inhibit early ovarian development, reduce the number of antral follicles in the ovary, inhibit follicular development, and stimulate AMH production (Uzumcu et al., 2006). In our study, mean adult female AMH concentrations between the three populations varied by only 0.5 ng mL^{-1}, and even though Georgia AMH levels were lowest, where ∑PCB concentrations were highest, it is unclear whether high contaminant body burdens affected AMH production in females.

It is possible that male bottlenose dolphins might show the earliest signs of adverse health effects. Contaminant levels in marine mammals can vary among individuals as a result of dietary preferences, age, sex, body size, reproductive history, and habitat (Aguilar et al., 1999). Male cetaceans generally harbor higher contaminant body burdens, due to life long bioaccumulation, and have no ability to depurate. On the contrary, females can offload toxic compounds to their offspring through gestation and lactation (Cockcroft et al., 1990; Borrell et al., 1995; Aguilar et al., 1999), and levels of organochlorines typically decline with female reproductive activity, where primiparous and multiparous females harbor lower organochlorine concentrations than nulliparous females (Wells et al., 2005). Our results confirm a similar decrease in ∑PCB and ∑PBDE concentrations with reproductive activity in females (Table 6). Thus, hormone concentrations in males could display the first signs of adverse reproductive effects resulting from higher contaminant body burdens.

CONCLUSIONS

This research successfully detected AMH, inhibin A, and inhibin B in bottlenose dolphins and represents the first report of circulating serum AMH and inhibin levels in free-ranging bottlenose dolphins from three southeastern U.S. estuaries. In addition, we investigated the use of these hormones as reproductive biomarkers and potential biomarkers of toxicant effects on reproduction. The following conclusions were reached through this study:

- AMH and inhibins appear to reflect a degree of gonadal function and allow us to assess fertility potential in bottlenose dolphins, to some extent. This study provides a benchmark of breeding season AMH and inhibin levels as a reference to assess change over time.
- AMH expression is related to maturity status in male dolphins and showed a significant, negative relationship with age, body length, body weight, and maximum girth. AMH appeared to be a stronger indicator of reproductive status in males than females, but with further investigation, might prove to be an indicator of declining fertility in females.
- Of the parameters used in this study, age was the best predictor of AMH levels in males, but body length might be a good alternative for individuals of unknown age.
- Inhibins in female dolphins seem to be an indicator of follicular and luteal estrous cycle phases and may therefore be a good marker of follicular development in

dolphins. Inhibins might be a useful complement to estradiol and progesterone measurements in females. The role of inhibins in males is not clear from this research and merits further examination.

- These findings suggest the possibility of toxicant effects on AMH and inhibin production in dolphins, but owing to the limited data set it is difficult to conclusively link these observations with contaminant exposure, as a host of stressors, not accounted for in this study could be affecting fertility. A better understanding of the mechanism(s) of action for contaminant-related reproductive toxicity could elucidate the diagnostic value of using these hormones as biomarkers for impacts of toxicant effects on reproduction in dolphins.

Further investigation of AMH and inhibins is imperative to advance our understanding of cycling patterns, seasonal changes, and relationships with sex steroids and gonadotropins (such as testosterone, estradiol, and follicle stimulation hormone). These efforts will require long-term and frequent sampling (perhaps with a captive population) from individuals of known health and reproductive status. Using AMH and inhibins in adjunct with the commonly measured sex steroids may improve our understanding of their synthesis and function in bottlenose dolphins. The capacity to evaluate the reproductive status of individuals and to potentially relate the effects of stressors to reproductive potential makes the assessment of AMH and inhibins a prospective tool for determining the true conservation of a species or population (Reynolds et al., 2009).

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