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Using Blubber to Assess Steroid Hormone Homeostasis in Bottlenose Dolphins (Tursiops truncatus) Exposed to Organochlorine Pesticides

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by

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A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies.

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Dedication

This dissertation is dedicated to two people who have been tremendously influential in my life and career. The first is Lou Guillette, my first major advisor who tragically passed away in August of 2015. His passion for and approach to science and research continue to inspire and motivate me. The second is my late grandfather, Dan Matarazzo ("Gramps"), who was perhaps my biggest fan and cheerleader during the first 22 years of my life. He always encouraged me to "go knock their socks off!" He is dearly missed by all of us who loved and were loved by him. This is for you, Gramps, I hope it knocks their socks off.

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Table of Contents

Key to Abbreviations

11βHSD.....11β-hydroxysteroid dehydrogenase
$17OHP_4$.....17-hydroxyprogesterone
$17OHP_5$.....17-hydroxypregnenolone
17βHSD.....17β-hydroxysteroid dehydrogenase
3βHSD.....3β-hydroxysteroid dehydrogenase
ABP.....androgen-binding protein
ACTH.....adrenocorticotropic hormone
AE.....androstenedione
B.....corticosterone
BCF.....bioconcentration factor
Cal.....calibration standard
CBG.....cortisol-binding globulin
CE.....collision energy potential
CHL.....chlordane
CL.....corpus luteum
CRH.....corticotropin-releasing hormone
CXP.....collision exit potential
CYP11A1 or P450scc.....cytochrome P450 11A1
CYP11B1.....cytochrome P450 11B1
CYP17.....cytochrome P450 17A1
CYP19.....cytochrome P450 19A1 aromatase
CYP21.....cytochrome P450 21
DDD.....dichlorodiphenyldichloroethane
DDE.....dichlorodiphenyldichloroethylene
DDT.....dichlorodiphenyltrichloroethane
DDx.....general DDT metabolites
DHEA.....dehydroepiandrosterone
DHT.....dihydrotestosterone
DOC.....11-deoxycorticosterone
DP.....declustering potential
E.....cortisone
E_1.....estrone
E_2.....estradiol
E_3.....estriol
EC50.....half-maximal effective concentration
EDC.....endocrine disrupting chemicals
EP.....entrance potential
F.....cortisol
FSH.....follicle stimulating hormone
GnRH.....gonadotropin releasing hormone
GPCR.....G-protein coupled receptor
HPA.....hypothalamo-pituitary-adrenal axis
HPG.....hypothalamo-pituitary-gonadal axis
IACUC.....Institutional Animal Care and Use Committee
IC50.....half-maximal inhibitory concentration

IS ..... internal standard
K_{oc} ..... soil organic carbon:water partitioning coefficient
K_{ow} ..... octanol:water partitioning coefficient
LC-MS/MS ..... liquid chromatography-tandem mass spectrometry
LH ..... luteinizing hormone
LOQ ..... limit of quantification
$MeSO_2$-DDE 3-methylsulfonyl-DDE
MMPA ..... Marine Mammal Protection Act of 1972
MS ..... mass spectrometry
NADP+ ..... nicotinamide adenine dinucleotide phosphate
NADPH ..... reduced NADP+
P_4 ..... progesterone
P_5 ..... pregnenolone
PBDE ..... polybrominated diphenyl ether
PCB ..... polychlorinated biphenyl
POP ..... persistent organic pollutant
RSD ..... relative standard deviation
RT ..... retention time
S 11-deoxycortisol
SALLE ..... salting-out assisted liquid-liquid extraction
SHBG ..... sex hormone binding globulin
sMRM ..... scheduled multiple reaction monitoring
SPE ..... solid phase extraction
StAR ..... steroidogenic acute regulatory protein
T ..... testosterone
UNEP ..... United Nations Environmental Programme

THOMAS MATTHEW GALLIGAN. Using Blubber to Assess Steroid Hormone Homeostasis in Bottlenose Dolphins (*Tursiops truncatus*) Exposed to Organochlorine Pesticides. (Under the direction of ASHLEY BOGGS and LORI SCHWACKE).

Abstract

The purpose of this dissertation is to examine the impacts of dichlorodiphenyltrichloroethane (DDT) and its metabolites (collectively DDTs or DDx) on steroid hormone homeostasis in the bottlenose dolphin (*Tursiops truncatus*) using only remotely collected blubber. First, I examined whether blubber is a suitable proxy for blood in the assessment of steroid hormone homeostasis in dolphins. To do so, I developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for the simultaneous measurement of multi-class steroid hormones in dolphin blood matrices. Then I quantified steroids in matched blood and blubber samples collected from reference populations, and modeled the relationships between the hormones in both matrices. I determined that while blubber hormone measurements are not sufficient to quantitatively predict circulating steroid hormones, blubber is still a useful matrix for endocrine assessment as it reflects physiological state. I examined the impacts of DDx exposure on steroid hormone homeostasis by using a free-ranging population that experiences high DDx exposure. For this population, only remotely collected blubber and skin biopsies are available, thus blubber was used for the measurement of persistent organic pollutants (POPs) and hormones, while skin was used to determine genetic sex. I observed negative correlations between testosterone and several DDTs in male bottlenose dolphins, and negative associations between cortisol and all DDTs in females. Notably though, these hormones are also negatively correlated with other POPs, which are positively correlated with the DDTs. Thus, it is impossible to definitively conclude whether DDTs are impacting steroid hormone homeostasis in this population. Nonetheless, these results indicate that endocrine disruption could be occurring, warranting further investigation. Finally, to examine potential

sources of disagreement between plasma and blubber hormone measurements, I examine the ability of blubber microsomes to metabolize F and E. Results suggest that blubber can interconvert cortisol and cortisone. However, these conclusions are limited by low sample size (n = 3). Further experimentation, especially with *ex vivo* study design, with more samples should be implemented to more conclusively test this hypothesis. This finding could potentially impact the interpretation of blubber hormones in relation to circulating values. Overall this dissertation advances our understanding of cetacean endocrinology and ecotoxicology.

CHAPTER 1: General Introduction & Literature Review

1. General Vertebrate Endocrinology

Vertebrates are complex organisms with highly specialized organ systems. To both maintain homeostasis and achieve the ultimate biological goal of reaching maturity and producing offspring, the body must coordinate a variety of complex biological processes involving multiple organ systems, such as development, sexual maturation, reproduction, and metabolism. As such, communication among organs is an absolute necessity. The endocrine system evolved as one such means of inter-organ communication. The organs comprising the endocrine system secrete signaling molecules, called hormones, to facilitate this communication.

Endocrine signaling specifically refers to hormonal signaling between cells that are not adjacent to one another. This process relies on the circulatory system to deliver hormones from source tissues to target tissues (reviewed in: [1]). In this way, endocrine signaling is distinguished from the other forms of hormonal signaling, autocrine (signal is produced and received within the same cell) and paracrine (signal is transmitted to a different cell in close physical proximity without need of the circulatory system) (reviewed in: [1]).

Vertebrate hormones can be divided into two major classes: those derived from amino acids and those derived from lipids. Amino acid-derived hormones are diverse and include tropic hormones (e.g. gonadotropins), tropic releasing hormone (e.g. gonadotropin-releasing hormone), thyroid hormones, and insulin, among others. Lipid-derived hormones include steroid hormones and eicosanoids. Steroid hormones are the focus of the discussion herein.

1.1 Steroid Hormone Structure and Signaling

Steroid hormones are categorized into four classes based on structure and function; they are progestogens (21 carbons), androgens (19 carbons), estrogens (18 carbons), and corticosteroids (21 carbons) (Figure 1.1). They exist within a common metabolic pathway (Figure 1.1, Table 1.1, Table 1.2) (reviewed in [1, 2]). Figure 1.1 illustrates the steroid hormone biosynthetic (steroidogenic) pathway as well as the chemical structures for the hormones. Table 1.1 lists the steroids of interest in this study with their classifications and common abbreviations. Table 1.2 lists the enzymes involved in steroidogenesis with their common abbreviations. These abbreviations will be used throughout this dissertation. As indicated in Figure 1.1, the precursor to all steroid hormones is cholesterol – a 27-carbon lipid with a four-ring "backbone" structure comprised of three cyclohexanes and one cyclopentane called the cyclopentanoperhydrophenanthrene ring (reviewed in: [1]). This backbone structure, also called the "steroid nucleus", is retained by all steroids (reviewed in: [1]).

Progestogens are typically associated with pregnancy and female reproductive cyclicity, but also serve as direct precursors to androgens and corticosteroids (reviewed in [1, 2]). Androgens are commonly referred to as "male sex steroids" as they are observed at high concentrations in males and regulate the expression of masculine traits (e.g. development of male external genitalia and secondary sex characteristics) and male reproduction, but they are important in female physiology as well (reviewed in [1, 2]). Estrogens, which are derived from androgens, are often called "female sex steroids" for analogous reasons and, similarly, are important in male physiology (reviewed in [1, 2]). Corticosteroids are involved in mediating the stress response, and are further divided into glucocorticoids, which regulate glucose homeostasis, and mineralocorticoids, which regulate ionic (Na^+/K^+) homeostasis (reviewed in [1, 2]).

Individual steroid hormones are structurally identical across vertebrate taxa, unlike peptide hormones, meaning, for example, testosterone produced by a human is indistinguishable from testosterone produced in a dolphin or mouse. This is important in the study of comparative endocrinology because the analytical methods used to measure a steroid hormone in one species can theoretically be used to measure that same hormone in another species, once matrix effects, which could vary by species, are considered. Matrix effects are impacts on analyte quantitation caused by the sample matrix (e.g. serum, plasma, adipose, etc.) itself through the presence of endogenous interfering compounds; considering that matrix composition could vary by species, different species could exhibit different matrix effects. Furthermore, this does not mean that the same hormones perform the same role in each species. For example, in humans, cortisol is the major active glucocorticoid and cortisone is the major inactive glucocorticoid, but in rodents, corticosterone and 11-dehydrocorticosterone are the active and inactive corticosteroids, respectively. Considerations about major/minor hormones within classes are important in the design of endocrinological experiments. As will be discussed later, investigators may choose to only measure the major hormone within the class of interest due to methodological limitations. Overcoming this limitation by using novel analytical methods is a key component of this dissertation.

Progestogens, androgens, and estrogens are predominantly produced in the gonads, while corticosteroids are produced in the adrenal gland (reviewed in: [1]). The adrenal also produces some androgens (reviewed in: [1]). Other peripheral tissues produce and/or metabolize steroids; these include but are not limited to the placenta, brain, skin, and adipose tissue (reviewed in: [1, 3]). Despite their differing structure, function, and source organs, the processes regulating steroid hormone homeostasis are similar across steroid classes. The gonads

and the adrenal glands are the terminal ends of two similar endocrine axes – the hypothalamic-pituitary-gonadal (HPG) and the hypothalamic-pituitary-adrenal (HPA) axes. As indicated by the axis names, gonadal and adrenal steroidogenic function is regulated ultimately by the hypothalamus and intermediately by the anterior pituitary gland (adenohypophysis). When the hypothalamus receives and integrates neuronal and hormonal signals indicating an increased need for progestogens, androgens, estrogens, or corticosteroids it secretes tropic hormone-releasing hormones (gonadotropin-releasing hormone [GRH] or corticotropin-releasing hormone [CRH]) to stimulate the anterior pituitary (reviewed in: [1]). Hypothalamic releasing hormones bind and activate their cognate receptors expressed by the gonadotropic or corticotropic cells in the anterior pituitary, which leads to the secretion of tropic hormones, including gonadotropins (follicle-stimulating hormone [FSH] and luteinizing hormone [LH]) or adrenocorticotropic hormone (ACTH) (reviewed in: [1]). FSH, LH, and ACTH are delivered by the circulatory system to the gonads/adrenal gland where they bind and activate their cognate receptors, ultimately inducing steroidogenesis and steroid secretion (reviewed in: [1]). Steroid hormones in circulation exert negative feedback on both the hypothalamus and the anterior pituitary, thereby regulating GnRH/CRH and FSH/LH/ACTH secretion, and, in turn, secretion of additional steroids from the gonads or adrenal glands (reviewed in: [1]).

Several other organ systems are important regulators of steroid hormone homeostasis, most notably the circulatory and hepatobiliary systems. As mentioned above, the circulatory system delivers hormones from source organs to target tissues. The liver performs two key functions in the endocrine system. First, the liver is largely responsible for the clearance of steroid hormones via phase I and II biotransformation, through which the lipophilic steroid hormones are rendered more water-soluble by the addition of polar moieties (i.e. hydroxides,

glucuronides, and sulfates); once water soluble, these steroid conjugates are more easily excreted in urine and bile (reviewed in: [1]). Second, the liver produces steroid-binding proteins that are secreted into the blood (reviewed in: [1]). These proteins include steroid-specific proteins, like sex hormone-binding globulin (SHBG) and cortisol-binding globulin (CBG) and non-specific proteins (albumin). When bound to these proteins, steroids are not biologically available to target tissues or clearance mechanisms, and roughly 98% of steroids in blood exist in protein-bound forms (reviewed in: [1, 4]). Thus, these binding proteins are thought to be critical in maintaining maximal steroid concentrations in blood, buffering against small changes in steroid hormone production/clearance, and in delivering hormones to target tissues (reviewed in: [1, 4]).

Steroid hormone signaling to target cells occurs through two pathways: the genomic and non-genomic pathways. In the genomic signaling pathway, steroid signaling regulates gene transcription by activating a nuclear receptor. When a steroid enters the cell, it binds its nuclear receptor (which is a ligand-mediated transcription factors), transactivating the receptor to bind target sequences in the promoter regions of target genes, called steroid response elements (reviewed in: [1]). This in turn can either stimulate or repress expression of target genes by facilitating or blocking the recruitment of transcriptional machinery to the transcription start site. Alternatively, the non-genomic pathway is mediated through G protein-coupled receptors (GPCR) expressed on the plasma membrane. GPCR activation leads to a secondary signaling cascade within the cell which ultimately impacts cellular physiology (reviewed in: [1]).

1.2 Steroid Hormones and Mammalian Reproduction

1.2.1 Mammalian Female Reproductive Cycle and Pregnancy

The ovary (female gonad) has two important, interrelated functions: oogenesis (production of viable ova) and steroidogenesis. In eutherian mammals, pregnancy is dependent, from the female perspective, on two discrete, sequential events occurring; first is the successful release of an ovum from the ovary (ovulation) and second is the successful implantation of an embryo in the uterus. To facilitate these events, female mammals exhibit distinct reproductive cycles underpinned by hormonal changes (reviewed in: [1]). There are two forms of female reproductive cycles in mammals; they are the estrous cycle and the menstrual cycle, which are essentially analogous aside from two key features which will be discussed below (reviewed in: [1]). Both the estrous and menstrual cycles can be divided into two phases; the first phase is defined by preparing for ovulation, and the second is defined by facilitating embryonic implantation in the uterus (reviewed in: [1]). The nomenclature for these phases differs between the two cycles; the estrous cycle phases are named for the defining ovarian events in each phase, while the menstrual cycle phases are named for the uterine events.

In the first phase (follicular phase or proliferative phase in the estrous and menstrual cycles, respectively), LH and FSH stimulate the development of ovarian follicles and simultaneously induce a progressive increase in estrogen secretion as the follicles mature (reviewed in: [1]). Steroidogenesis involves two ovary-specific cells: theca interna and granulosa cells. Initially, LH stimulates the synthesis of androgens in theca interna cells, and FSH stimulates granulosa cells to convert these androgens into estrogens (reviewed in: [1]). As a follicle reaches maturity, the elevated circulating estrogen concentrations stimulate the secretion of an LH surge from the anterior pituitary which facilitates ovulation (reviewed in: [1]). Additionally, estrogens stimulate proliferation of the endometrium, the luminal tissue of the uterus in which a developing embryo will implant (reviewed in: [1]). The first difference between estrous and

menstrual cycle animals becomes apparent during this phase. Estrous cycle animals are only receptive to sexual intercourse at the end of the follicular phase (immediately preceding/during ovulation), while menstrual animals are receptive throughout the entire menstrual cycle (reviewed in: [1]).

The second phase (luteal phase or secretory phase, respectively) begins after ovulation (reviewed in: [1]). In this phase, under the influence of the LH surge, the ruptured follicle is transformed into a corpus luteum (CL), a process called luteinization (reviewed in: [1]). The cells of the CL secrete primarily progestogens (P_4 in most mammals) and some estrogens, which stimulates the uterus to secrete materials required for embryonic implantation and sustainment of the developing embryo (reviewed in: [1]). The resultant high circulating concentration of P_4 inhibits gonadotropin secretion, preventing follicular development (reviewed in: [1]). The second difference between the estrous and menstrual cycles becomes apparent at the end of the second phase if an embryo does not implant – in this scenario, animals with the estrous cycle will resorb the excess uterine tissues that were produced to facilitate embryo implantation while menstrual cycle animals will slough these tissues and discharge them (reviewed in: [1]).

If the ovum is successfully fertilized and implants in the uterus, the secretory function of the uterus that begins in the luteal/secretory phase must be maintained throughout pregnancy (reviewed in: [1]). This is facilitated by sustained secretion of progestogens from the CL, leading to major increases in circulating progestogen concentration during pregnancy [5-8] (reviewed in: [1]). Estrogen secretion also tends to increase during pregnancy, potentially to support elevated progestogen secretion [5, 7-11]. Circulating concentrations of androgens, particularly testosterone and androstenedione, also increase during pregnancy in many mammals (including killer whales, bottlenose dolphins, humans, baboons, rats, mice, and horses) to support elevated

ovarian progestogen and estrogen secretion, either by directly stimulating progestogen synthesis or by serving as substrate for estrogen production [5, 6, 11-23]. Conversely, dosing rats and hamsters in the early stages of pregnancy with androstenedione inhibited embryo implantation, suggesting that the function of androgens in female reproductive physiology are potentially dependent on the stage of pregnancy [24, 25].

The placenta, the tissue interface between fetus and mother, plays a key role in facilitating sustained steroid secretion during pregnancy by secreting unique gonadotropins, called chorionic gonadotropins, which sustain the CL, and directly secreting progestogens, estrogens, and androgens [12-16, 21, 26, 27] (reviewed in: [1]). This function of the placenta may vary by species, though, because androgens and estrogens were not detected in the hamster placenta [7].

1.2.2 Steroid Hormones in Parturition

Parturition (birth) is also a hormonally-facilitated process. The fetal HPA axis and the placenta both play important roles (reviewed in: [1]). The placenta secretes CRH which stimulates the fetal adrenal gland to produce corticosteroids (reviewed in: [1]). Fetal corticosteroids increase placental estrogen secretion which induces expression of oxytocin receptors (OR) in the uterus (reviewed in: [1]). Oxytocin from the maternal posterior pituitary gland (neurohypophysis) can then bind and activate the OR to stimulate uterine contractions and induce labor (reviewed in: [1]). Fetal corticosteroids stimulate increased CRH secretion from the placenta in a positive feedback loop (reviewed in: [1]). Furthermore, CRH acts directly on the maternal uterus to stimulate production of prostaglandins which also stimulate contraction of the myometrium (reviewed in: [1]).

1.2.3 Mammalian Male Reproductive Physiology

The testis (male gonad) has two primary functions which are analogous to those of the ovary: spermatogenesis (production of viable spermatozoa) and steroidogenesis (primarily androgens) (reviewed in: [1]). As with oogenesis and ovarian steroidogenesis, these two testicular processes are interrelated and regulated by LH and FSH (reviewed in: [1]). Spermatogenesis requires high local concentrations of androgens (reviewed in: [1]). There are two cell types involved in testicular steroidogenesis: the Leydig cell and Sertoli cell (reviewed in: [1]). Leydig cells produce testosterone (T) and are regulated by LH; Sertoli cells are regulated by FSH and metabolize Leydig cell-derived T into the more potent androgen, dihydrotestosterone (DHT), or into estrogens (reviewed in: [1]). Sertoli cells also promote Leydig cell steroidogenesis through paracrine signaling, and produce androgen-binding protein (ABP), which helps maintain high concentrations of androgens within the seminiferous tubules where spermatogenesis occurs (reviewed in: [1]).

T is the primary circulating androgen in mammals, but others, including androstenedione (AE) and DHT may be observed in circulation (reviewed in: [1]). DHT is more potent than T, thus many androgen target tissues metabolize circulating T into DHT to generate a more potent, local androgen signal (reviewed in: [1]). Alternatively, some androgen target tissues, including the brain, metabolize circulating T into estradiol (E_2), meaning that androgen-controlled processes within these tissues are actually more directly mediated by estrogen signaling (reviewed in: [1]). This demonstrates the point discussed above – that the standard classifications of steroid hormones (e.g. "androgens are male sex steroids, while estrogens are female sex steroids") are not categorically true; "female sex steroids" are critical to male physiology and vice versa.

1.2.4 Puberty

Puberty is the process by which immature mammals become sexually mature, or capable of sexually reproducing. Hormonally, this process is generally mediated and marked by increased secretion of GnRH and increased sensitivity of the anterior pituitary to GnRH, which induces increased adenohypophyseal secretion of gonadotropins (reviewed in: [1]). This, in turn, stimulates gametogenesis and increased secretion of both gonadal and adrenal androgens, termed gonadarche and adrenarche, respectively (reviewed in: [1]).

1.3 HPA/HPG crosstalk

There is significant crosstalk between the HPG and HPA axes. Figure 1.2 presents these complex crosstalk relationships graphically. Stress tends to suppress reproductive function in mammals (reviewed in: [28-30]). Signaling from the HPA axis can modulate gonadal steroidogenesis by exerting effects at all levels of the HPG axis (reviewed in: [28-30]). CRH and glucocorticoids can both directly inhibit the GnRH secretion, and glucocorticoids can suppress gonadotropin secretion by impacting adenohypophyseal expression of GnRH-receptor and gonadotropins (Figure 1.2) (reviewed in: [28-30]). Cortisol reduces testosterone (T) biosynthesis in the testis, potentially by affecting expression of LH-receptor and steroidogenic enzymes and/or by inducing apoptosis of steroidogenic cells (Figure 1.2) (reviewed in: [28-30]). Adrenal insufficiency is associated with testicular insufficiency (diminished testicular steroidogenesis) (Figure 1.2) (reviewed in: [30]). The testis also expresses the enzyme 11β-hydroxysteroid dehydrogenase (11βHSD) which catalyzes the interconversion of active/inactive glucocorticoids; the testis is thought to metabolize glucocorticoids in the dehydrogenase (deactivating) direction (cortisol [F] to cortisone [E]) by 11βHSD2 (Figure 1.2) (reviewed in: [30]).

The ovary is also sensitive to glucocorticoid signaling – glucocorticoids inhibit both LH-mediated and FSH-mediated ovarian steroidogenesis (Figure 1.2) (reviewed in: [30]). The ovary

modulates the effects of glucocorticoids on steroidogenesis by locally metabolizing glucocorticoids (Figure 1.2). During the follicular phase, 11βHSD2 is expressed and high concentrations of NADP+ are present (Figure 1.2). Thereby, the ovary deactivates active glucocorticoids present, suppressing the glucocorticoid signal and, thus, preventing glucocorticoids inhibiting ovarian steroidogenesis (Figure 1.2) (reviewed in: [30]). Conversely, during the LH surge 11βHSD1 is expressed and high concentrations of NADPH are present, thereby locally elevating concentrations of active glucocorticoids (Figure 1.2) (reviewed in: [30]).

The HPA axis is sensitive to signaling from gonadal steroids at various levels (reviewed in: [31]). Female mammals tend to exhibit higher secretion of ACTH and glucocorticoids than males, both basal and in response to stress stimuli (reviewed in: [31]). Given that these differences are ablated by gonadectomy, they may be due to either suppressive effects of androgens in males and/or stimulatory effects of estrogens in females (Figure 1.2) (reviewed in: [31]). Indeed in female rats, ACTH and glucocorticoid concentrations tend to increase during the follicular phase when estrogen secretion increases (Figure 1.2) (reviewed in: [31]). In order for estrogen to cause this stimulatory effect, though, P_4 concentrations must be low – high P_4 concentrations inhibit this response, potentially by downregulating estrogen receptor expression in HPA axis tissues (Figure 1.2) (reviewed in: [31]). The CRH gene has estrogen response elements in its promoter region, meaning CRH expression in the hypothalamus may be directly regulated by estrogen signaling (Figure 1.2) (reviewed in: [31]). P_4 also seems to directly impact the HPA axis, inhibiting ACTH secretion in ewes and disrupting the HPA axis' negative feedback mechanisms (Figure 1.2) (reviewed in: [31]). T inhibits glucocorticoid synthesis and ACTH responsiveness in the adrenal gland, and ACTH secretion in the anterior pituitary gland

(Figure 1.2) (reviewed in: [31, 32]). Rodents with lower circulating T tend to exhibit a more robust HPA response to stress stimuli (reviewed in: [31, 32]).

Clearly, the interactions between the HPA and HPG axes are complex and bidirectional. The message to take is this: modulation or disruption of one of these axes is likely to impact the other. This point is particularly important in the study of endocrinology in wildlife because the act of collecting biological samples, which typically necessitates capture, handling, and restraint, is stressful and therefore likely to induce an HPA axis response. This will not only impact corticosteroid measurements but also gonadal steroid measurements. Therefore, it is important to keep in mind that "baseline" steroid hormone concentrations measured in captured wildlife may not be the true physiological baseline.

1.4 Endocrine Disruption

A wide variety of exogenous chemicals have been demonstrated to disrupt the normal function of vertebrate endocrine systems. These compounds are commonly called endocrine disrupting chemicals (EDCs). Notable examples include organochlorine pesticides, like dichlorodiphenyltrichloroethane (DDT) – the focus of this dissertation – and polychlorinated biphenyls (PCBs). As outlined above, homeostatic control of endocrine signaling is complex and involves many tissues. As such, there are a variety of mechanisms by which EDCs can disrupt the endocrine system, including disrupting hormone biosynthesis, transport/delivery, peripheral metabolism/clearance, or direct agonism/antagonism of hormone receptors. These mechanisms of disruption will be discussed in greater detail within the context of DDT exposure in the next section.

1.5 Summary

The scope of the above review is intended to provide sufficient understanding of HPA and HPG physiology to facilitate later discussion herein; this should not be regarded as a comprehensive review of HPG and HPA physiology.

2. DDT

Dichlorodiphenyltrichloroethane (DDT) is a synthetic organochlorine insecticide that was developed in the mid-1800s and first mass-produced in the 1940s for agricultural pest and disease vector control. In 1972, due to evidence that it was dangerous to wildlife and potentially posed a threat to human health, the use of DDT was banned in the United States. Ecologist and author Rachel Carson is largely credited with bringing the risks posed by DDT to the forefront of public attention through the publication of her book *Silent Spring* in 1962. DDT was among the first 12 compounds classified by the United Nations Environmental Programme (UNEP) as a persistent organic pollutant (POP). Despite having been banned from use in most developed countries for decades, DDT still constitutes a risk to the health of wildlife, humans, and ecosystems due to its environmental persistence and propensity to bioaccumulate and biomagnify.

2.1 Chemical and Biological Properties of DDT

Technical grade DDT is primarily composed of the *p,p'*-DDT isomer (85%), but also contains the *o,p*-DDT (15%) and *o,o'*-DDT (trace amounts) isomers [33]. It may also be contaminated with the metabolites dichlorodiphenyltrichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD; see Figure 1.3 for chemical structures [33].

DDTs are persistent in the aquatic environment and prone to bioaccumulation in organisms and biomagnification in aquatic food webs. Persistence and bioconcentration factor

(BCF) vary by isomer and by a variety of biotic and abiotic factors (reviewed in: [34-36]). With soil organic carbon:water partitioning coefficients (K_{oc}) values between ~26,000 and 130,000, DDTs strongly adsorb to soil and, in the aquatic environment, will partition to suspended solids and sediment (reviewed in: [34-36]). In sediment, DDT can be biodegraded into its two most prevalent metabolites, DDE and DDD, neither of which undergo appreciable biodegradation in marine sediments (reviewed in: [34-36]). DDTs are 6 to 7 orders of magnitude more soluble in lipid than water ($\log K_{ow}$ = 6-7) (reviewed in: [34-36]). This, combined with long biological half-lives, means DDTs are prone to bioaccumulate and biomagnify (reviewed in: [34-36]). Collectively, these traits mean that DDTs are still a significant threat to wildlife and humans. Indeed, as will be discussed in greater detail later, bottlenose dolphins (*Tursiops truncatus*), the species of interest in this dissertation, in southeastern United States still experience considerable exposures to DDTs (Fig. 1.3, Table 1.3).

DDTs have been shown to cause a variety of acute and chronic deleterious health effects in a wide variety of animals. Importantly for this study, it has been well established that DDTs disrupt vertebrate steroid hormone axes/signaling through a variety of mechanisms which are discussed in detail below and summarized in Table 1.4, which also contains dosing information (not explicitly discussed in the body of sections 2.2 to 2.6 for the sake of brevity). This section and accompanying table are not an exhaustive review of DDx-mediated endocrine disruption; rather they are intended to illustrate two points: 1) each DDx has the capacity to disrupt steroid hormone axes through several specific mechanisms, and 2) these mechanisms are generally conserved across vertebrate taxa. Please refer to Table 1.4 throughout the reading of sections 2.2 to 2.6.

2.2 DDTs Impact Adrenal Steroidogenesis and Are Directly Adrenotoxic

Several DDTs have been shown to modulate adrenal steroidogenesis in vertebrates, which may impact circulating concentrations of corticosteroids and, thus, signaling at target tissues (Table 1.4). Of the DDTs examined for potential effects on adrenal steroidogenesis, *o,p*-DDD has most consistently (i.e. across taxa) been shown to disrupt adrenal steroidogenesis. Treating domesticated dogs with *o,p*-DDD led to diminished adrenal steroidogenesis by two mechanisms: 1) inhibition of ACTH-mediated induction of pregnenolone (P_5) production from cholesterol, and 2) inhibition of 11β-hydroxylation of 11-deoxycortisol (S) to F [37, 38]. Similarly, Lund et al. 1994 demonstrated that this same metabolite irreversibly binds mitochondrial protein in the adrenal gland of the gray seal (*Halichoerus grypus*) and specifically inhibits the activity of CYP11B, the enzyme which catalyzes 11β-hydroxylation (Figure 1.1, Table 1.2) [39]. o,*p*-DDD also inhibited the activity of CYP450scc (conversion of cholesterol to P_5), CYP21 (conversion of P_4 and 17-hydroxyprogesterone [17OHP_4] to 11-deoxycorticosterone [DOC] and S, respectively), and 11βHSD in bovine adrenal microsomal and mitochondrial isolates (Figure 1.1, Table 1.2) [40]. Rainbow trout (*Oncorhynchus mykiss*) interrenal cells (analogous to adrenocortical cells in other vertebrates) treated o,*p*-DDD exhibited diminished ACTH-dependent steroidogenesis; the *p,p'* DDD isomer also produced similar results in this assay [41, 42]. Similarly, *o,p*-DDD, impaired the ACTH response *in vitro* in interrenal cells from *Sarotherodon aureus*, another freshwater teleost fish [43]. *In* vivo, *o,p*-DDD initially caused an increase in baseline plasma cortisol in *S. aureus* followed by an eventual decrease and persistently suppressed ACTH responsiveness [44].

p,p'-DDT also impairs adrenal steroidogenesis in many vertebrates, though not in all species tested. *p,p'*-DDT inhibited the activity of, CYP21, and 11βHSD in bovine adrenal microsomal and mitochondrial isolates [40]. Rats fed food containing 50 parts per million (ppm)

p,p'-DDT for 47 days exhibited diminished 11β-hydroxylation compared to control animals [40]. Treating rainbow trout interrenal cells with *p,p'*-DDT produced effects similar to those described above for *o,p*-DDD treatment. However, it did not impact ACTH responsiveness in the *S. aureus* interrenal cells [43].

Lastly, in regard to other DDx isomers, *o,p*-DDT treatment did not alter adrenal steroidogenesis in the domesticated dog [45], while *p,p'*-DDE did disrupt steroidogenesis in *S. aureus* interrenal cells [43].

Collectively, these results demonstrate several things. First and foremost, several DDTs affect adrenal steroidogenesis through mechanisms conserved across species, and, second, that these effects vary by DDx. All species tested exhibited impaired adrenal steroidogenesis following treatment with *o,p*-DDD. *p,p'*-DDT also impacted adrenal steroidogenesis in most species, with *S. aureus* being the only exception. By disrupting steroidogenesis, these DDTs reduce adrenal corticosteroid production, which means that circulating concentrations may be reduced below normal levels. Considering the important role that corticosteroids play in maintaining health and fitness, particularly in response to stress stimuli, exposure to these DDTs may indirectly affect survival and precipitate effects at higher orders of biological division (i.e. populations, communities, ecosystems). However, as will be discussed later, DDTs also impact steroid hormone clearance; thus, contaminant-mediated changes in central hormone production do not necessarily produce the hypothesized analogous changes in circulating steroid concentrations.

In addition to direct effects on adrenal steroidogenesis, DDTs are also directly toxic to the adrenal gland, as has been demonstrated in mice, chickens, mink, and Atlantic cod [46-50]. The DDT metabolite 3-methylsulfonyl-DDE ($MeSO_2$-DDE) caused necrosis of the *zona fasciculata*,

the region responsible for glucocorticoid production, in the adrenal gland of suckling, fetal, and adult mice [46, 47]. Mink (*Mustela vison*) dosed once with *p,p'*-DDD or *o,p*-DDD exhibited necrosis and bleeding in the *zona fasciculata* and *zona reticularis*, the region responsible for adrenal androgen production [49]. Both $MeSO_2$-DDE and *o,p*-DDD caused similar effects in the chicken [48]. Comparable adrenal abnormalities were observed in beluga whales from the St. Lawrence Estuary, a population which is characterized by DDx exposure (Table 1.5), and Hudson Bay, but it remains unclear whether this phenomenon is causally linked to contaminant exposure [51, 52]. Interestingly, it seems that these DDT metabolites are bioactivated by cytochrome P450 enzymes within the adrenal, meaning this observed toxicity is mediated by the adrenal gland itself [39, 46-49].

This direct adrenal toxicity may constitute an additional mechanism by which DDTs could disrupt adrenal steroidogenesis. It stands to reason that DDx-caused necrosis of the *zona fasciculata* and *zona reticularis* and, thus, loss of the cells responsible for glucocorticoid and androgen production, would inevitably reduce adrenal glucocorticoid and androgen secretion. As before, this may result in lower circulating glucocorticoid concentrations.

2.3 DDTs Impact Gonadal and Placental Steroidogenesis

Several DDTs have been shown to disrupt estrogen and progestogen production, as with the corticosteroids. High doses of *o,p*-DDT and both DDE isomers (at 400 and 4,000 ng/mL) increased E_2 secretion from porcine ovarian cells compared to control and lower doses (0-40 ng/mL), while the same doses of *p,p'*-DDT reduced E_2 secretion [53]. Furthermore, all of these DDTs increased conversion of T to E_2 at 4,000 ng/mL doses, suggesting a stimulatory effect on P450arom activity (Figure 1.1, Table 1.2) [53]. However, in human placental explants, all doses (1, 10, 100, 1000 ng/mL) of all four of these DDTs (except 1 ng/mL of *o,p*-DDE) decreased

conversion of DHEA to E_2 compared to control, which may have been due to an observed reduction in aromatase activity [54]. Importantly though, the effect of DDx treatment on P450arom activity was only tested at 100 ng/mL for each compound; therefore, it is not clear whether this effect occurs at other doses [54]. Additionally, no consideration is given to the possibility that DDx treatment impacts the activity of the other steroidogenic enzymes required for conversion of DHEA to E_2, namely 3βHSD (DHEA to AE) and 17βHSD (AE to T, or E_1 to E_2) (Figure 1.1, Table 1.2). *p,p'*-DDE also induced hepatic P450arom expression in adult male rats [55]. Thus, DDTs have been shown to impact estrogen synthesis in several tissues from various vertebrate species in laboratory experiments, which may impact female reproductive success.

Furthermore, beyond laboratory dosing experiments, wild juvenile American alligators (*Alligator mississippiensis*) from a site characterized by heavy DDx contamination (multiple DDTs, but primarily DDE) demonstrate altered gonadal steroidogenesis *ex vivo* – the testis exhibits elevated E_2 secretion, while E_2 secretion from the ovary is reduced. The authors noted that this pattern of altered gonadal estrogen production does not match the pattern of circulating steroid hormone concentrations observed in these animals, which they hypothesize may be due to impacts of DDTs on hepatic steroid clearance [56].

P_4 synthesis is also impacted by DDTs. *p,p'*-DDT, *o,p*-DDT, and *p,p'*-DDE at doses of 4,000 ng/mL reduced P_4 secretion in porcine ovarian cells [53]. Conversely, *o,p*-DDT, *p,p'*-DDE, and o,*p'*-DDE at 100 or 1,000 ng/mL stimulated P_4 secretion from human placental explants [53, 54]. These paradoxical results may stem from the dose-, tissue-, and/or species-dependent differences. Another study demonstrated that a lower dose (10 ng/mL) of *p,p'*-DDE stimulated P_4 secretion from porcine ovarian cells with a concomitant increase in expression of P450scc, giving credence to the hypothesis that the divergent results listed above arise from differences

in dosing schemes [57]. This final result suggests that *p,p'*-DDE may increase P_4 secretion by stimulating P450scc expression, thereby facilitating increased conversion of cholesterol to P_5, the immediate precursor to P_4 (Figure 1.1, Table 1.2). From these three independent studies, collectively, one can only conclude that DDx may impact P_4 secretion – the direction and magnitude of that effect remains unclear given the disagreement among studies. By impacting progestogen production, female reproductive success may be impacted; furthermore, since progestogens are precursors to the other steroids, other steroid hormone concentrations and steroid-mediated processes could be affected.

2.4 DDTs Impact Hepatic Steroid Hormone Metabolism

DDx have been demonstrated to impact steroid hormone clearance, providing another mechanism by which circulating steroid hormone concentrations may be altered by DDx. *p,p'*-DDD and *p,p'*-DDE treatment elevated the rate of estrogen metabolism in rat livers, leading to a systemic reduction in estrogen activity, as indicated by uterotropic assay (i.e. diminished estrogen-induced increase in uterine weight in rats dosed with DDTs); chronic treatment with technical grade DDT, *p,p'*-DDE, and *p,p'*-DDD increased hepatic metabolism of T, estrone (E_1), E_2, P_4, and 11-deoxycorticosterone (DOC) in rats [58, 59]. *p,p'*-DDE treatment increased hydroxylation of T in rat hepatocyte microsomes [60]. In chickens, *p,p'*-DDT treatment led to elevated T metabolism, while *o,p*-DDT increased metabolism of E_2 and AE [61]. Treating guinea pigs with technical grade DDT, *p,p'*-DDT, or *o,p*-DDD increased metabolism of cortisol without impacting adrenal production of cortisol [62, 63]. In the domesticated dog, *o,p*-DDT treatment increased the activity of hepatic phase I/II biotransformation enzymes responsible for steroid hormone clearance [45]. In contrast, *o,p*-DDD treatment diminished hepatic cortisol metabolism

in the teleost *Sarotherodon aureus* [44]. Nonetheless, this mechanism of disruption is conserved in vertebrates.

Collectively, these studies demonstrate DDx exposure leads to elevated hepatic clearance of steroid hormones in vertebrates. This mechanism would hypothetically lead to diminished circulating concentrations of steroid hormones; however, one must also take into consideration the potential effects on steroid hormone synthesis, discussed in above sections, before formulating mechanistic hypotheses. Should DDx exposure simultaneously stimulate hormone synthesis and clearance, there may not be any observed change in systemic hormone concentrations. For this reason, assessments of endocrine disruption based solely on circulating/systemic hormone measurements could generate a false negative – i.e. disruption is occurring at two independent points in the system, but they cancel each other out such that the system appears to be functioning normally.

2.5 DDTs as Steroid Receptor Agonists and Antagonists

Several DDTs have been shown to directly affect steroid hormone signaling by acting as steroid hormone receptor agonists or antagonists. Several DDTs (*p,p*-DDT, *o,p*-DDT, *o,p*-DDE, and *o,p*-DDD) have been demonstrated to bind and transactivate the human and rodent estrogen receptors, and produce *in vivo* estrogenic effects in rats [64-66]. Conversely, technical grade DDT induced changes in reproductive tract development that indicate it acted as an antiestrogen in the larval tiger salamander, while *p,p'*-DDE was estrogenic [67].

Some DDTs (*p,p'*-DDT, *o,p'*-DDT, and *p,p'*-DDE) can also act as xenoandrogens. *p,p'*-DDE is an especially potent anti-androgen – it binds the rat androgen receptor and inhibits signaling, thereby ablating the expression of androgen-mediated phenotypes at all life stages [66, 68] Importantly, these *in vivo* effects arose without any change in circulating levels of T or the

activity of 5α-reductase, meaning they occurred independently of potential DDE-induced changes in androgen production and clearance [68]. Alternatively, *p,p'*-DDE did not act as an antiandrogen in larval tiger salamanders, suggesting that these antiandrogenic effects may be taxa- and/or dose-dependent [67].

Unlike the other mechanisms of disruption, which directly impact circulating steroid hormone concentrations and, thereby, indirectly alter hormone signaling at target tissues/cells, hormone mimicry acts directly at the level of hormone signaling and indirectly on steroid production. By directly binding steroid hormone receptors, DDTs may aberrantly stimulate receptor activation in the absence of endogenous signal, or block receptor activation (by occupying the ligand binding site) in the presence of endogenous signal. Thus, steroid synthesis could be affected via hormone mimicry through the negative feedback loops inherent to the HPA and HPG axes. For example, if *p,p'*-DDT binds and transactivates ER expressed in the hypothalamus and/or anterior pituitary gland, this signal will be construed as negative feedback, inhibiting the secretion of GnRH and LH and FSH. This in turn would impact gonadal steroidogenesis. Nonetheless, hormone mimicry may also be similarly hindered by the "false negative" issue discussed in the previous section – that is, we cannot ascertain effects on target tissues by measuring systemic hormone concentrations. Examination of these effects requires target tissue- or cell-specific endpoints.

2.6 DDTs May Impact Steroid Hormone Transport

Steroid hormone transport may be affected by DDx exposure through two mechanisms. First, DDTs impact the expression and secretion of the steroid binding proteins directly. Human adrenocortical carcinoma patients undergoing treatment with *o,p'*-DDD (called "mitotane" in the clinical setting) exhibit significantly elevated circulating SHBG and CBG levels [69, 70].

Furthermore, treating human hepatic cells *in vitro* with *o,p*-DDD led to increased SHBG and CBG expression and secretion in a dose-dependent manner [70]. Conversely, neither current burden of *p,p'*-DDE nor cumulative past exposure to DDT were significantly correlated with current circulating SHBG concentrations in human men occupationally exposed to DDT [71]. These differing results suggest that impacts on hormone binding protein expression differ by DDx, which is consistent with the other mechanisms of disruption. Increasing production of SHBG and/or CBG would increase the capacity of blood to store steroids.

Second, DDx may impact steroid hormone transport through hormone mimicry. Logically it follows that if a contaminant is sufficiently hormone-like to bind to a hormone receptor, it may also interact with other proteins to which the hormones bind, like the circulating steroid-binding proteins. By inhibiting endogenous hormones from binding to circulating binding globulins, DDTs would decrease the amount of bound hormone and simultaneous increase the amount of free hormone. However, findings from studies examining this mechanism are mixed. One study showed that several DDTs inhibited steroid binding to human and rat steroid binding proteins (specifically human SHBG and rat ABP), while another showed that several of these same DDTs failed to impact steroid binding to human SHBG, rat ABP, or rainbow trout SHBG [66, 72]. Therefore, I suspect this mechanism is not likely to major contributor to endocrine disruption in vertebrates.

2.7 DDTs and Wildlife

For the most part, the literature cited in sections 2.2 through 2.6 are controlled laboratory studies that utilize laboratory model species. These types of experiments are useful for defining causal mechanisms of endocrine disruption, but cannot directly predict the effects on free-ranging wildlife for several reasons.

In general, laboratory studies fail to aptly replicate real-world exposure scenarios and fail to account for the multitude of mechanisms by which contaminants can impact endocrine function. Laboratory studies tend to focus on a single mechanism of disruption mediated by discrete, acute doses of a single contaminant (e.g. how a specific dose of *p,p*-DDT may impact ovarian steroidogenesis *in vitro*). Yet, as discussed in the preceding sections, each DDx has the capacity to disrupt endocrine function at various points in the endocrine system, meaning multiple mechanisms of disruption may occur simultaneously. Thus, it is difficult to predict the systemic effects of even a single dose of a single contaminant. Free-ranging animals will be exposed to not only multiple DDTs simultaneously but also other contaminants and exogenous factors that may impact steroid hormone homeostasis. Additionally, exposures in wildlife may not be acute high doses, but rather chronic low-dose exposures over the course of their entire lifetimes, which may be years to decades. Furthermore, often with wildlife, exposure is estimated by body burden, which cannot be easily translated into an equivalent laboratory-administered dose. Collectively, these factors make it difficult to utilize laboratory experiments to predict how organismal physiology will ultimately be affected by real-world exposure.

This issue is well illustrated in the work by Guillette et al. (1995) discussed in section 2. 3. Alligators from an organochlorine pesticide-contaminated site exhibited impaired gonadal E_2 secretion, with the ovary secreting less E_2 and the testis secreting more E_2 compared to reference animals [56]. This finding might lead one to predict that circulating E_2 concentrations should similarly be diminished in females and elevated in males from this contaminated site, but this was not the case [56]. Rather, circulating E_2 concentrations were normal in males (instead of elevated) and higher in females (rather than diminished) [56]. These discordant results suggest that other mechanisms of steroid hormone regulation, in addition to steroidogenesis, were

simultaneously affected by exposure; alternatively, it is possible that the alligator endocrine system was able to compensate for the changes in steroidogenesis induced by DDx exposure, thus producing normal systemic hormone levels.

In light of these results, one could argue that systemic measures of endocrine function are poorly suited to the study of endocrine disruption. However, for ethical and/or legal reasons, often these are the only types of assessments that can be complete d. Lethal sampling of wildlife is not always possible, thus internal organs cannot be collected and tissue-specific, mechanistic endpoints cannot be analyzed. Investigators must rely on non-lethal, system-level sample matrices to assess endocrine function. Blood matrices are commonly used because, in theory, circulating hormone concentrations reflect systemic homeostasis, (i.e. the status of the equilibrium between hormone secretion, storage, transport, and clearance). While changes in circulating hormone concentrations cannot in and of themselves point to a specific mechanism of disruption (e.g. impacts on hormone production versus impacts on clearance), perturbation of circulating hormone concentrations following contaminant exposure provides indication that endocrine disruption is occurring at some level in the system. Therefore, blood measures are a useful starting point in the investigation of endocrine disruption. However, as seen with the male alligators in Guillette et al., disruption of endocrine organ physiology can occur without observable changes in circulating hormone concentrations [56].

Despite the difficulties presented by the exclusive use of blood (or other systemic matrices) for endocrine assessment, systemic measurements have been able to identify endocrine disruption in both laboratory and wild mammals. Female rats dosed with technical grade DDT (diet containing 150 ppm for 36 weeks) exhibited significantly diminished plasma progesterone concentrations; whether this results from altered secretion, elevated clearance, or

both is unclear, but nonetheless disruption has occurred [73]. Ciesielski et al. (2017) recently demonstrated that circulating dihydrotestosterone concentrations were negatively correlated with circulating POP concentrations in male polar bears (*Ursus martimus*) from Svalbard, Norway [74]. Similarly, plasma testosterone and cortisol have previously been shown to be negatively related to POP burden in male polar bears from this same site [75, 76].

In laboratory experiments, investigators create control (unexposed) and experimental (exposed) populations through dosing with contaminants. Conversely, when studying ubiquitous contaminants like DDTs in free-ranging wildlife, the identification of suitable unexposed/reference populations may prove challenging due to the ubiquitous presence of many of these contaminants. Furthermore, the generation of an exposed group through direct dosing in wildlife may not be feasible, ethical, or legal, meaning investigators must identify populations that experience incidental contaminant exposures. Therefore, reference and exposed animals will often come from independent populations, which presents several issues. Laboratory animals used in an individual experiment are likely closely related and have similar/identical life histories (i.e. same age, habitats, diet, etc.), minimizing several potential sources of intraspecies variation in basic endocrine function and response to contaminants. While free-ranging animals may also be related and have comparable life histories, it would be inappropriate to assume so without collecting supporting evidence. In the absence of such data, investigators cannot definitively conclude that differences between reference and exposed populations are due exclusively to contaminant exposure and not resulting, at least in part, from differences in genetics, diet/nutritional status, habitat, age, season, life history, etc. Indeed, returning to the Guillette et al. (1995) study discussed above, the differences in gonadal steroidogenesis were not explicitly linked to contaminant exposure, rather the authors relied on

the assumption that the only meaningful difference between the reference and experimental populations was pesticide exposure [56]. In section 4.4 below and subsequent chapters I will discuss this issue as it specifically relates to my selected reference and exposed populations of bottlenose dolphins.

Contaminant exposure can persistently impact health following the cessation of exposure. Exposure to DDTs and other endocrine-disrupting contaminants at early life stages can induce organizational (developmental) changes that permanently influence health, including steroid hormone homeostasis and reproductive success (reviewed in: [77-80]). Thus, with cross-sectional studies, such as the one that will be described in Chapter 4 of this dissertation, where full-lifetime exposure histories are unknown, it is impossible to determine whether a current phenotype/effect is caused by a current exposure or stems from exposures earlier in life. This is pertinent to bottlenose dolphins since nursing calves likely experience acute POP exposure via milk because females offload significant portions of their POP burden through lactation; this exposure occurs while calves are still developing and thus may be susceptible to permanent organizational disruption [81, 82].

Assessments at even higher orders of biological division, like the organismal or population levels, may also provide evidence of endocrine disruption in wildlife. For example from a laboratory study, Jonsson et al. (1975) demonstrated that dosing female rats with technical grade DDT (diet containing 150 ppm for 36 weeks) led to impaired reproductive success [73]. Since reproductive success relies heavily on endocrine signaling, this result may indicate that endocrine disruption has occurred. The relationship between POP burden and reproductive success has been assessed in some cetaceans, including *T. truncatus*, but none of these studies explicitly examined endocrine disruption as a potential mediating mechanism.

Semi-domesticated female bottlenose dolphins who had unsuccessful pregnancies (i.e. stillborn or failure of calf to survive > 6 months) had significantly higher preparturient ∑DDT (29.58 ppm), total DDD (1.72 ppm), and total DDE (26.91 ppm) burdens compared to those who had successful pregnancies (9.363, 0.655, and 8.237 ppm, respectively) [83]. PCB concentrations were also higher in females with unsuccessful pregnancies [83]. Notably, 67% of the calves that died in this study were from primiparous (first-time) mothers, while only one primiparous mother was reproductively successful [83]. Poor first-time calf survival may be linked to contaminant-mediated impacts on maternal health and/or POP exposure in the calf in utero or via milk [81, 83]. Similarly, reproductive failure has also been blamed on POP exposure in other free-ranging marine mammals, including beluga whales, polar bears, and sea lions. Poor recruitment in the beluga whale population inhabiting the St. Lawrence estuary (the same population with adrenal pathologies discussed in section 2.2) has been anecdotally suggested to be related to organochlorine contamination [51]. Polar bear mothers who lost their cubs exhibited higher concentrations of POPs (including DDTs) in their milk compared to those whose cubs survived [84]. Several cub morphometrics, which may influence cub fitness, were correlated with cub POP burdens [84]. Abortion and premature parturition were linked to DDx and PCB exposure in California sea lions in the 1960s and 70s – prematurely parturient females exhibited significantly higher contaminant concentrations than those that had full-term births [85-87]. It was suggested that this effect may have been mediated through contaminant-induced alterations to reproductive steroid hormone homeostasis and/or immunosuppression (which could also be hormonally-mediated, considering there is a clear link between the endocrine and immune systems) [86]. By the 1990s, DDx burdens in this population of California sea lions had dropped significantly and the population had grown significantly, leading some to

suggest that diminishing DDx exposure (related to the cessation of DDT use after 1972) was responsible for the rebounding population [88, 89]. However, this conclusion has been contested due to a lack of evidence explicitly demonstrating that contaminants played a role in sea lion reproductive success and population dynamics, which, as discussed above, is difficult to ascertain in free-ranging wildlife [90]. Considering that organismal/population health are ultimately the endpoints of interest to conservation efforts, these studies are valuable. However, as with studies of systemic endpoints, these studies have little capacity to conclusively demonstrate contaminant-mediated endocrine disruption, due simply to the fact that no endocrine-specific endpoints are measured.

In conclusion, the study of endocrine disruption in free-ranging wildlife presents several unique challenges that limit researchers' ability to conclusively demonstrate cause-effect relationships between exposure and health outcomes. Whereas laboratory studies are useful for characterizing causal mechanisms of endocrine disruption due to their ability to control confounders, but are not directly translatable to the real world; field studies are more informative to real-world issues because natural variation is not eliminated, but gathering conclusive, mechanistic evidence is not feasible.

2.8 Summary

DDTs have a broad range of effects on vertebrate endocrine systems – these include effects on steroid hormone synthesis/secretion, transport, metabolism, and signaling – that are largely conserved across vertebrate taxa. Therefore, it is reasonable to hypothesize that other vertebrates in which analogous testing has not been carried out will likely experience similar effects following DDx exposure. Studies in laboratory models are useful for understanding

mechanisms of disruption, while field studies are more useful to wildlife/environmental policy makers and resource managers.

3. Bottlenose Dolphin (*Tursiops truncatus*) Endocrinology

3.1 Female Reproductive Endocrinology

Like most mammals, female dolphins must undergo puberty before they are capable of reproducing, which may occur as early as 8-9 years of age [91]. According to a review of cetacean endocrinology, females in many cetacean species exhibit an increase in circulating P_4 at the onset of sexual maturity, with sexually immature individuals exhibiting concentrations < 1 ng/mL of blood (reviewed in: [92]). Implicit in this is the conclusion that sexually mature females should have P_4 concentrations > 1 ng/mL. However, this threshold is misleading because P_4 concentrations will vary significantly by pregnancy status and during the estrous cycle (see Section 1.2 above). In reality, circulating P_4 concentrations only rise above this threshold when a female is pregnant or in the luteal phase of the estrous cycle [93, 94]. As such, a circulating P_4 concentration < 1 ng/mL cannot be considered a marker of sexual immaturity, but simply indication that the female has not recently ovulated and/or is not pregnant.

Several populations of *T. truncatus* have been shown to exhibit seasonal breeding cycles, meaning that they are only reproductively active during specific seasons of the year [93-97]. In these populations, mature females are anestrus (noncycling) throughout most of the year, then exhibit spikes in P_4 production consistent with having recently ovulated between the spring and fall – though they may be anestrus for a year or more [93-95]. The gestational period for *T. truncatus* is 11-12 months; therefore, in seasonally breeding stocks, calves are born concurrent with breeding season [91, 97, 98]. Evidence suggests that *T. truncatus* are

spontaneous rather than induced ovulators, e.g. they do not require copulation to ovulate [93, 94]. *T. truncatus* is polyestrous, meaning females may cycle more than once per breeding season, with each cycle lasting roughly 30-36 days [94, 95, 99]. Following ovulation and during pregnancy, circulating P_4 concentrations rise above 3 ng/mL; for ovulation without pregnancy, P_4 concentration returned to < 1 ng/mL within one month, while persistent P_4 concentrations > 3 ng/mL for 6 or more weeks is considered sufficient to diagnose pregnancy [93, 94]. The preovulatory surge in E_2 and LH typical of the mammalian estrous cycle has been observed in captive dolphins by monitoring both circulating hormones and excreted hormones/hormone conjugates (per Yoshioka et al. 1986, baseline circulating E_2 was typically < 50 pg/mL, while surge values were generally between 50 and 100 pg/mL), providing evidence that the female mammalian reproductive cycle is conserved in *T. truncatus* [95, 99]. Robeck et al. characterized the estrous cycle of *T. truncatus* in great detail, and concluded that the cycle is roughly 36 days long with an 8 day follicular phase and 19 day luteal phase [99]. They found that the E_2 surge occurred about 8 hours before the preovulatory LH surge, and ovulation, diagnosed by ultrasound, occurred 17-40 hours thereafter [99]. Interestingly, ovariectomized female dolphins exhibited circulating concentrations of P_4 and E_2 comparable to baseline values in intact adult females, indicating that female gonadal steroids are produced in peripheral tissues – the authors suggest the adrenal as a potential source [93]. F, T, and estrogens have been shown to increase during pregnancy in addition to P_4 [11].

3.2 Male Reproductive Endocrinology

Immature males are characterized by smaller testes and circulating T concentrations < 1 ng/mL (reviewed in: [92]). Based on gonadal morphology, male dolphins become sexually mature between the ages of 9 and 13 years old (reviewed in: [100]). T concentrations in sexually

mature males increase during breeding season. Seasonal variation in circulating T was observed in a captive 19 year-old male, ranging from 1.1 ng/mL and 54 ng/mL with a gradual increase beginning in April and peaking in June [101]. Similarly, circulating T concentrations appear to be elevated in both Spring and Fall in a male in another study, though sample size is limited and values are reported by individual with no summary statistics or hypothesis tests [102].

3.3 Stress Endocrinology

As with other mammals, stress stimuli will induce secretion of corticosteroids in *T. truncatus.* Baseline corticosteroid concentrations are difficult to ascertain because the act of capturing and handling is sufficient to induce a stress response in *T. truncatus*. Handling and transport stress led to increased plasma F concentrations in a 19 year-old male bottlenose dolphin [101]. Similarly, capture stress induced an increase in F from a baseline level of 11 µg/mL (30 nmol/mL) to 40 µg/mL (110 nmol/mL) in an hour, while aldosterone, the primary mineralocorticoid, rose from < 100 ng/mL (280 pmol/mL) to 678 ng/mL (1880 pmol/mL) within three hours [103]. Supplementation with additional ACTH did not lead to higher F or aldosterone concentrations, meaning that capture and handling stress was sufficient to produce maximal stimulation of the HPA axis [103]. In a comparison of free-ranging dolphins and semi-domesticated dolphins (distinguished from "captive" animals because, according to the authors, semi-domesticated animals "voluntarily accompany humans on exercises at sea and choose to remain with their handlers"), free-ranging dolphins exhibited significantly higher mean concentrations of F (free-ranging = 26 ng/mL, semi-domesticated = 19 ng/mL) and aldosterone (free-ranging = 0.116 ng/mL, semi-domesticated = 0.028 ng/mL) (these are averages of all animals, irrespective of variation in restraint time) [104]. The semi-domesticated dolphins had been trained to voluntarily submit to blood collection and thus were conditioned to the

stimulus, so it is assumed that the sampling process is not as stress-inducing as in free-ranging animals. The authors claimed that free-ranging animals that were sampled within 1 hour of net encirclement, as opposed to those that were sampled between 1 and 4 hours, had cortisol (F) values statistically indistinguishable from the semi-domesticated animals, but these data were not displayed in the paper [104]. Nonetheless, if we accept that conclusion as true, the circulating F and aldosterone values reported in the semi-domesticated dolphins are likely to be as close to physiological baseline as is conceivably possible, but sampling within an hour of the onset of the capture process should facilitate measurement of baseline F concentrations in wild dolphins. Circulating corticosteroid concentrations were not correlated with capture or restraint time in a population of wild bottlenose dolphins, which may not be surprising considering that all capture times in this study were under the 1hr threshold established by Suzuki et al 1998 [105].

In addition to capture, restraint, and handling stress stimuli, exposure to cold also induced the HPA stress response in captive bottlenose dolphins conditioned to blood sample collection. Serum F and aldosterone concentrations were negatively correlated with water temperature – the colder temperatures were significantly associated with higher F and aldosterone concentrations [106].

3.4 DDT-mediated Endocrine Disruption in Cetaceans

T. truncatus is a long-lived apex predator in the marine environment. Like other marine mammals, *T. truncatus* maintains large lipid reserves in the form of blubber, a specialized form of subcutaneous adipose tissue [107]. Due to these traits, in addition to the propensity of POPs to biomagnify, dolphins bioaccumulate high body burdens of DDTs and other POPs; some of the highest ΣDDT burdens ever reported in wildlife come from odontocetes [79]. Given the body of

evidence summarized above indicating that DDTs impact vertebrate endocrinology, it stands to reason that bottlenose dolphins are very likely to present negative endocrinological effects stemming from their exposure to DDTs.

Remarkably, we have only identified two studies that investigated the effects of DDx exposure on endocrine function in cetaceans – circulating T concentrations were negatively correlated with DDE burden in male Dall's porpoise (*Phocoenoides dalli*) [108], and E_1 was negatively associated with *p,p*-DDE in female pilot whales [109]. This lack of study limits our understanding of, and ability to manage/mitigate, the potential risks posed by DDTs to cetaceans. The overall purpose of this dissertation is to examine the impacts of DDx exposure on steroid hormone homeostasis in one cetacean, the common bottlenose dolphin, in order to better understand the risks posed to the health of these animals by DDTs and, thereby, facilitate better cetacean conservation.

3.5 Dolphins as Sentinels for Ecosystem and Human Health

Understanding the impacts of DDx exposure on *T. truncatus* health has implications beyond cetacean conservation. Marine mammals are considered sentinels of marine ecosystem health. Marine mammals are long-lived, apex predators, thus they likely experience among the highest POP exposure in their food webs due to bioaccumulation and biomagnification (reviewed in: [79]). Since marine mammals are charismatic megafauna, significant changes in population health will likely be noticed by resource managers and the general public and met with public concern [110]. These factors, combined with the fact that bottlenose dolphins often exhibit high site fidelity, makes dolphins excellent indicators of long-term local contamination, i.e. if dolphins are impacted by local DDx contamination, the other local organisms may also be affected [111]. This may be helpful to conservation of less-charismatic/conspicuous animals at

lower trophic levels, for which effects of contaminant exposure may go unnoticed. Additionally, this may be informative for mitigating the risk to other organisms at upper trophic levels, like humans (especially considering that roughly half of the world's human population inhabits coastal areas), that also consume organisms from these ecosystems [110]. Therefore, the impact of this dissertation will not be limited to marine mammal conservation, but may be helpful to general marine ecosystem conservation human and public health.

4. Experimental Design Considerations

4.1 Limitations Posed by the Marine Mammal Protection Act of 1972

The lack of studies investigating the effects of DDx exposure on cetacean endocrine systems is likely at least partially due to the legal and logistical challenges associated with working with marine mammals. Marine mammals in the United States are federally protected under the Marine Mammal Protection Act of 1972 (MMPA), restricting any action considered harassment of marine mammals, including collection of biological samples and dosing with exogenous chemicals [112, 113]. These regulations require that the least invasive sampling methods available be used for collection of biological tissues and prohibits lethal sampling [112, 113]. Ideally, we would design a laboratory-like study in which DDx exposures are controlled via direct dosing, and effects on the endocrine system are assessed directly by collecting and studying internal organs. However, this is unfeasible given the protected status of marine mammals under the MMPA. Internal organs can be collected opportunistically from dead, stranded animals, but this sampling paradigm is inherently biased towards unhealthy and stressed animals, and tissues that are often too decomposed to be useful for many analyses. Therefore, use of stranded animals for studies of endocrine disruption is not ideal. Additionally,

irrespective of these legal concerns, study of cetaceans is further hindered by their size, lifespan, habitat, and nutritional needs, which restrict their captive husbandry to highly specialized facilities and make wild sampling efforts very labor intensive and logistically challenging. Therefore, determining the effects of DDTs on cetacean endocrinology necessitates an approach that uses non-lethal, minimally invasive sampling techniques in animals that are incidentally exposed to DDTs through their diet/habitat.

As discussed in Section 2.8, blood matrices are often used. Unfortunately, collecting blood from free-ranging cetaceans requires capture and restraint, which is an invasive and stressful event for the animal, and is expensive and logistically challenging – particularly in deeper waters – limiting the number of animals that can be sampled. Use of an alternative matrix, which could be collected remotely, would minimize stress to the animals, reduce labor costs for researchers, and allow for collection of samples from more animals, which in turn would aid in endocrine assessment of wild cetaceans. One proposed alternative matrix is blubber.

4.2 Blubber as a Matrix for Endocrine Assessment

There is great interest in the use of blubber as a matrix for endocrine assessment in marine mammals because blubber can be collected remotely by dart biopsy. Blubber is a specialized form of subcutaneous adipose tissue, which contains steroid hormones [107, 114-121]. Blubber hormone measurements have already been used in qualitative diagnostic capacities. Blubber cortisol concentrations were shown to be associated with fatality type in short-beaked common dolphins (*Delphinus delphis*) – beach-stranded animals, the fatality type qualitatively characterized as being more stressful, had higher blubber F concentrations than those killed via fisheries bycatch, (24.3 ng/g versus 3.99 ng/g, respectively) [114]. The authors

consider this indication that blubber F measurements, at least qualitatively, reflect HPA axis stimulation [114]. Adult male *D. delphis* exhibited significantly higher blubber T concentrations (14.3 ng/g) compared to pubertal (2.5 ng/g) and immature males (2.2 ng/g) [115]. Furthermore, average blubber T concentrations in adult male *D. delphis* bycaught in the California gillnet fishery were higher in the summer months (53.9 ng/g) compared to the rest of the year (7.9 ng/g), suggesting that summer is the reproductive seasons for *D. delphis* [115]. Blubber P_4 measurements have been used to diagnose pregnancy in many cetaceans, including *T. truncatus* (pregnant: 54.82 ng/g [n=2], non-pregnant: 6.16 ng/g [n=9]), other odontocetes, and baleen whales [116-119]. Notably, this published work only reports measurements for a small subset of steroid hormones (F, T, and P_4) [114-120]. Boggs et al. (2017) recently measured several other steroid hormones in *T. truncatus*, including 17-hydroxyprogesterone ($17OHP_4$), 11-deoxycorticosterone (DOC), 11-deoxycortisol (S), cortisone (E), and androstenedione (AE), but these measurements have not been linked to any health parameters as of yet [121]. As such, many questions about the endocrinological role of blubber remain unanswered.

While some studies have shown the utility of blubber steroids for qualitative assessment of physiological changes, it is currently unclear whether steroid hormone concentrations in blubber reflect systemic endocrine status. It is uncertain whether blubber steroids are entirely of central origin (e.g. if they were exclusively produced in the gonads/adrenal and delivered by the circulatory system to the blubber) or if some are directly produced by the blubber. The fact that blubber hormone patterns tend to qualitatively match those observed in blood (e.g. increase in P_4 during pregnancy, higher T in reproductively active adult males, higher F in more stressed animals) suggests that blubber hormones are of a central source, unless blubber is responsive to pro-steroidogenic gonadotropin and ACTH signaling. Champagne et al. 2016

reported a positive correlation between F concentrations in blood and blubber in *T. truncatus*, suggesting that F is delivered from a central source to the blubber [120]. Though, with only 57% of the variation in blubber F explained by circulating F concentrations, one must consider whether F may also be produced or metabolized in blubber, either through *de novo* steroidogenesis or through metabolism of circulating precursors [120]. In humans and rodents, adipose tissue expresses 11βHSD and metabolizes corticosteroids, providing preliminary support for the hypothesis that blubber may be a peripheral site of glucocorticoid metabolism [122-124](reviewed in: [3]). Furthermore, Boggs et al. measured E in *T. truncatus* blubber and observed a positive correlation between blubber F and blubber E concentrations, which further suggests that there may be F-E interconversion occurring within the blubber [121] (Boggs et al. unpublished). Therefore, regarding Champagne et al. 2016, perhaps collectively blubber F and E measurements could provide a better estimate of circulating cortisol.

In general, I hypothesize that this question (e.g. whether blubber hormones are of central or local origins) can be better answered by quantifying a larger subset of the steroid hormone pathway in blood and blubber and assessing the relationships between all hormones in both matrices. By answering this question, I will be able to determine whether blubber is an acceptable matrix for quantitative endocrine assessments in *T. truncatus*. Additionally, assessing the steroidogenic/steroid metabolizing function of blubber would improve understanding of the endocrinological role of blubber and facilitate better use of blubber as an endocrinologically-relevant matrix.

4.3 Steroid Hormone Assay Methods

To date, steroid hormones in cetacean blood and blubber have been measured by immunoassays, which utilize antibodies to detect hormones of interest [11, 93-95, 99, 101-106,

114-120, 125-127]. Immunoassays are indirect-detection methods, meaning the quantified endpoints (radioactivity, color change, or light production) are secondary signals generated by the binding of an antibody to a target. Since steroid hormones are structurally similar, steroid hormone immunoassays may be hampered by a lack of specificity –i.e. an antibody for one steroid hormone may cross-react with other similarly structured hormones, thereby artificially inflating the signal. Furthermore, antibodies may interact with components within the matrix, which could interfere with antibody binding the target and/or could artificially inflate the signal through non-specific binding. This is the caveat about cross-species hormone assay methods alluded to in the first section of this chapter – steroid hormones are identical across species, but matrices are not. Human blood and dolphin blood (or human adipose and dolphin blubber) are different matrices, and, therefore, may contain variable interfering compounds, which could alter the applicability of analytical methods across species. Therefore, assay methods need to be validated for different species and different matrices before use.

Another key limitation of immunoassays is that only a single compound can be measured per assay. Thus, to gain a comprehensive understanding of endocrine status in an individual, investigators are required to run an independent immunoassay for each individual hormone. Due to these limitations, investigators typically take a targeted approach to endocrine assessments when using immunoassays, measuring only a small subset of steroids; generally, only the presumptive major hormone within the class of interest will be measured (i.e. when interested in pregnancy, only P_4 will be measured, ignoring the other progestogens and other hormone classes). While utilitarian, this approach disregards potentially interesting and biologically-relevant inter- and intra-class hormonal relationships, especially considering that all steroids fall within a common metabolic pathway.

Unlike immunoassays, mass spectrometry (MS) is a direct-detection method, wherein compound-specific fragmentation patterns are identified and quantified, which eliminates the specificity (cross-reactivity) issues associated with immunoassays detailed above. Matrix-specific interferences still exist and need to be addressed, but they are not related to antibody binding; rather, interferences in MS arise either through presence of compounds with similar fragmentation patterns or enhancing/suppressing ionization of target analytes. Different matrices may require different extraction protocols to sufficiently extract steroids and remove interferences. Use of chromatographic methods before MS enables separation of multiple steroids in a sample extract so multiple steroids can be quantified in a single assay. Therefore, coupling chromatography to MS improves the quantity and quality of endocrinological data generated from a single sample aliquot compared to immunoassays. Boggs et al. recently demonstrated the feasibility of using a reversed phase solid phase extraction (SPE) to liquid-chromatography tandem MS (LC-MS/MS) method to simultaneously quantify 17 steroid hormones spanning all four classes of vertebrate steroid hormones in a single 2 mL aliquot of human serum or plasma [128]. Additionally, the same LC-MS/MS method with a different extraction protocol (salting-out assisted liquid-liquid extraction [SALLE] to dispersive SPE) has been used to measure this same suite of hormones in *T. truncatus* blubber [121]. By employing this LC-MS/MS method we will be able to quantify a larger subset of steroid hormones in both blood and blubber with specificity and feasibility unattainable by immunoassay. In the next chapter, I will discuss the development of SPE to LC-MS/MS methods to measure steroid hormones in dolphin blood matrices (plasma and serum).

4.4 Study Populations

This dissertation utilizes samples from four populations of free-ranging bottlenose dolphins with varying contaminant exposure profiles (Figure 1.4, Table 1.3). Three of these populations (Barataria Bay, LA; Sarasota, FL; Brunswick, GA) were selected as reference populations due to their comparatively low burdens of DDTs (Table 1.3) [129, 130] (Balmer et al. unpublished). We intend to use these populations to characterize steroid hormone profiles in the absence of high DDx exposure in *T. truncatus*. The fourth population, St. Andrews Bay, FL, is characterized by comparatively higher burdens of DDTs and similar or lower concentrations of other endocrine disrupting persistent organic pollutants (Table 1.3). We intend to use this population to examine the effect of elevated DDT exposure on steroid hormone homeostasis in bottlenose dolphins.

Unfortunately, as is the case with most ecological studies, our reference populations are not true references. Animals from all three reference populations exhibit quantifiable burdens of DDTs, likely due to the ubiquitous nature of DDT contamination and *T. truncatus*' propensity for bioaccumulating POPs (Table 1.3). Additionally, these populations experience differential exposure to other contaminants/stressors, which may impact steroid hormone homeostasis. Barataria Bay received heavy oiling following the 2011 Deepwater Horizon (DWH) Oil Spill in the Gulf of Mexico, and the population of dolphins here have exhibited a variety of diseased phenotypes, including impacts on adrenal function, following the spill [131]. Therefore, we cannot define reference/baseline corticosteroid concentrations with this population. Furthermore, considering the HPA/HPG axis cross-talk discussed above (section 1.3, Figure 1.2), these impacts on adrenal function in Barataria Bay dolphins may also precipitate effects on gonadal steroid concentrations. Dolphins from Brunswick, GA have high burdens of PCBs due to local contamination, which, like DDTs, are well-known POPs and endocrine disruptors in

vertebrates (through disruption mechanisms comparable to those of DDTx) (Table 1.3) [61, 73, 132-134] (reviewed in: [135, 136]). Dolphins from Sarasota Bay exhibit greater ∑DDT burdens than Barataria Bay and Brunswick (Table 1.3) [82, 129-131](Balmer et al. unpublished). Therefore, none of these three populations are ideal references. Alternatively, we could consider using captive dolphins as a reference, but dietary exposure to DDTs and other POPS is still relevant in captive dolphins because their diets consist of wild-caught fishes, which will likely contain DDTs. Additionally, they experience different exposures and stressors stemming from their very different habitats and lifestyles. The overall goal of this project is to study free-ranging dolphin endocrinology; therefore, I argue that despite the issues associated with our reference populations, it is more important to minimize the impacts of confounding variables associated with free-ranging versus captive dolphins rather than those associated with our reference sites. In addition, our use of three reference populations rather than a single reference population reduces the potential confounding of any individual site-specific stressors.

St. Andrews Bay, Florida, is a designated EPA Superfund site due to high concentrations of DDTs measured in the sediment. The population of *T. truncatus* inhabiting this site exhibit higher ∑DDT burdens than those in our reference populations, while concentrations of other POPs are comparable to or lower than in reference populations (Table 1.3) [129, 130] (Balmer et al. unpublished). Importantly, the DDx burdens observed in this population are comparable to those observed in St. Lawrence estuary beluga whales where poor recruitment and adrenal pathologies have been anecdotally linked to elevated POP exposure (Tables 1.4 and 1.5) [51, 52]. Samples collected from this population therefore provide an opportunity to examine the impacts of high ∑DDT exposure on dolphin endocrinology without necessitating dosing with DDTs.

5. Hypothesis and Aims

Hypothesis and Rationale: ΣDDT burden is associated with altered systemic progestogen, androgen, estrogen, and corticosteroid concentrations in *T. truncatus*. This hypothesis is derived from the following facts detailed in the previous sections of this chapter: 1) general mammalian HPA and HPG physiology is evolutionarily conserved in *T. truncatus*, 2) DDTs disrupt steroid hormone homeostasis in a diverse variety of vertebrates via mechanisms conserved across taxa, 3) bottlenose dolphins maintain high concentrations of DDTs due to bioaccumulation and biomagnification through the marine food web.

Aim 1: Develop methods for the prediction of circulating steroid hormone concentrations from blubber steroid hormone measurements in *T. truncatus*

1.1 Develop SPE to LC-MS/MS steroid hormone method for dolphin blood

1.2 Quantify steroid hormones in matched blood and blubber samples collected from free-ranging reference (i.e. low ΣDDT exposure) populations of *T. truncatus* by LC-MS/MS

1.3 Model the relationships between steroid hormone concentrations in blood and blubber

Aim 2: Characterize the relationships between steroid hormone concentrations and ΣDDT burden

2.1 Measure steroid hormones in blubber biopsies collected from St. Andrews Bay dolphins, and estimate circulating hormone concentrations using the model developed in Aim 1.c

2.2 Assess relationships between ΣDDT burden (measured by collaborators) and steroid hormone concentrations in both blubber (measured) and blood (predicted)

Aim 3: Examine the capacity of *T. truncatus* blubber to interconvert cortisol and cortisone

3.1 Develop a technique for the extraction of microsomes from *T. truncatus* blubber

3.2 Treat microsomes isolated from *T. truncatus* blubber with cortisol or cortisone and quantify conversion to the cortisone or cortisol, respectively by LC-MS/MS

Table 1.1 Steroid hormone classification and abbreviations

Class	Name	Abbrev.
Progestogens	Progesterone	P_4
	17-Hydroxyprogesterone	$17OHP_4$
	Pregnenolone	P_5
	17-Hydroxypregnenolone	$17OHP_5$
Androgens	Testosterone	T
	Dihydrotestosterone	DHT
	Androstenedione	AE
	Dehydroepiandrosterone	DHEA
Estrogens	Estrone	E_1
	Estradiol	E_2
	Estriol	E_3
Corticosteroids	Cortisol	F
	11-Deoxycortisol	S
	Corticosterone	B
	11-Deoxycorticosterone	DOC
	Cortisone	E

Table 1.2 Names, common abbreviations, cellular location, and steroidogenic steps catalyzed by vertebrate steroidogenic enzymes.

Name	Other Names	Abbrev.	Reactions	Location
Steroidogenic acute regulatory protein		StAR	Cholesterol transport (rate limiting)	Mitochondria
Cytochrome P450 11A1	Cholesterol side-chain cleavage enzyme	CYP11A1; P450scc	Cholesterol → P_5	Mitochondria
Cytochrome P450 17A1	17,20 lyase; 17α-hydroxylase	CYP17	P_5 → $17OHP_5$ → DHEA P_4 → $17OHP_4$ → AE	Microsomes
3β-hydroxysteroid dehydrogenase		3βHSD	P_5 → P_4 $17OHP_5$ → $17OHP_4$ DHEA → AE	Microsomes
Cytochrome P450 21	21-hydroxylase	CYP21	P_4 → DOC $17OHP_4$ → S	Microsomes
Cytochrome P450 11B1	11β-hydroxylase	CYP11B1	DOC → B S → F	Mitochondria
11β-hydroxysteroid dehydrogenase 1		11βHSD1	F → E	Microsomes
11β-hydroxysteroid dehydrogenase 2		11βHSD2	E → F	Microsomes
17β-hydroxysteroid dehydrogenase		17βHSD	AE → T E_1 → E_2	Microsomes
Cytochrome P450 19A1	Aromatase	CYP19; P450arom	AE → E_1 T → E_2	Microsomes
5α-reductase			T → DHT	Microsomes

Table 1.3 Contaminant concentrations (µg/g lipid) measured in male bottlenose dolphin blubber from each of the four populations analyzed in this dissertation. From Kucklick et al. 2011, Balmer et al. 2015, and Balmer et al. unpublished [129, 130].

	Barataria Bay	Sarasota	Brunswick	St. Andrews Bay
∑DDT	16.2	36.5	26.1	67
∑PCB	51.4	71.6	450	70
∑Chlor	3.69	23.0	4.97	2.6
∑PBDE	2.69	1.91	3.61	1.5
Dieldrin	0.52	1.39	0.41	0.2
Mirex	0.17	1.96	2.89	0.2
HCB	0.07	0.086	0.055	0.0

Table 1.4 Review of laboratory studies examining mechanisms of DDx-mediated endocrine disruption. Row colors indicate mechanism of disruption: gray fill = effects on hepatic hormone metabolism; green fill = adrenal steroidogenesis and toxicity; pink fill = ovarian steroidogenesis; orange fill = placental steroidogenesis; yellow fill = estrogen signaling; blue fill = androgen signaling; white fill = steroid binding proteins. *IC50: half maximal inhibitory concentration; **EC50: half maximal effective concentration.

	Target (mechanism)	**Species** (design if not *in vivo*)	**Dose** (length of time)	**Effect**	**Ref.**
Technical Grade DDT	Liver (steroid metabolism and clearance)	Rat	25 or 50 mg/kg twice daily (10 days)	Increased metabolism of T, E_2, P_4, and DOC	[59]
		Guinea pig	150 mg/kg/day (1 week)	Increased metabolism of cortisol	[62]
	Estrogen Signaling	Tiger salamander	Immersed in 0.01 ppm (28 days)	Antagonized systemic effects of E_2	[67]
p,p'-DDT	Adrenal Gland	Cow (*in vitro*)	IC50* = $1.25 \cdot 10^{-5}$ - $3.7 \cdot 10^{-4}$ M	Inhibited adrenal steroidogenic enzyme activity	[40]
		Rat	Food containing 50 ppm (47 days)		[40]
		Rainbow trout (*ex vivo*)	50-100 mg/L		[41]
	Ovary	Pig (*in vitro*)	400 and 4000 ng/mL	Reduced E_2 secretion; increased conversion of T to E_2 (4000 ng/mL only); reduced P_4 secretion (4000 ng/mL only)	[53]
	Placenta	Human (*ex vivo*)	1, 10, 100, 1000 ng/mL	Reduced conversion of DHEA to E_2	[54]
	Liver (steroid metabolism and clearance)	Chicken	1 mg/day (1 week)	Increased metabolism of T	[61]
		Guinea pig	150 mg/kg/day (1 week)	Increased metabolism of cortisol	[62]
	Estrogen Signaling	Human (*in vitro*)	EC50** = 1 µM	Transactivated ER (i.e. estrogenic)	[64]
		Rat	10 and 100 µM	Inhibited E_2 binding by approx. 20%	[65]
	Androgen Signaling	Rat (*in vitro*)	100 µM	Inhibited binding of DHT to AR by 80%	[66]
	Steroid Binding Proteins	Rat (*in vitro*)	100 µM	Inhibited DHT binding to ABP by approx. 60%	[66]

o,p-DDT	Ovary	Pig (*in vitro*)	400 and 4000 ng/mL	Increased E_2 secretion; increased conversion of T to E_2 (4000 ng/mL only); reduced P_4 secretion (4000 ng/mL only)	[53]
	Placenta	Human (*ex vivo*)	1, 10, 100, 1000 ng/mL	Reduced conversion of DHEA to E_2; increased P_4 secretion (100 and 1000 ng/mL only)	[54]
	Liver (steroid metabolism and clearance)	Chicken	1 mg/day (1 week	Increased metabolism of E_2 and AE	[61]
		Domesticated dog	50 mg/kg/day (32 days)	Increased activity of phase I/II biotransformation enzymes responsible for steroid hormone clearance	[45]
	Estrogen Signaling	Human (*in vitro*)	EC50 = 193 nM	Transactivated ER (i.e. estrogenic)	[64]
		Rat (*in vitro*)	0.1-100 µM; 100 µM	Inhibited E_2 binding to ER (dose responsive: min. approx. 10% reduction at 0.1 µM, max. approx. 75% reduction at 10 and 100 µM); inhibited binding of E_2 to ER by 60%	[65, 66]
	Androgen Signaling	Rat (*in vitro*)	100 µM	Inhibited binding of DHT to AR by 90%	[66]
	Steroid Binding Proteins	Rat (*in vitro*)	100 µM	Inhibited DHT binding to SHBG by approx. 30%	[66]
p,p'-DDE	Adrenal Gland	*Sarotherodon aureus* (*in vitro*)	50-150 mg/L	Inhibited adrenal steroidogenic enzyme activity	[43]
	Ovary	Pig (*in vitro*)	400 and 4000 ng/mL	Increased E_2 secretion; increased conversion of T to E_2 (4000 ng/mL only); reduced P_4 secretion (4000 ng/mL only)	[53]
			10 ng/mL	Increased P4 secretion	[57]
	Placenta	Human (*ex vivo*)	1, 10, 100, 1000 ng/mL	Reduced conversion of DHEA to E_2; increased P_4 secretion (100 and 1000 ng/mL only)	[54]
	Liver (steroid metabolism and clearance)	Rat	100 mg/kg/day (7 days); 25 mg/kg/day (7 days); 500 µmol/kg (once)	Induced P450arom expression/activity; increased rate of estrogen metabolism and reduced systemic estrogenic activity (uterotropic assay); increased metabolism of T	[55, 58, 60]

	Estrogen Signaling	Tiger salamander	Immersed in 0.01 ppm (28 days)	Produced systemic estrogenic effects	[67]
	Androgen Receptor	Rat (*in vitro* and *in vivo*)	100 µM (*in vitro*); IC50* = 5 µM (*in vitro,* binding), IC50* = 0.2 µM (*in vitro*, transactivation), 100 mg/kg/day (5 days) (*in vivo,* pregnant dams), 100 mg/kg/day (36 days) (*in vivo,* pubertal male), 200 mg/kg/day (4 days) (*in vivo,* adult males)	Inhibited DHT binding to AR by 100%; inhibited DHT binding to AR (*in vivo*), inhibited AR transactivation (*in vitro*), and antagonized systemic androgenic effects (i.e. acted as an anti-androgenic) in fetal, pubertal, and adult males	[66, 68]
	Steroid Binding Proteins	Rat (*in vitro*)	100 µM	Inhibited DHT binding to ABP by 20%	[66]
o,p-DDE	Ovary	Pig (*in vitro*)	400 and 4000 ng/mL	Increased E_2 secretion; increased conversion of T to E_2 (4,000 ng/mL only)	[53]
	Placental	Human (*ex vivo*)	10, 100, 1000 ng/mL	Reduced conversion of DHEA to E_2; increased P_4 secretion (100 and 1000 ng/mL only)	[54]
	Estrogen Signaling	Human (*in vitro*)	EC50** = 91 nM	Transactivated ER (i.e. estrogenic)	[64]
		Rat (*in vitro*)	0.1-100 µM	Inhibited E_2 binding to ER (dose responsive: min. <10% reduction at 0.1 µM, max. approx. 50% reduction at 100 µM)	[65]
$MeSO_2$-DDE	Adrenal Gland	Mouse	12.5, 25, 50, and 100 mg/kg (once); 25 and 50 mg/kg (once) (pregnant dam)	Necrosis of the *zona fasciculata*	[46, 47]
		Chicken	0.25 mmol/kg (once)	Necrosis of the *zona fasciculata* and *zona reticularis*	[48]

p,p'-DDD	Adrenal Gland	Mink	125 mg/kg (once)	Necrosis of the *zona fasciculata* and *zona reticularis*	[49]
	Liver (steroid metabolism and clearance)	Rat	25 mg/kg/day (7 days)	Increased rate of estrogen metabolism and reduced systemic estrogenic activity (uterotropic assay)	[58]
o,p-DDD (Mitotane)	Adrenal Gland	Domesticated dog	60 mg/kg (once)	Inhibited adrenal steroidogenic enzyme activity/secretion	[37, 38]
		Gray seal (*ex vivo*)	40 µM		[39]
		Cow	IC50* = $4 \cdot 10^{-5}$ - $6 \cdot 10^{-4}$ M		[40]
		Rainbow trout (*ex vivo*)	25-100 mg/L; 75-200 µM		[41, 42]
		Sarotherodon aureus (*in vivo* and *in vitro*)	50 mg/kg (once) (*in vivo*), 0.023 -1 mg/L (*in vitro*); 50 mg/kg (once) (*in vivo*)		[43, 44]
		Mink	125 mg/kg (once)	Necrosis of the *zona fasciculata* and *zona reticularis*	[49]
		Chicken	80 and 100 mg/kg (once)		[48]
	Liver (steroid metabolism and clearance)	Guinea pig	300 mg/kg/day (5 days) then 50 mg/kg/day (7 more days)	Increased cortisol metabolism	[63]
		Sarotherodon aureus	50 mg/kg (once)	Reduced cortisol metabolism	[44]
	Estrogen Signaling	Human (*in vitro*)	1 µM	Transactivated ER (i.e. estrogenic)	[64]
		Rat (*in vitro*)	0.1-100 µM	Inhibited E_2 binding to ER (dose responsive: min. approx. 10% reduction at 0.1 µM, max. approx. 50% reduction at 100 µM)	[65]
	Steroid Binding Proteins	Human	Oral dose sufficient to sustain ≥20 mg/L fasting plasma concentration (daily, 20-24 months); 4-6 g/day (>6 months)	Increased CBG and SHBG expression (liver) and concentration (blood)	[69, 70]

Table 1.5 DDx concentrations (μg/g blubber) by wet weight in blubber from stranded beluga whales in the St. Lawrence estuary (adapted from [51])

	Mean	Std. Dev.	Range	Median
∑DDT	55.59	55.05	1.166 - 225.6	41.18
DDT	13.64	15.26	0.233 - 53.80	10.30
DDE	33.23	34.73	0.530 - 130.0	22.49
DDD	10.91	10.63	0.403 - 35.80	7.36

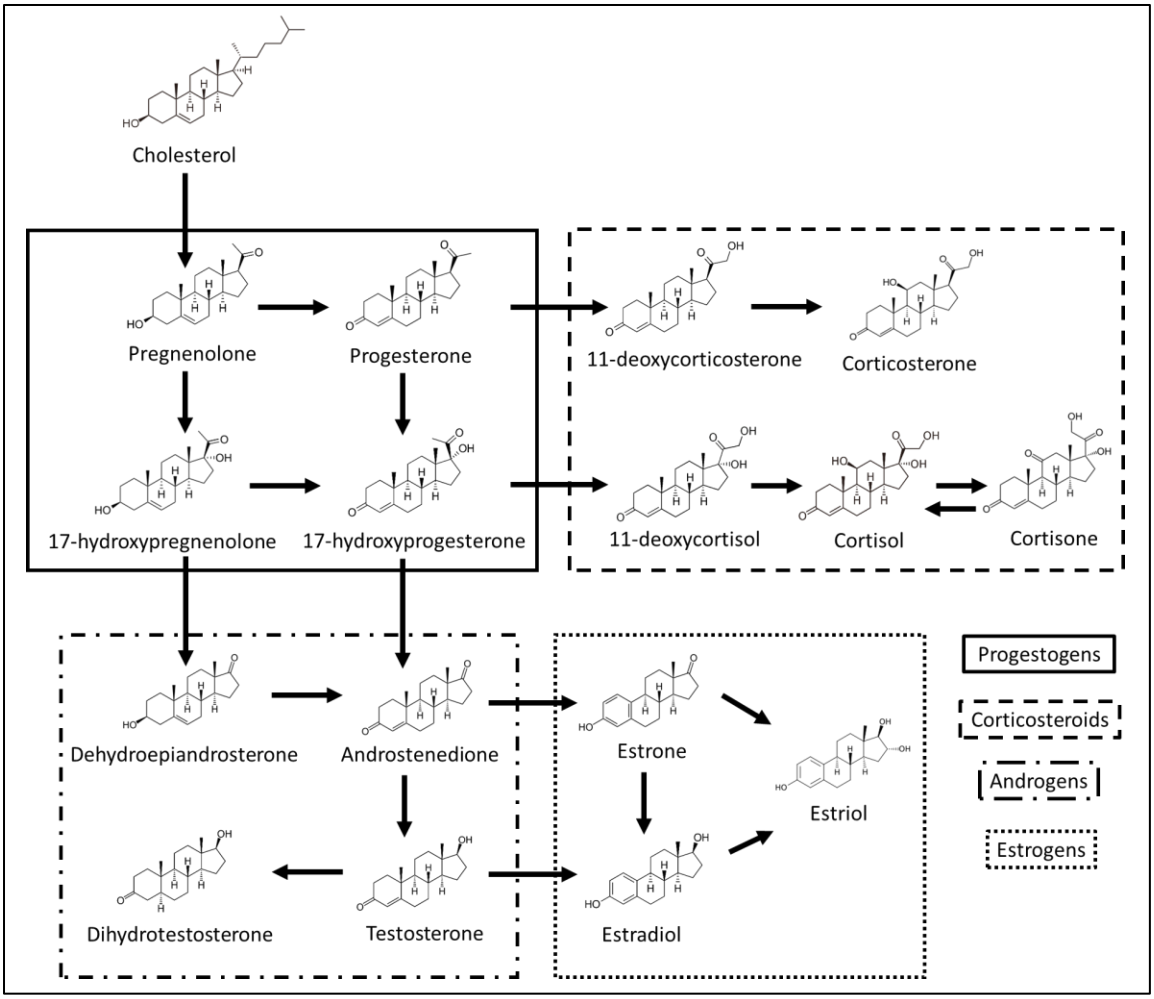

Figure 1.1 The steroidogenesis pathway with steroid structures (from Wikimedia Commons) and names.

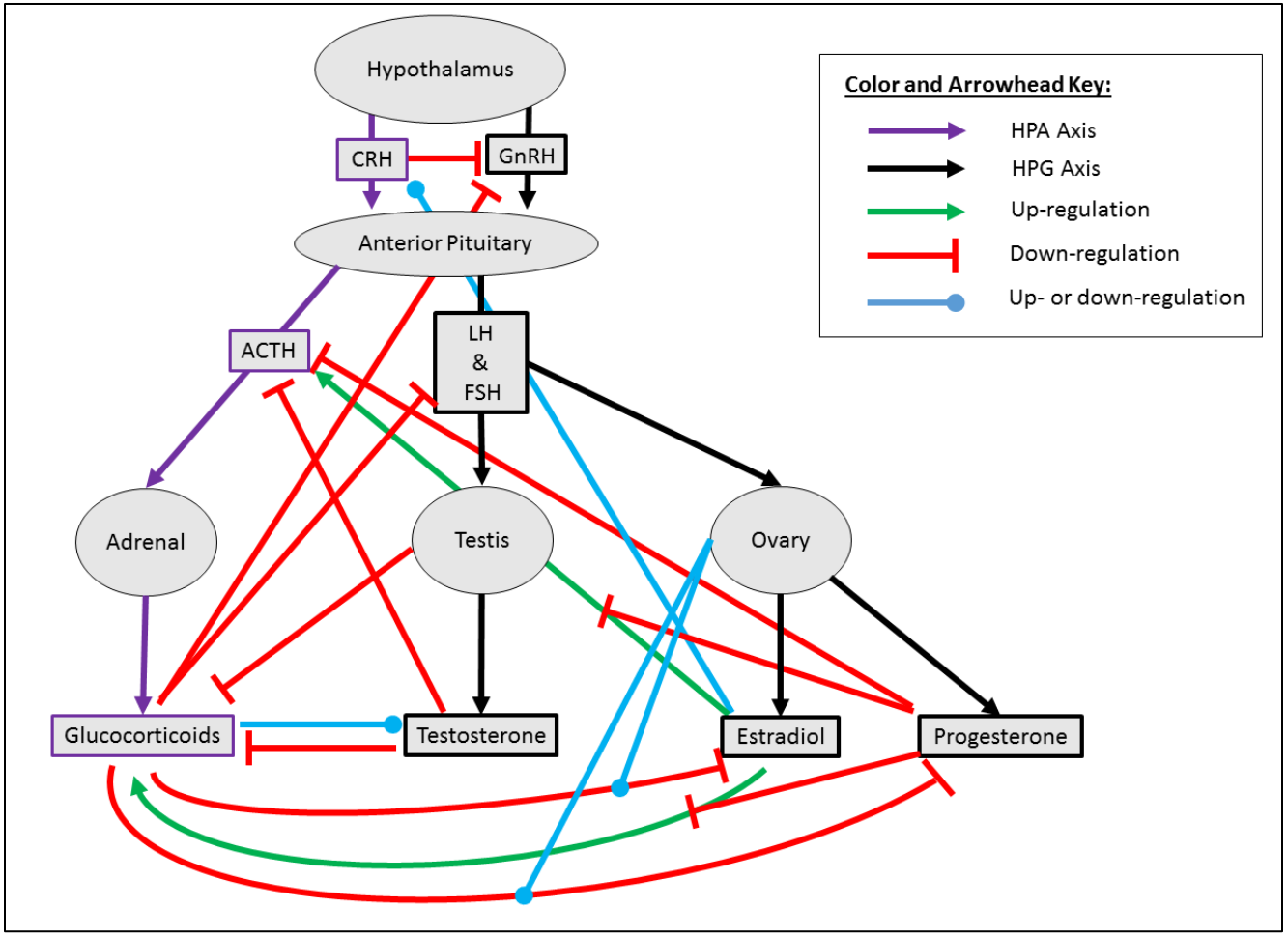

Figure 1.2 Mechanisms of cross-talk between the HPA and HPG endocrine axes. For clarity, intra-axis feedback loops are not included. HPA = hypothalamo-pituitary-adrenal, HPG = hypothalamo-pituitary-gonadal CRH = corticotropin-releasing hormone, ACTH = adrenocorticotropic hormone, GnRH = gonadotropin-releasing hormone, LH = luteinizing hormone, FSH = follicle stimulating hormone

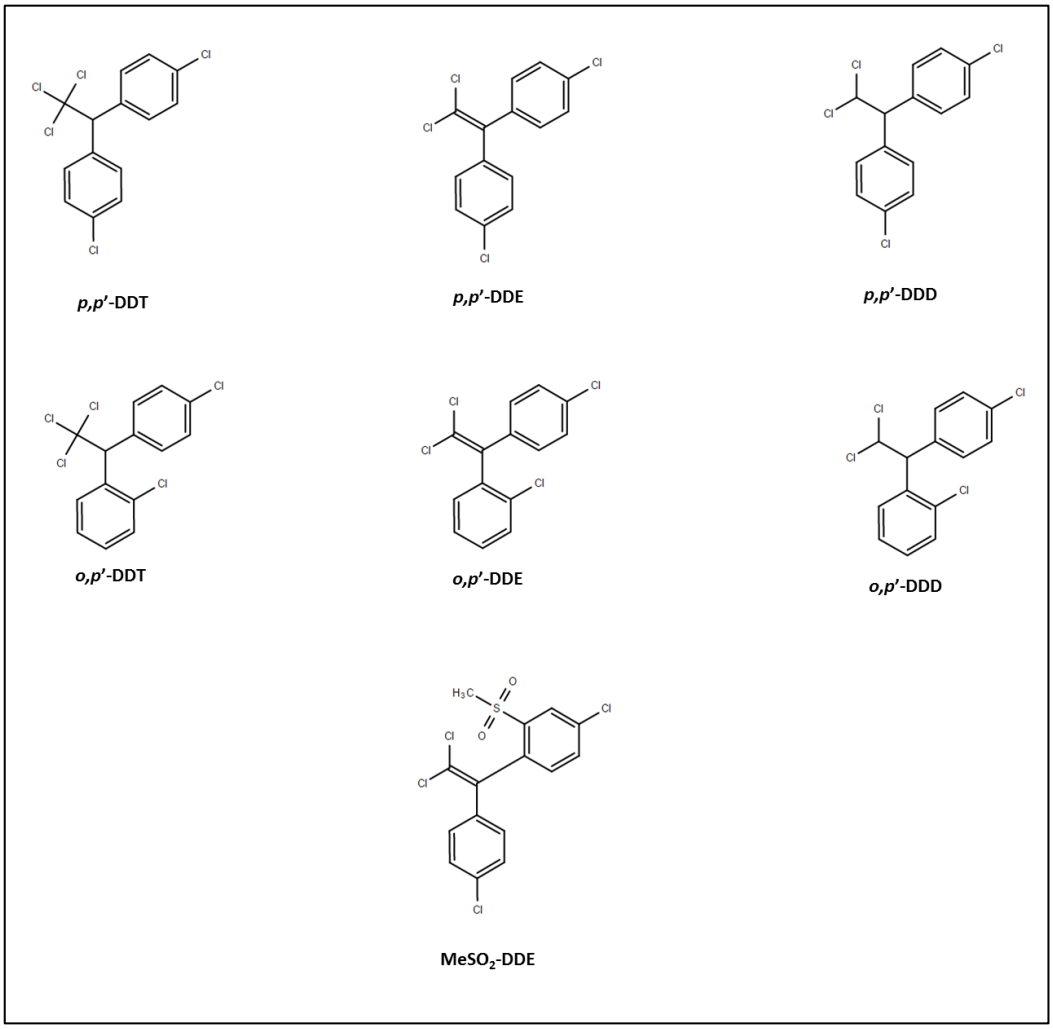

Figure 1.3 DDx chemical structures (produced with ChemSpider.com)

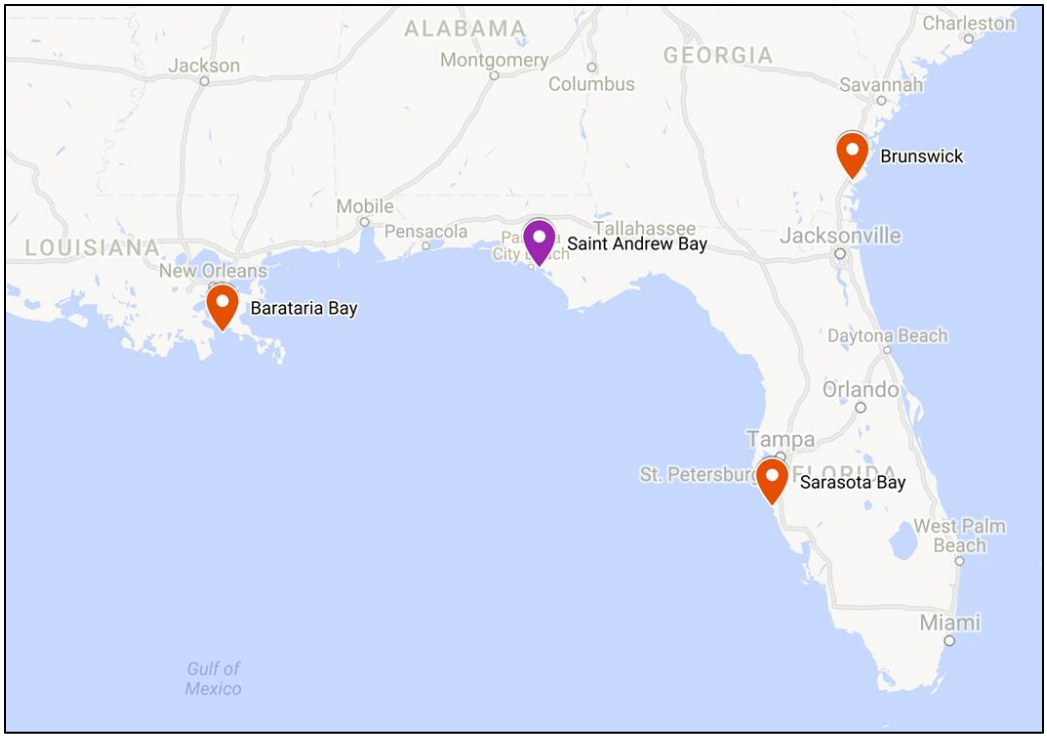

Figure 1.4 Map of study population locations. Orange markers indicate reference populations; the purple marker indicates contaminated population. Made with Google Maps.

CHAPTER 2: Development of a Solid Phase Extraction (SPE) to LC-MS/MS Method for the Measurement of Steroid Hormones in *T. truncatus* Blood

1. Introduction

LC-MS/MS methods provide considerable improvement over traditional steroid hormone quantification methods (immunossays), as discussed in Chapter 1 section 4.3. Thus, I intend to use LC-MS/MS for all hormone measurements in this dissertation. LC-MS/MS methods have been used to measure steroid hormones in several biological matrices, including bottlenose dolphin blubber and human blood, but not bottlenose dolphin blood [121, 128]. Matrix-dependent interferences have the potential to impact quantification in LC-MS/MS. Therefore, in this chapter I aim to validate the use of these methods with bottlenose dolphin blood matrices. Because steroid hormones are identical across species, the instrumental methods for steroid separation and detection will not be altered from previous studies – rather this is an examination of whether a selected extraction method can sufficiently minimize the effects of interferences in bottlenose dolphin blood matrices (plasma and serum) such that accurate and precise steroid hormone measurements can be made by LC-MS/MS. I performed three distinct experiments to assess method and matrix validity. Method accuracy was examined with a spike-recovery experiment, in which I spiked known quantities of steroids into blood matrices and examined the ability of this method to accurately and precisely extract and measure those quantities. Method precision was further assessed by examining variation in repeated measures of endogenous hormone concentrations in subsamples of pooled matrices. These two experiments were performed with both serum and plasma to initially assess matrix suitability. I further compared matrix suitability by comparing measurements made in individual-matched plasma and serum.

2. Materials and Methods

2.1 Animals and Samples

2.1.1 Pooled Samples

Blood samples were collected from adult bottlenose dolphins maintained at the U.S. Navy Marine Mammal Program (Space and Naval Warfare Systems Center Pacific, San Diego, CA) via the arteriovenous plexus of the ventral fluke on various dates in October and November 2012. Sample collections performed at the U.S. Navy Marine Mammal Program were performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the Biosciences Division, Space and Naval Warfare Systems Center Pacific and the Navy Bureau of Medicine and Surgery, and followed all applicable U.S. Department of Defense guidelines for the care and use of laboratory animals. All samples were collected under trained, voluntary participation of the dolphins to avoid issues of handling-induced stress. Plasma was produced by centrifugation of whole blood collected in sodium-heparin vacutainers to prevent coagulation. Serum was produced by centrifugation of whole blood that was allowed to clot for 45 minutes. Samples from each date were pooled by matrix and sex. Pools were frozen in approximately 5 mL aliquots at - 80 ^{o}C, shipped frozen on dry ice to Hollings Marine Laboratory (Charleston, SC), and stored at - 80 ^{o}C until analysis.

2.1.2 Individual-Matched Serum and Plasma

Blood was collected from free-ranging bottlenose dolphins from three sites (Fig. 1.4) in the southeastern United States during capture-release health assessments, including: Barataria Bay, Louisiana (June 2014); Sarasota Bay, Florida (May 2013, 2015, and 2016); and Brunswick, Georgia (September 2015). Methods for the temporary capture and blood collection have been previously described [137-139]. Sarasota Bay sampling was performed under National Marine Fisheries Service (NFMS) Scientific Research Permit No. 15543 and annually renewed IACUC approvals through Mote Marine Laboratory. Barataria Bay and Brunswick sampling was conducted under NMFS permit no. 932-1905/MA-009526 with protocols reviewed and approved by National Oceanic and Atmospheric

Administration IACUC. This sample set includes pregnant (or suspected pregnant) and non-pregnant females (n = 4 and 5, respectively), subadult and adult males (n = 6 and 5, respectively), and samples collected at two different time points during collection, T1 (collected as soon as possible following restraint) and T6 (collected at the end of sampling, immediately preceding release of the animal) (n = 17 and 3, respectively). Pregnancy was diagnosed by ultrasound. Age was determined either through lifelong observation (i.e. known birth date) or through examination of growth layer patterns in teeth using methods that have been described previously [140, 141]. Age classification (i.e. subadult or adult) was defined by age (individuals ≥ 10 years old were classified as adults) or length (individuals ≥ 240 cm total length classified as adult), in the absence of age data. Serum and plasma were produced from whole blood as described above for pooled samples. Aliquots (1 to 2 mL) were frozen and shipped in nitrogen dry shippers to the National Institute of Standards and Technology (NIST) Environmental Specimen Bank (ESB) at Hollings Marine Laboratory (Charleston, SC) where they were stored at - 80 °C until analysis.

2.2 Calibration and Internal Standards

Calibration and isotopically-labeled internal standards were acquired from various manufacturers for use in isotopic dilution quantification (Table 2.1). Calibration (Cal) and internal standard (IS) mixture solutions were diluted in methanol, with the concentration of each compound calculated gravimetrically (ng compound/g mixture). Average masses of IS compounds amended to each tube are reported in Table 2.2; Cal ranges used for quantification are reported in Tables 2.3, 2.4, and 2.5.

2.3 Reverse Phase Solid-Phase Extraction

Steroid hormones were extracted via a method modified from Boggs et al. [128]. IS mixture (100 or 150 µL) was added to clean borosilicate culture tubes, and was dried under nitrogen gas (N_2) at 1.0 to 1.3 bar in a water bath at 40 °C to prevent potential precipitation of blood proteins by the methanol

associated with the IS. Approximately 2 mL of serum or plasma (thawed at room temperature for approximately 30-40 minute), or 0.5 to 1.0 mL of calibration standard was added. The masses of IS and sample matrix (serum, plasma, or calibration standard) were tracked gravimetrically. IS-only blanks were also included, but received no additional matrix. Sodium acetate buffer (4 mL, 0.01 M, pH 5) was added to each tube, vortexed briefly, and incubated at room temperature for 1 hour. During sample incubation, Supelclean LC-18, 6 mL capacity, 1 g bed weight solid-phase extraction columns (Sigma Aldrich; St. Louis, MO) were arranged on a vacuum SPE manifold and conditioned sequentially with 5 mL of methanol, 5 mL of MilliQ water, and 1 mL of sodium acetate buffer (0.01 M, pH 5). After incubation, the sample/buffer mixture was loaded onto the conditioned SPE columns. A vacuum (- 33.3 kPa) was applied as necessary to facilitate the flow of sample through the column. Columns were washed with 12 mL of MilliQ water followed by 5 mL of 80:20 MilliQ water:acetonitrile (volume fraction). A vacuum was applied to ensure removal of all wash solution. Samples were eluted into clean borosilicate culture tubes with 2.5 mL of methanol. Eluent was dried under N_2 at 100 to 130 kPa in a water bath at 40 °C, reconstituted in 200 µL of methanol, and transferred to amber autosampler vials with 250 µL glass inserts.

2.4 Dansyl Chloride Derivatization for Measurement of Estrogens

Dansyl chloride derivatization was performed using methods modified from Nelson et al. [142]. SPE extract (approximately 50 µL) in methanol was transferred to borosilicate tubes containing 200 µL of acetone and 500 µL of sodium bicarbonate buffer (0.1 M, pH 10.5) and was vortexed for 1 min. Dansyl chloride solution (500 µL of a 1 mg/mL; Sigma Aldrich; St. Louis, MO) in acetone was added, and vortexed for 1 min. This mixture was incubated for 3 min in a heat block at 60 °C, and then dried under N_2 at 100 to 130 kPa in a water bath at 40 °C. Dried samples were reconstituted in 2 mL of methanol, filtered by UniPrep 0.2 µm PTFE syringeless filter (Whatman Inc, Piscataway, NJ) to remove excess salts,

and transferred into new borosilicate tubes. Filtered samples were dried as before, reconstituted in 50 µL of methanol, and transferred into a new amber autosampler vial with a 250 µL glass insert.

2.5 Instrumental Methods

Instrumental methods used here have been described previously by Boggs et al. [128]. Three different chromatographic separations were performed: 1) biphenyl separation of underivatized steroids, 2) biphenyl separation of derivatized estrogens, and 3) C18 separation to improve detection of corticosteroids. Instrumental and compound parameters were consistent across methods. I used an Agilent (Santa Clara, CA) 1200 Series HPLC system with a binary pump and an autosampler linked to an AB Sciex (Framingham, MA) API4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer. Instrument parameters for the first biphenyl and C18 separation are the same, where collision gas pressure = 13.8 kPa, curtain gas pressure = 138 kPa, nebulizer gas pressure = 207 kPa, drying gas pressure = 473 kPa, temperature = 700 °C, and ion spray potential = 4500 V; for the dansyl chloride biphenyl separation collision gas pressure = 41.1 kPa, curtain gas pressure = 138 kPa, nebulizer gas pressure = 621 kPa, drying gas pressure = 345 kPa, temperature = 500 °C, and ion spray potential = 4500 V. Compound parameters for each analyte is reported in Table 2.1. Separation of androgens, progestogens, and estrogens was conducted using a Restek (Bellefonte, PA) Ultra Biphenyl column (250 mm x 4.6 mm, 5 µm particle size) and a gradient of acetonitrile and methanol (both containing 0.1% formic acid) beginning with 80% methanol which was decreased to 65% methanol over 20 min, then decreased to 0% methanol over 1 min, held for 5 min, increased to 80% methanol over 0.1 min, and held for 9.9 min. Prior to the C18 separation for corticosteroid measurement, extracts were solvent exchanged into 50:50 methanol:water (volume fraction) by transferring 50 µL of extract into a clean borosilicate culture tube, drying under N_2 at 1.0 to 1.3 bar in a water bath at 40 °C, reconstituting in 50:50 methanol: water solution (volume fraction), and transferring to a new autosampler vial with glass

insert. An Agilent Eclipse Plus C18 column (21 mm x 150 mm, 5.0 µm particle size), and a gradient of methanol and MilliQ water (both with 0.1% acetic acid) held at 46 % methanol for 10 min, increased to 82.5 % methanol over 10 min, then increased to 83.3 % methanol over 5 min was used to separate the corticosteroids. The column was then washed with 100% methanol for 5 min, and re-equilibrated to 46:54 methanol:water (volume fraction) for 10 min. Scheduled multiple reaction monitoring (sMRM) was used. Two transitions were monitored per compound in all separations – the transition with the largest signal was used for quantification, while the other was used for qualitative identity confirmation (Table 2.1).

2.6 Quantification

Chromatographic peaks for target compounds and internal standard compounds were integrated using Sciex Analyst software (Version 1.5; Framingham, MA). Target compound peak areas were divided by the peak area of the matched isotopically labeled internal standard (F-*d*4 was used for S, B, and DOC due to a lack of suitable commercially available isotopically labeled standards). These area ratios were interpolated on regressions calculated from extracted calibration standards (Tables 2.3-5). The standard curve for each compound was comprised of at least three calibration standards, which fully encompassed the range of sample values (Tables 2.3-5). Observed limits of quantification (LOQ_{obs}) are defined as the lowest calibration standard used in the calibration curve; calculated limits of quantification (LOQ_{calc}) were calculated as three times the standard deviation of the mean plus the mean of the extracted blanks as suggested by Ragland, Liebert [143] (Table 2.6).

2.7 Accuracy Assessment: Spike Retrieval

I performed a spike recovery experiment to assess method accuracy and precision in both serum and plasma. I did not have sufficient female serum to perform all experiments in this study, thus I did not include female serum in this experiment. Matrices used in this experiment were charcoal-stripped to

remove endogenous hormones to simplify analysis. Charcoal stripping was performed as follows, based on the method provided in the dextran coated charcoal product information sheet from Sigma Aldrich [144]. Roughly 25 mL of serum and plasma pools were thawed at room temperature and re-pooled by matrix and sex in 50 mL falcon tubes, approximately 500 mg of dextran-coated charcoal (Sigma Aldrich; St. Louis, MO) was added to each, and mixtures were incubated, rocking at 4 °C for 12 hours. Serum coagulated during incubation. Thus, to ensure charcoal stripping of serum was successful, the serum pools were sonicated briefly (1 to 2 min), vortexed for 30 to 60 s to disperse the clots, and incubated rocking at 4 °C for an additional 24 hrs. Charcoal was pelleted by centrifugation at 2,000 x g for 15 min. Stripped serum/plasma was transferred to a new 50 mL falcon tube and used immediately or stored at 4 °C until use (within 24 hours).

The extraction method was slightly modified for this spike retrieval experiment. Following addition of IS, 500 µL of a calibration standard mixture was gravimetrically amended to 9 tubes (n = 3 each for male serum, male plasma, and female plasma) to constitute the steroid spike. Then both the IS and spike were dried, after which sample matrix was added (2 mL; n = 3 per matrix) and extraction proceeded as described above. Calibration standards (n = 6) were not spiked.

Eleven hormones, progesterone (P_4), 17-hydroxyprogesterone ($17OHP_4$), androstenedione (AE), testosterone (T), estradiol (E_2), estrone (E_1), 11-deoxycortisol (S), cortisol (F), cortisone (E), 11-deoxycorticosterone (DOC), and corticosterone (B), were included in this experiment. Method accuracy was determined by calculating percent recovery of each hormone per the following equation:

$$\%\,Recovery = \frac{Recovered\ Hormone\ Mass}{Expected\ Hormone\ Mass} \times 100 = \frac{(a\ \times\ b)}{(c\ \times\ b) + (d\ \times\ e)} \times 100$$

Where “a” is the measured hormone concentration (ng hormone/g sample; this is any residual hormone not removed by charcoal stripping, or, otherwise, the LOQ), “b” is the sample mass (g), “c” is the mean hormone concentration in three unspiked matrix samples (ng/g), “d” is the hormone concentration in the spike mixture (ng/g), and “e” is the spike mass (g). Relative standard deviations (RSDs) of percent recoveries were calculated by sex and matrix to assess method precision. A percent recovery between 70 % and 120 % with an RSD below 15 % was considered comparable to existing techniques for accuracy and precision [114-116, 118, 120, 145].

2.8 Precision Assessment: Comparison of Endogenous Steroid Concentrations in Plasma and Serum Pools

Due to a lack of sufficient serum from any single sampling date, serum pools from multiple sampling dates were thawed and re-pooled (by sex) to provide adequate volume of a homogenous pool for analysis (serum: n = 5 per sex, plasma: n = 4 per sex, calibration standard solution: n = 7, and blanks: n = 3). Upon addition of sodium acetate buffer to female serum samples, the serum coagulated preventing it from mixing with the buffer. Thus, after addition of buffer, these samples were sonicated for 1 to 2 min and then vortexed for 30-60 s in an attempt to disperse clots. This sonicating-vortexing cycle was repeated one to two times until the clots appeared entirely dispersed or showed no improvement in dispersal. Any remaining solid debris was not transferred to the SPE column due to potential for clogging.

2.9 Matrix Assessment: Comparison of Individual-Matched Plasma and Serum

Individual-matched plasma and serum (n=20), calibrants (n=10), and blanks (n=4) were extracted as described above. Serum coagulation occurred in six samples, and was remedied as before by repeated sonication and vortexing.

2.10 Statistical Analysis

Statistical analyses were performed with IBM SPSS Statistics 23 or 24 (IBM, North Castle, NY, USA). For all hypothesis tests, $\alpha = 0.05$. Pearson's (r) or Kendall's tau-b (τ_b) correlations were utilized for the matrix assessment experiment to examine the relationship between hormone measurements in matched serum and plasma. Kendall's tau-b was used for P_4, $17OHP_4$, T, and AE because these variables are left-censored (i.e. contain measurement values below LOQ), and, rather than substituting arbitrary values for measurements below the LOQ, I censored values below LOQ to the same value below LOQ and utilized this non-parametric test. The same censoring value was to ensure that all values below LOQ would be tied in rank-based statistical tests. E measurements were not left censored, but neither raw nor $\log_{10}$ transformed values met the assumptions of Pearson's correlation, thus this relationship was also analyzed by Kendall's tau-b. F was not censored and met the assumptions of Pearson's correlation once $\log_{10}$ transformed; therefore, the relationship between plasma and serum F was assessed by Pearson's correlation.

3. Results

3.1 Accuracy Assessment: Spike Retrieval

I performed a spike recovery experiment to examine method accuracy and precision in female plasma, male serum, and male plasma. Eight of the eleven hormones met the criteria of acceptable accuracy (70 % to 120 % recovery) and precision (< 15 % RSD) in all three matrices tested (Fig. 2.1). These were P_4, $17OHP_4$, AE, T, E_1, E_2, F, and E (Fig. 2.1) Extraction efficiencies for S, B, and DOC were highly variable, and generally did not meet the criteria for acceptance. Only DOC in female plasma met the criteria, with 106 % recovery and 8 % RSD (Fig. 2.1). S was within the acceptable range for female plasma (75 % recovery) but had an RSD of 22 % (Fig. 2.1).

3.2 Precision Assessment: Comparison of Endogenous Steroid Concentrations in Plasma and Serum Pools

I assessed method precision by measuring endogenous hormone concentrations in pooled male and female plasma and serum in quadruplicate (plasma) or quintuplicate (serum), and calculating RSDs for each hormone within each matrix. As in the extraction efficiency experiment, an RSD less than 15 % is considered acceptable precision. Endogenous P_4 concentrations was not detected in any of the matrices. AE was only detected in male matrices and exhibited RSD less than 15 % in both serum and plasma (Fig. 2.2). $17OHP_4$, T, E, and F were detectable in both matrices from both sexes (Fig. 2.2). $17OHP_4$, T, E, and F were below the 15 % RSD threshold in female plasma, male plasma, and male serum, but not in female serum (Fig. 2.2). RSDs for all quantifiable hormones were lower in plasma than in serum, regardless of sex (Fig. 2.2).

3.3 Matrix Assessment: Comparison of Individual Matched Plasma and Serum

I assayed endogenous steroids in individual-matched serum and plasma from free-ranging bottlenose dolphins, and examined the relationships between hormone concentrations in each matrix. Hormone measurements in serum compared to plasma were significantly ($p < 0.05$) and positively correlated for all six detectable hormones ($17OHP_4$ [$\tau_b = 0.730$], P_4 [$\tau_b = 0.465$], T [$\tau_b = 0.644$], AE [$\tau_b = 0.674$], F [$r = 0.822$], and E [$\tau_b = 0.758$]) (Fig 2.3). Note that unlike in the previous experiments, which utilized pooled blood matrices, P_4 was quantifiable in several samples in this sample set.

4. Discussion

The purpose of this study was to validate the use of SPE to LC-MS/MS methods to measure circulating steroid hormone profiles in bottlenose dolphins. Method accuracy and precision, and matrix suitability were tested with three experiments. Through the method accuracy (spike recovery)

experiment, I demonstrated that spiked quantities of eight of the eleven tested hormones (P_4, $17OHP_4$, T, AE, E_2, E_1, F, and E) can be accurately and precisely extracted and quantified by SPE to LC-MS/MS in bottlenose dolphin serum and plasma. I suspect that the failure of S, DOC, and B to meet the criteria for acceptance stems from the lack of commercially available isotopically labeled standards for these compounds. As such, I used alternative internal standard compounds to quantify these hormones by isotopic dilution; I tested both F-d_4 and E-$^{13}C_3$, but neither produced acceptable results, thus illustrating the importance of using matched isotopically labeled internal standards for measurement in complex biological matrices. The difference in retention time in the C18 chromatography between the target analytes (B = 20.0 min, S = 20.5 min, DOC = 22.3 min) and internal standards (E-$^{13}C_3$ = 12.4 min and F-d_4 = 16.0 min) indicates that E-$^{13}C_3$ and F-d_4 are poor internal standards for these analytes. Should isotopically labeled standards for S, DOC, or B become available, it would be worthwhile to repeat this experiment using those internal standards. Without such standards, this method should not be used to measure S, DOC, or B in dolphin blood matrices. However, the utility for qualitative assessments (i.e. absence/presence) is acceptable.

Interestingly, I observed that charcoal-stripping did not completely remove endogenous hormones from blood matrices. Specifically, endogenous T and F were detectable in stripped male matrices. This did not impact the calculation of percent recoveries because the equation accounts for the potential presence of endogenous hormones. In the future, the charcoal stripping methods used here should be modified to ensure complete removal potentially by using more charcoal, extending treatment time, or both.

Having established that this method can accurately and precisely measure known quantities of several steroid hormones in both serum and plasma, I examined precision of endogenous hormone measurements in both matrices. Five hormones were detected: $17OHP_4$, T, AE, F, and E. For all five

hormones, plasma exhibited lower RSDs than serum in both sexes. Measurements made in female serum were imprecise, exceeding the 15% RSD threshold by 8.9% to 14%, which may stem from the difficulties that arose during extraction (i.e. the coagulation issues discussed in section 2.8). Because I was unable to fully disperse the clots and the remaining solid debris was not loaded onto the SPE columns, variable quantities of hormones could have been retained in the solid debris and thus left unextracted. This loss could be accounted for because the IS mixture was added before the buffer and concentrations were calculated by isotopic dilution, so long as the IS has sufficient time to equilibrate/bind with the residual protein in the unextracted clot; if this equilibrium is not reached, then the IS would not account for this loss. Nonetheless, this could have introduced additional variation to female serum measurements, contributing to lower precision.

To my knowledge, this is the first time $17OHP_4$, AE, and E have been measured in dolphin blood. Furthermore, because the chromatographic method enables me to do so, I screened for endogenous concentrations of eleven other hormones, including pregnenolone (P_5), 17-hydroxypregnenolone ($17OHP_5$), P_4, DOC, B, S, dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), E_1, E_2, and estriol (E_3). However, none of these eleven were found at detectable concentrations in the pooled matrices. This is unsurprising for numerous reasons. P_4 and estrogens have been measured in bottlenose dolphin blood in other studies, and circulating concentrations of these hormones depend on reproductive status [91, 93-95, 125, 127]. P_4 should only be elevated in female individuals that are pregnant or in the luteal phase of the estrous cycle, while estrogens would be elevated in female individuals in the follicular phase of the estrous cycle, particularly immediately preceding ovulation [91, 93-95, 99, 125, 127]. None of the females in this portion of the study were pregnant. Bottlenose dolphins have been shown to exhibit somewhat seasonal patterns in reproductive activity, with the reproductively active season typically falling between spring and early fall [93-95, 146]. Therefore, because sampling occurred in mid-

fall, it is unlikely that any of the females sampled to produce the blood matrix pools were actively cycling. Furthermore, because these are pooled samples, even if an individual had elevated P_4 or estrogens, these hormones may be diluted to below LOQ by pooling with other, non-cycling animals. Thus, low P_4 and estrogen concentrations are to be expected in these blood matrix pools. Through the method accuracy experiment, I demonstrated that I am able to accurately and precisely measure spiked quantities of P_4, E_2, and E_1. Thus, this method could potentially be used for quantification of these hormones. As for the ten remaining hormones that were not detected in this study (P_5, $17OHP_5$, DOC, B, S, DHEA, DHT, and E_3), these hormones have not been measured in dolphin blood or blubber. I suspect P_5, $17OHP_5$, DOC, B, S, and DHEA are only present in steroidogenic tissues to serve as precursors to other hormones, meaning very little would be secreted and evident in circulation. DHT and E_3, if produced, may be formed by metabolism of other hormones in peripheral tissues, once again meaning very little would be secreted into the blood stream. Overall, this method provides improvement over traditional methods by allowing for the simultaneous measurement of at least five (and potentially eight) steroid hormones at endogenous concentrations. This will allow investigators to more thoroughly assess steroid hormone homeostasis and characterize relationships between hormones within and among the various steroid hormone classes in bottlenose dolphins.

For several reasons, I conclude that plasma is the preferred matrix for future applications of this method. First, plasma steroid measurements exhibit better precision than serum measurements. Second, plasma is unaffected by the coagulation issue observed in serum making plasma easier to process. Furthermore, upon thawing serum pools, I found that a significant portion (roughly 20-50 %) of the volume of serum within the tube was coagulated before the addition of buffer. This coagulation precluded mixing of the aliquot and caused difficulty when transferring serum into the culture tube. Again, this makes processing more difficult and potentially introduces additional variation. Therefore,

due to concerns over precision and feasibility, plasma is better suited to this method. Because measurements in female serum all had RSDs greater than the 15 % threshold, future applications of this method to female blood should use plasma. Some investigators might be hesitant to use plasma due to the use of anti-coagulant additives (sodium-heparin, in this case) in the production of plasma, which introduces the potential for plasma-specific interferences. However, if plasma-specific interferences were problematic, they would have been evident in the method accuracy experiment. Plasma measurements were within the acceptable percent recovery range, meaning if plasma-specific interferences were present, they did not significantly impact method accuracy.

It is important to note that I did not compare hormone concentrations across matrices in the endogenous precision experiment because each matrix was derived from a separate pool. In other words, the blood used to produce the serum pools was collected on different dates from the plasma pools (roughly two weeks apart) which also means the pools may have been comprised of samples collected from different animals. Considering that hormone concentrations could vary temporally and by individual, comparing hormone concentrations across matrices in the endogenous precision experiment with these pools would be inappropriate. Since several previous studies of bottlenose dolphin endocrinology have used serum while we, instead, recommend using plasma, it is important that I characterize the relationship between hormone measurements in both matrices.

Thus, I used individual-matched plasma and serum samples from free-ranging bottlenose dolphins to assess and compare serum and plasma hormone measurements. I found that measurements were significantly and positively correlated across matrices. For F and E, these relationships seemed the strongest at low-to-mid plasma concentrations, while high plasma values were not well matched in serum. This could potentially be due to loss of hormone associated with coagulation during processing and/or extraction, or poorly matched IS masses. Nonetheless, I have demonstrated that serum and

plasma hormone values, as measured by SPE to LC-MS/MS, are in good agreement, providing assurance that measurements made in plasma are sufficiently comparable to serum. Future experiments with larger samples sizes and wider ranges of endogenous values or different IS masses could potentially yield quantitative models that can be used to predict plasma hormone concentrations from serum measurements (or vice versa).

This sample set included pregnant and non-pregnant females, subadult and adult males, and samples collected at different time points for specific individuals at either end of the sampling process (T1 and T6). P_4 secretion increases during pregnancy; thus, the inclusion of pregnant animals allowed me to detect and quantify endogenous P_4, whereas it was undetectable in pooled samples [93, 94]. However, in this experiment each sample was only extracted and measured once (as opposed to in replicate) due to limited sample volume, thus I cannot assess precision of these P_4 measurements. T is a marker of sexual maturity in male bottlenose dolphins [101, 102]. Therefore, I included adult and subadult males in this study to broaden the range of T concentrations measured. Capture and handling stimulates the hypothalamic-pituitary-adrenal axis, leading to elevated secretion of F in bottlenose dolphins [103, 104]. I included T1 and T6 samples to widen the range of elapsed time in this sample set with the intention of also widening the range of F observed. I refrain from discussing how hormone values vary by these demographic variables in this chapter because these relationships will be examined in the next chapter with greater sample size.

As in the previous experiments, endogenous estrogens were not detected in any matched serum/plasma samples. In this experiment, the lowest calibration standard, with E_2 and E_1 concentrations of 45.3 and 74.4 pg/g respectively, had distinct peaks for each, indicating I should detect concentrations this low. In bottlenose dolphins, baseline circulating E_2 concentrations have been measured at less than 50 pg/mL, while concentrations during the preovulatory surge at the end of the

follicular phase of the estrous cycle tend to fall between 50 and 100 pg/mL [95, 99]. Thus, I conclude that this method likely has the capacity to detect and quantify E_2 surge values, but potentially cannot be used to measure baseline values as currently defined within the literature. Granted, these baseline and surge values were established by immunoassay in serum, meaning they could potentially be different in plasma and in measurement made by LC-MS/MS, given the limitations of immunoassays (discussed in Chapter 1 section 4.3) and considering potential differences in serum and plasma.

Overall, I have demonstrated that use of an SPE to LC-MS/MS method allows for the simultaneous measurement of multi-class steroid hormones in bottlenose dolphin blood matrices, including not only hormones that have been measured previously by immunoassay (P_4, T, E_2, and F) but four hormones that, to my knowledge, have not been reported for dolphin blood ($17OHP_4$, AE, E_1, and E). Therefore, this SPE to LC-MS/MS method allows for more thorough assessment of steroid hormone homeostasis in bottlenose dolphins with an efficiency and specificity that was previously reported.

Table 2.1 Calibration and internal standard compound manufacturer, purity information, and monitored transitions with mass spectrometry compound parameters (DP = declustering potential, EP = entrance potential, CE = collision energy potential, CXP = collision exit potential, RT = retention time). The first transition (product ion) listed for each analyte is the quantitative transition, while the second transition was used for qualitative identity confirmation.

Analyte	**Manufacturer**	**Stated Purity**	**Precursor ion** (m/z)	**Product Ion** (m/z)	**DP** (V)	**EP** (V)	**CE** (V)	**CXP** (V)	**RT** (min)
P_4	Sigma Aldrich	≥ 99 %	314.7	109.2	100	10	30	15	13.5
				97.2	125	10	25	12	
$17OHP_4$	Sigma Aldrich	≥ 95 %	331	97.2	75	15	35	10	8.9
				109.2	100	15	35	20	
AE	Steraloids	≥ 98 %	287.1	97.2	50	10	35	15	11.8
				109.2	100	15	30	20	
T	Sigma Aldrich	≥ 98 %	288.9	109.2	100	10	30	15	9.2
				97.1	125	5	35	10	
E_1	Sigma Aldrich	≥ 99 %	504.5[a]	171.3[a]	25	5	60	20	181.7
				440.1[a]	125	5	30	20	
E_2	Sigma Aldrich	≥ 98 %	506.2[a]	170.9[a]	100	5	60	15	13
				442.3[a]	125	10	30	30	
F	Sigma Aldrich	≥ 98 %	363.2	121.3	50	5	25	15	16
				267.3	25	5	30	30	
E	Sigma Aldrich	≥ 98 %	361.1	163.3	100	5	30	12	12.4
				121.3	75	10	60	15	
S	Steraloids	99.10%	347.3	109.2	25	5	35	10	20.5
				97	100	5	25	20	
B	Sigma Aldrich	≥ 98.5 %	347.3	135	25	5	35	15	20.1
				121	125	15	30	12	
DOC	Steraloids	≥ 98 %	331.1	97.1	25	10	25	15	22.3
				109.2	100	15	25	10	

P_4-$^{13}C_3$	Cambridge Isotopes	98%	318.3	100	75	10	30	20	13.5
				112.1	50	15	30	20	
17OHP$_4$-$^{13}C_3$	Cerilliant	99.99%	334.1	112.1	50	10	35	10	8.9
				100	100	10	35	30	
AE-$^{13}C_3$	Cerilliant	99.99%	290.2	100.3	100	15	35	10	11.8
				112.2	100	15	30	15	
T-$^{13}C_3$	Cerilliant	99.9 9%	292.1	112	75	10	35	12	9.2
				100	75	10	35	20	
E_2-$^{13}C_3$	Cerilliant	99.99%	509.4[a]	170.9[a]	25	5	60	15	13
				NA[b]					
F-d_4	Cerilliant	99.99%	367.3	121.2	25	5	60	10	16
				271.5	25	5	20	10	
E-$^{13}C_3$	Sigma Aldrich	98%	364.2	166.5	75	15	60	10	12.4
				124.1	50	15	60	5	

[a] These are the dansyl chloride-derivatized values

[b] No suitable secondary fragment was identified for E_2-$^{13}C_3$ (i.e., intensities of potential secondary transitions were poor under instrumental parameters utilized)

Table 2.2 Internal standard (IS) mixture concentrations and approximate range of masses amended to samples across all experiments

Compound	**Concentration in IS Mix** (ng/g)	**Approximate Spike Mass Range** (ng)
P_4-$^{13}C_3$	236	18.7 to 28.1
17OHP_4-$^{13}C_3$	214	17.0 to 25.4
F-d_4	222	17.6 to 26.5
E-$^{13}C_3$	218	17.3 to 26.0
T-$^{13}C_3$	244	19.4 to 29.0
AE-$^{13}C_3$	241	19.1 to 28.6
E_2-$^{13}C_3$	245	19.5 to 29.2

Table 2.3 Calibration curve information for accuracy assessment

Compound	Cal Range (ng)	Curve Points	Curve	Intercept	Slope or a, b Coefficients	r^2
P_4	10.8 - 0.464	4	Quadratic	0.00	0.0033, 0.00	1.000
$17OHP_4$	110 - 0.539	6	Quadratic	0.0426	0.0831; 0.1739	1.000
DOC	5.69 - 0.118	3	Quadratic	0.1675	90.9877; -11.476	1.000
B	21.7 - 0.452	4	Quadratic	-0.0404	-0.5972; 2.5464	0.998
S	8.40 - 0.175	4	Quadratic	-0.2051	-58.219; 43.070	0.999
F	41.4 - 0.862	4	Linear	0.0126	0.0126	0.999
E	9.71 - 0.202	3	Linear	-0.0082	2.1937	1.000
T	24.0 - 0.587	4	Quadratic	0.0012	0.1002; 0.303	1.000
AE	2.65 - 0.0551	4	Linear	0.055	1.3518	0.998
E_2	8.81 - 0.183	4	Linear	0.0088	0.7251	0.998
E_1	4.98 - 0.104	4	Linear	-0.0003	1.9421	1.000

Table 2.4 Calibration curve information for precision assessment

Compound	Cal Range (ng)	Curve Points	Curve	Intercept	Slope or a, b Coefficients	r^2
$17OHP_4$	26.1 - 0.114	4	Quadratic	-0.0024	-0.2896; 0.6818	1.000
F	41.7 0 - 00.0903	4	Linear	0.0144	0.6697	0.997
E	4.04 - 0.0836	4	Quadratic	-0.0166	-15.695; 6.0083	0.999
T	252 - 0.259	6	Linear	-0.0104	0.1886	0.999
AE	9.46 - 0.00973	4	Quadratic	0.0305	15.6366; -0.838	1.000

Table 2.5 Calibration curve information for matrix assessment

Compound	**Cal Range** (ng)	**Curve Points**	**Curve**	**Intercept**	**Slope**	**r^2**
P_4	113 - 0.116	6	Linear	0.000	0.0032	1.000
$17OHP_4$	10.9 - 0.114	5	Linear	-0.0029	0.4855	1.000
F	87.4 -0.853	5	Linear	-0.1882	1.7236	0.999
E	81.0 - 0.200	5	Linear	-0.2434	4.6262	1.000
T	251 - 0.259	7	Linear	0.0007	0.1293	1.000
AE (high)	9.44 - 0.460	3	Linear	-0.2435	9.2937	0.999
AE (low)	0.929 - 0.00971	5	Linear	-0.0026	3.0056	0.999

Table 2.6 Limits of quantification (ng) by experiment. Observed limits of quantification (LOQ_{obs}) determined by the lowest calibration standard used in the calculation of the standard curve. Calculated limits of quantification (LOQ_{calc}) were calculated as three times the standard deviation of blank measurements plus mean of blank measurements

Compound	**Accuracy Assessment**		**Precision Assessment**		**Matrix Assessment**	
	LOQ_{obs}	**LOQ_{calc}**	**LOQ_{obs}**	**LOQ_{calc}**	**LOQ_{obs}**	**LOQ_{calc}**
P_4	0.464	0.927	NQ	NQ	0.116	0.270
$17OHP_4$	0.539	-	0.114	0.105	0.114	0.107
DOC	0.118	2.23	NQ	NQ	NQ	NQ
B	0.452	0.327	NQ	NQ	NQ	NQ
S	0.175	0.102	NQ	NQ	NQ	NQ
F	0.862	-	0.0903	NA	0.853	1.87
E	0.202	0.0771	0.0836	NA	0.200	0.845
T	0.587	-	0.259	1.64	0.259	-
AE	0.0551	-	0.195	-	0.00971	0.0164
E_2	0.183	-	NQ	NQ	NQ	NQ
E_1	0.104	0.00701	NQ	NQ	NQ	NQ

- = Negative value
NQ = analyte not detected in experiment
NA = could not be calculated

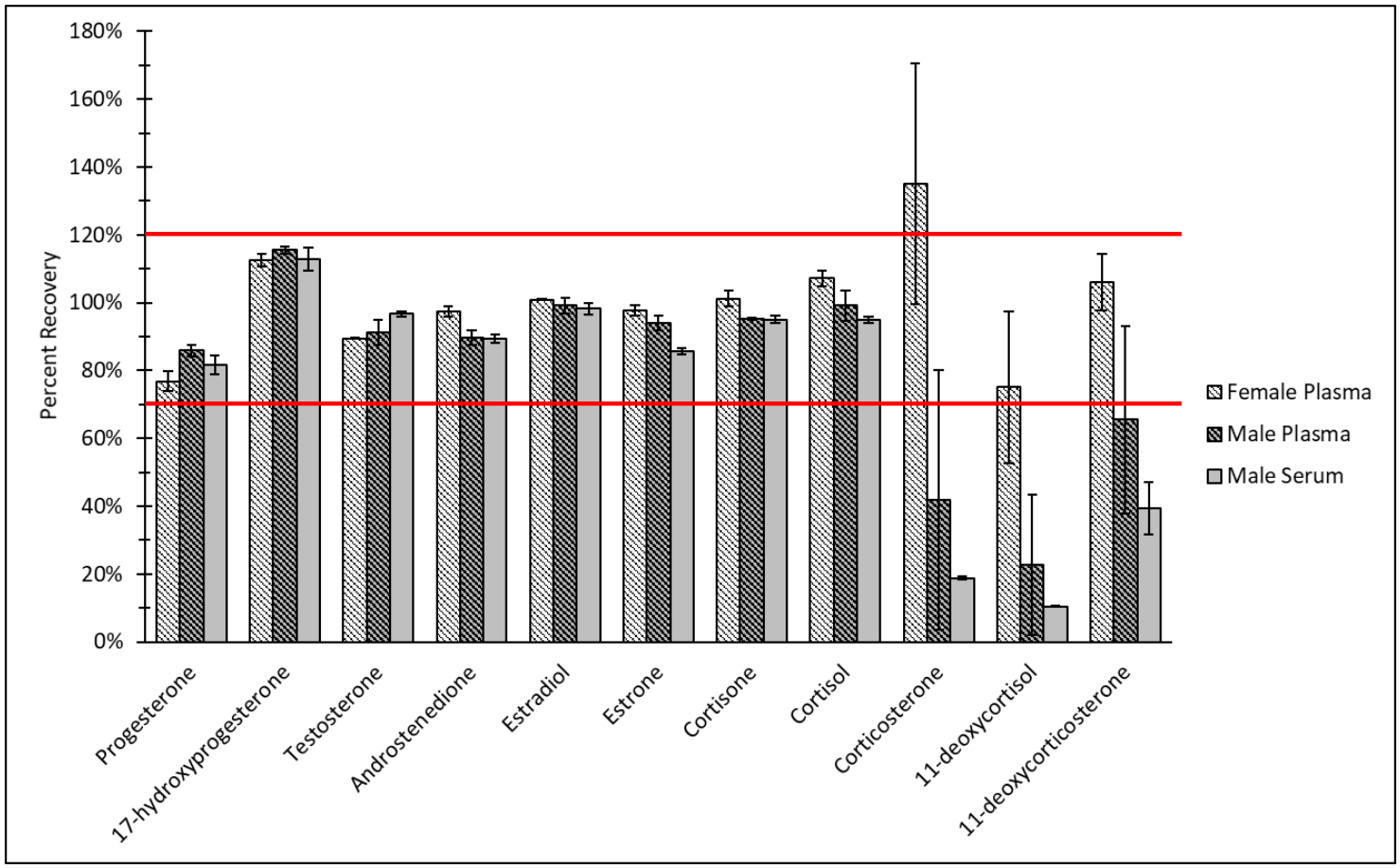

Fig. 2.1 Average percent recovery of each steroid hormone by sample matrix. Error bars indicate standard deviation, solid red lines in dicate the upper and lower threshold values for acceptable accuracy (between 70 and 120 %

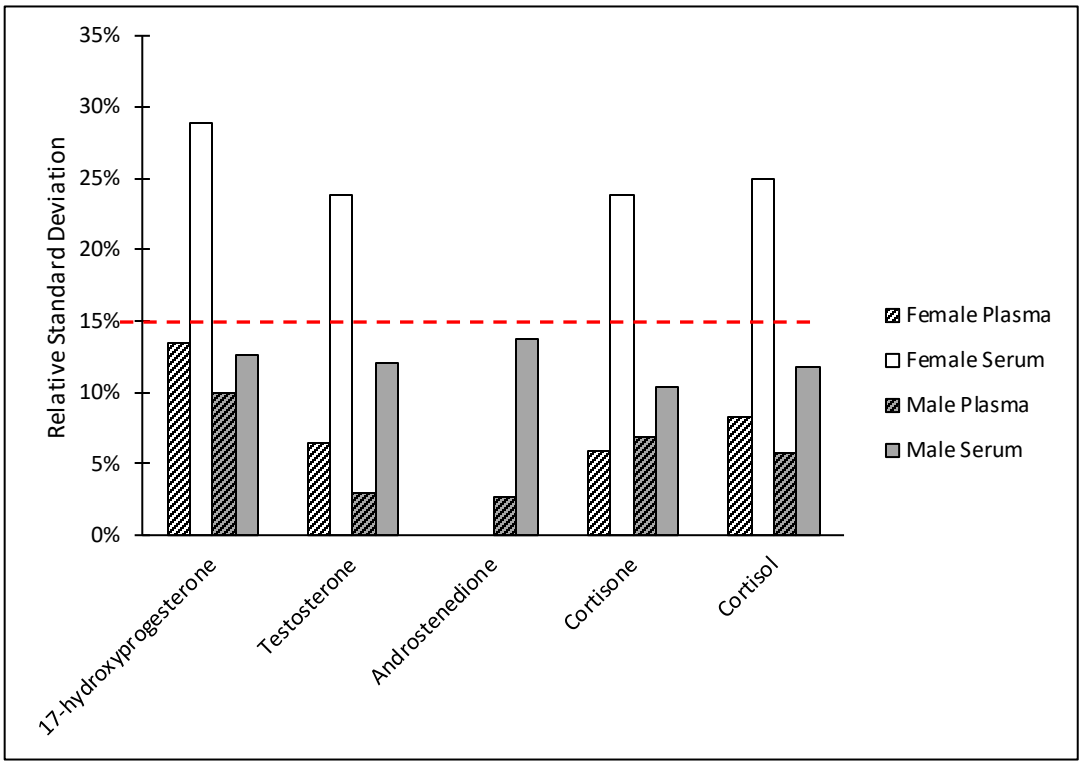

Fig. 2.2 Percent RSD of endogenous steroid hormone measurements made in quadruplicate or quintuplicate. Dashed red line indicates the threshold for acceptable precision (< 15 % RSD)

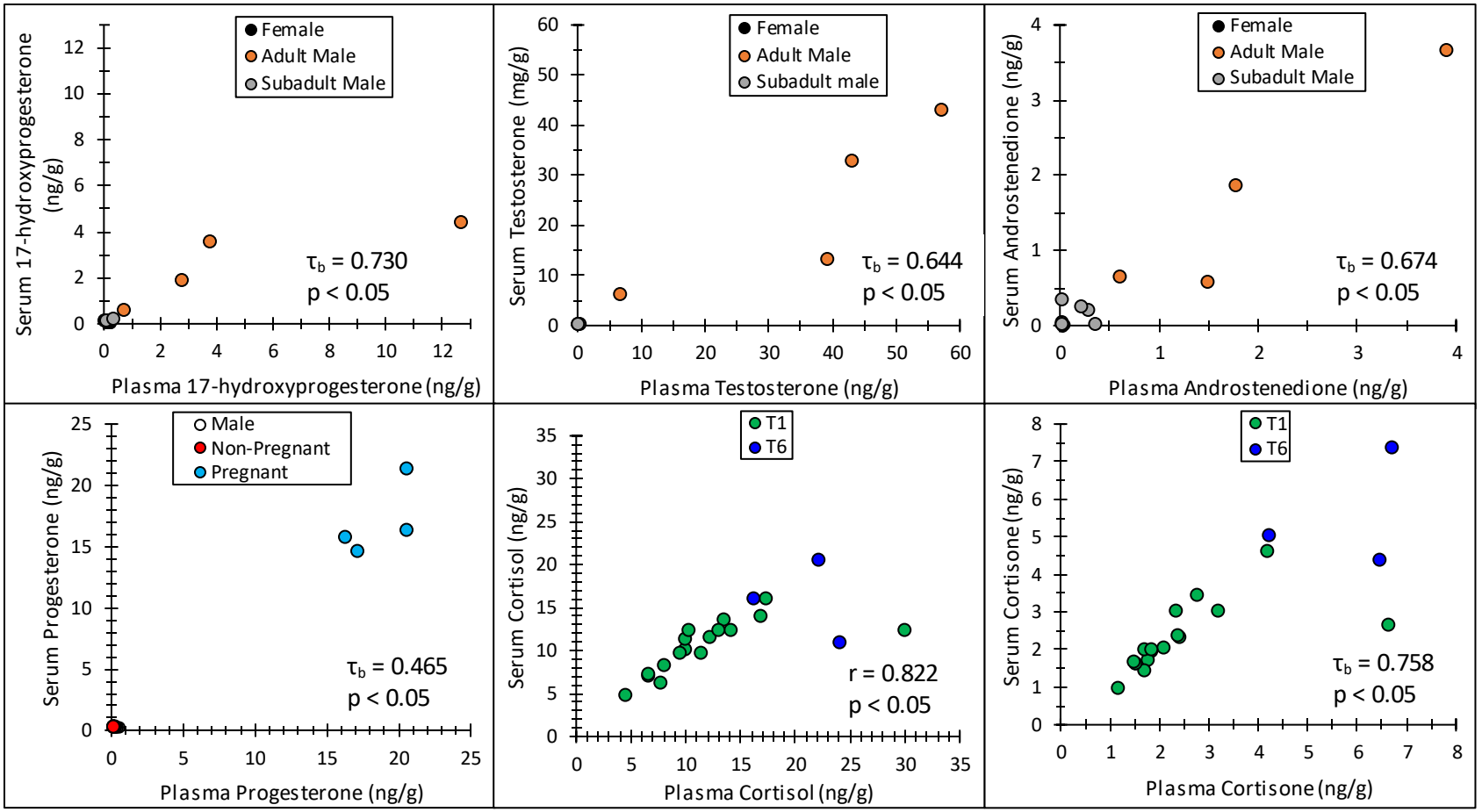

Fig. 2.3 Relationships between steroid hormone measurements in individual-matched plasma and serum. All relationships are statistically significant ($p < 0.05$) per Kendall's tau-b or Pearson's correlation.

CHAPTER 3: Can blubber steroid hormone measurements be used to predict circulating hormone concentrations?

1. Introduction

Blubber steroid hormones have been used for qualitative diagnosis of physiological states associated with changes in circulating steroid hormone profiles (pregnancy, sexual maturity, and stress), and blubber biopsies can be collected remotely, establishing blubber as a useful matrix for endocrine assessments in cetaceans [114-119]. The fact that blubber hormone patterns tend to qualitatively match those observed in blood suggests that blubber could be a good proxy for blood in quantitative assessment of systemic steroid hormone homeostasis. However, this has not been conclusively tested. Only one study has directly compared blubber and circulating steroid hormones in matched samples, and that study only looked at cortisol (F) [120]. Champagne et al. 2016 reported that blubber F values are only marginally related to circulating F values ($R^2 = 0.57$), suggesting that blubber cannot be used as a quantitative proxy for blood in cetaceans [120]. In this chapter I improve and expand upon this study to better address this question. I accomplish this by using LC-MS/MS to simultaneously quantify eight steroids in plasma and eleven in blubber, and then assessing the relationships among all hormones in both matrices. Ultimately, I aim to produce models that facilitate the prediction of circulating steroid hormone concentrations from blubber measurements.

This is also the first study to examine the potential physiological roles of 17-hydroxyprogesterone ($17OHP_4$), androstenedione (AE), 11-deoxycortisol (S), and cortisone (E) in bottlenose dolphins. Thus, I will test the following hypotheses: 1) the hormones in the Δ_4-androgen biosynthesis pathway will be elevated in both plasma and blubber adult males because sexual maturity is marked by an increase in T secretion in cetaceans [101, 102, 115]; 2)

progestogens will be elevated in both tissues in pregnant females, as has been observed previously [93, 94, 116-119]; 3) androgens will also be elevated in both tissues in pregnant animals as seen previously in captive bottlenose dolphins and killer whales [11, 23]; 4) the hormones in the glucocorticoid pathway will be positively correlated with elapsed time to sample collection because capture and handling stress has been shown to induce the HPA axis [103, 104, 114]; 5) animals from Barataria Bay will have lower corticosteroids than animals from other sites due to the incidence of adrenal insufficiency observed in this population following the Deepwater Horizon oil spill [131].

2. Materials and Methods

2.1 Animals, Field Data Collection, and Sample Collection

Matched blubber and blood samples were collected from free-ranging bottlenose dolphins from three locations in the southeastern United States (Barataria Bay, LA (2013, 2014) n = 34; Brunswick, GA (2015) n = 16; and Sarasota Bay, FL (2013-2016) n = 31). Methods for the temporary capture been previously described [137-139]. Briefly, a sein net was deployed encircling an animal or group of animals, and, if necessary, the radius of the space enclosed by the net would be reduced to force the animal to become entangled or enable handlers to safely restrain the animal without entanglement. When an animal entangled itself in the net, handlers would immediately restrain the animal and disentangle it. Sarasota Bay sampling was performed under National Marine Fisheries Service (NFMS) Scientific Research Permit No. 15543 and annually renewed IACUC approvals through Mote Marine Laboratory. Barataria Bay and Brunswick sampling was conducted under NMFS permit no. 932-1905/MA-009526 with protocols reviewed and approved by National Oceanic and Atmospheric Administration IACUC.

This sample set includes subadult and adult males; pregnant, suspected pregnant, and non-pregnant females (adult and subadult) (Table 3.1). Age was determined either through lifelong observation (i.e. known birth date) or through examination of growth layer patterns in teeth using methods that have been described previously [140, 141]. Age class was dictated by age, if known (individuals ≥ 10 years old were classified as adults), or total length if age was not known (individuals with total length ≥ 240 cm were classified adults). Blubber samples were collected by surgical or punch biopsy, and skin was removed. Blood was collected via the arteriovenous plexus of the ventral fluke into sodium heparin vacutainers, and plasma was produced by centrifugation at site of capture. Blubber and plasma (in 1-5 mL aliquots) were immediately frozen in a liquid nitrogen dry shipper, and shipped to the National Institute of Standards and Technology (NIST) Environmental Specimen Bank at Hollings Marine Laboratory (Charleston, SC), where they were stored at -80 °C until analysis.

2.2 Calibration and Internal Standards

The same calibration and isotopically-labeled internal standards from the previous chapter were used here (Table 2.1). Calibration (Cal) and internal standard (IS) mixture solutions were diluted in methanol, with the concentration of each compound in the final mixture calculated gravimetrically (ng compound/g mixture). Average masses of IS compounds amended to each tube are reported in Table 3.2; Cal ranges used for quantification are reported in Tables 3.3 and 3.4.

2.3 Blubber Hormone Extraction

Blubber hormone extraction was completed using the methods described in Boggs et al. (2017) with a kit (Agilent, Santa Clara, CA, USA) that utilizes a salting-out assisted liquid:liquid extraction (SALLE) to dispersive solid phase extraction (SPE) process [121]. Clean, empty 2mL

garnet bead (0.7 mm) homogenization tubes (Mo Bio, Carlsbad, CA, USA) were labeled and weighed, and masses were recorded. Approximately 100 µL of an internal standard (IS) mixture containing isotopically-labeled steroid hormones in methanol was added to each tube, after which tube masses were measured and recorded again. Frozen blubber samples (roughly 400 mg) were minced with acetone- and hexane-rinsed razor blades in similarly rinsed glass beakers resting on dry ice. Minced blubber samples or 1 mL of calibration standard (Cal) mixtures (n = 7) containing unlabeled steroids in methanol were transferred into their homogenization tube containing the IS mixture, and masses were recorded once again. IS blanks (n = 4) did not receive additional matrix. Samples, calibration curve standards, and IS blanks were processed identically hereafter. Each tube was filled with MilliQ water (0.5 mL to 2 mL), and was homogenized in a Precellys 24 bead homogenizer (Bertin Instruments; Montigny-le-Bretonneux, France) four times for 30 s at 6500 rpm; two 30 s and one five min rest intervals between homogenization cycles were used. During the five min rest, samples were removed from the homogenizer and placed in a chilled tube rack. Homogenates were decanted into clean 50 mL falcon tubes. The homogenizer tube was rinsed twice with approximately 1.5 mL of MilliQ water, each time the rinse was decanted to the falcon tube containing the homogenate. Then the falcon tube was vortexed for 10 s. The homogenizer tube was then rinsed three times with acetonitrile, and each time the rinse was added to the falcon tube. Additional acetonitrile was added until the total volume was 15 mL, and then the falcon tube was shaken vigorously by hand for 30 s. The salt packet from the extraction kit was added to the falcon tube, and the tube was shaken again for 1 min. Samples were centrifuged at 29000 x g for 5 min at 4 °C. The upper (acetonitrile) phase (roughly 9 mL) was transferred to the C18 dispersive SPE tube provided in the kit, which was then vortexed for 1 min before being centrifuged at 20000 x g and 4 °C for 3

min. Liquid was transferred from the SPE tube into clean, labeled borosilicate culture tubes using glass Pastuer pipettes, and was dried at 35 °C under nitrogen (100-130 kPa bar) in a Biotage TurboVap (Uppsala, Sweden). Extracts were reconstituted in 2mL 80:20 water:acetonitrile (volume fraction), sonicated for 9 min, and then filtered through a 0.22 µm cellulose spin filter at 12000 x g for 1 min. Filtered extracts were transferred to new borosilicate tubes, and dried under N_2 at 35 °C. Dried extracts were reconstituted in 200 µL of methanol and transferred into amber autosampler vials containing glass inserts.

2.4 Blood Hormone Extraction

Plasma hormones were extracted by methods developed and described in Chapter 2 Section 2.3 of this dissertation.

2.5 Instrumental Methods and Quantitation

Instrumental methods and quantitation methods for both blubber and plasma extracts are identical to those described in Chapter 2 of this dissertation. As before, the standard curve for each compound in each matrix was comprised of at least three calibration standards, which fully encompassed the range of sample values (Tables 3.3 and 3.4). Observed limits of quantification (LOQ_{obs}) are defined as the lowest calibration standard used in the calibration curve; calculated limits of quantification (LOQ_{calc}) were calculated as three times the standard deviation of the mean plus the mean of the extracted blanks (Table 3.5).

2.6 Statistics

Statistical analyses were performed with IBM SPSS Statistics 23 (IBM, North Castle, NY, USA). For all hypothesis tests, $\alpha = 0.05$. Principle component analyses (PCA) were performed to examine relationships between hormones and demographic/sampling variables. Only hormones were included in the analysis, but component scores were analyzed by de mographics. Hormone

values below LOQ were substituted with a random value between 0 and LOQ. Data were $\log_{10}$ transformed before being mean centered and unit scaled. Suitability for PCA was confirmed by ensuring all variables had at least one correlation with $R^2 > 0.3$ and Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy > 0.5; that the KMO measure of adequacy for the whole set was also > 0.5; and that Bartlett's test of sphericity was significant ($p < 0.05$) [147]. Factors with eigenvalues greater than 1.0 were extracted. Varimax rotation was utilized to simplify interpretation.

Differences in hormone measurements by demographic variables were assessed using non-parametric methods due to left censoring; for these tests, values below LOQ were censored to the same value below LOQ to ensure that all such values would be tied in rank-based statistical tests. Mann-Whitney U test was used to examine the differences in gonadal steroid concentrations by age class in adult males (stratified by month); Kruskal-Wallis test with Bonferroni post hoc was used to examine differences in gonadal steroid measures by pregnancy status in females; Kendall's tau-b correlation was used to assess relationships between hormone measurements and elapsed time to sample collection (i.e. time in minutes between onset of capture process [i.e. deployment of the net] to sample collection), and between hormones within each matrix.

Since elapsed time was likely to impact corticosteroid measurements, variation in corticosteroids by demographic variables (maturity, site, pregnancy status) were performed by analysis of covariance (ANCOVA) in which elapsed time was included as the covariate. For males, a two-way ANCOVA was performed to examine potential impacts of month, since seasonal variations in T has been previously observed. For analysis by pregnancy status, only Barataria Bay animals were included in a one-way ANCOVA since site effects likely exist (given the

evidence that Barataria Bay animals exhibit hypoadrenocorticism) and all but one pregnant females were from Barataria Bay; thus, inclusion of non-pregnant animals from all sites would confound the ANCOVA [131]. Analysis by site was performed by one-way ANCOVA and included all animals. Plasma F or E did not require censoring. There was minimal censoring in blubber F and E (12 % and 14 % censoring, respectively), and these values were substituted with random values between LOQ and 0. Values were $\log_{10}$ transformed, if necessary, to meet the assumptions of ANCOVA.

Multiple linear regression was used to build predictive models, with plasma steroid hormone concentrations as the dependent variables and blubber hormones as predictor variables. Values below LOQ were substituted with a random value between 0 and LOQ. Hormone values were $\log_{10}$ transformed to meet assumptions of multiple linear regression. Models were built stepwise, with entry/removal determined by probability of F distribution (entry = 0.05, removal = 0.10).

3. Results

Six hormones were detected and quantified in both blubber and plasma, these were: P_4, $17OHP_4$, AE, T, S, F, and E, and a seventh, S was also detected and quantified in only blubber.

3.1 Principle Components Analyses

I performed two PCAs; the first included both plasma and blubber hormone measurements, while the second included only blubber hormones. Blubber S was removed from both analysis due to a poor KMO measure. In the first PCA, four components with eigenvalues > 1 were extracted explaining 29.315 %, 21.941 %, 12.116 %, and 8.932 % of the variance individually (72.305 % cumulatively). Plasma T, AE, and $17OHP_4$ and blubber T and AE load

strongly onto PC1 (Table 3.6). Based on scores, PC1 seems to generally delineate adult males from subadult males and females (Fig 3.1A). P_4 (both matrices) and plasma F and E and measurements load heavily to PC2, while blubber F and E loaded to PC3 (Table 3.6). PC2 scores separate pregnant and suspected pregnant females from the other demographic groups. Furthermore, PC2 scores are significantly and positively correlated with elapsed time to blood collection ($\tau_b = 0.282$), while PC3 scores are significantly correlated to elapsed time to blubber collection ($\tau_b = 0.344$) (Fig 3.1B). Blubber T and $17OHP_4$ load to PC4. PC4 scores indicate some separation of adult males similar to that observed with PC1 (Fig 3.1A). Furthermore, PC2 and PC4 scores are significantly correlated to age, weight, and length in males (Fig. 3.2)

In the second PCA (blubber hormones only), three PCs were extracted explaining 36.148 %, 26.854 %, and 16.670 % of the variance individually (79.672 % cumulatively). Variable loading patterns were similar to the first PCA. Blubber F and E load strongly to PC1; blubber T, AE, and $17OHP_4$ load to PC2; and Blubber AE and P_4 load to PC3 (Table 3.6). Per score plots, adult males and pregnant females are somewhat separated from each other and the remaining demographic groups in the PC2/PC3 dimensions, though they are not fully delineated (Fig 3.3). As in the first PCA, the PC characterized by T, AE, and $17OHP_4$ (PC2) is significantly and positively correlated with age, length, and weight in males (Fig 3.4).

Examining PCA scores by due date in pregnant animals yielded no significant correlations (Fig 3.5)

3.2 Demographics and Steroid Hormones

I examined hormone measurements by various sampling and demographic variables: age class (males), pregnancy status (females), elapsed time, and site. Distributions of several blubber and plasma hormones differed significantly by age class in males sampled in both May

and September including plasma T, AE, and $17OHP_4$ and blubber T which were higher in adult males compared to subadults (Fig 3.6). Blubber AE and $17OHP_4$ were significantly elevated in adult males in May but not September, though the only males with quantifiable AE and $17OHP_4$ in September were adults (Fig 3.6). Plasma F and E also vary by age class, but not site nor the interaction between site and age class, in males after correcting for differences in elapsed time by two-way ANCOVA; blubber F and E did not vary significantly by age class, site, or the interaction between age class and site in males (Fig 3.7). Also in males, plasma T, AE, and $17OHP_4$ were significantly and positively correlated with one another, as were blubber T, AE, and $17OHP_4$ (Fig 3.8). Plasma T and E were also significantly correlated.

In females, distributions of plasma P_4, $17OHP_4$, and AE and blubber P_4 and AE differed by pregnancy status (Fig 3.9). P_4 is elevated in pregnant and suspected pregnant animals compared to non-pregnant, while $17OHP_4$ (plasma only) and AE are only elevated in pregnant females, but not suspected pregnant females (Fig 3.9). Plasma F and blubber E differed by pregnancy status in Barataria Bay animals after correcting for elapsed time, while plasma E and blubber F did not (Fig. 3.10).

Inclusive of all animals (both sexes), elapsed time to blood collection was significantly and positively correlated with plasma F ($\tau_b = 0.468$), E ($\tau_b = 0.273$), and T ($\tau_b = 0.251$), while elapsed time to blubber collection was significantly and positively correlated with blubber S ($\tau_b = 0.281$), F ($\tau_b = 0.430$), E ($\tau_b = 0.416$), and P_4 ($\tau_b = -0.198$) (Fig 3.11). F and E are positively correlated with one another in both matrices ($\tau_b = 0.609$ and 0.841 in plasma and blubber, respectively) (Fig 3.12). Blubber S is also correlated with blubber F and E ($\tau_b = 0.402$ and 0.412, respectively) (Fig 3.13). Plasma F, but not blubber F, was significantly lower in animals from Barataria compared to Brunswick and Sarasota after correcting for elapsed time (Fig 3.14).

Plasma and blubber E values violated the assumptions of the ANCOVA and, thus, were not analyzed as such.

3.3 Model Building

The final models are reported in Table 3.7. In all models, b_0 is the intercept term, while $b_{1...n}$ are the slope coefficients for each independent variable included in the final models. Parameter estimates are reported in Table 3.8.

For plasma P_4, blubber P_4 was the only significant predictor. Plasma $17OHP_4$ only includes blubber AE and T as predictors, with blubber $17OHP_4$ notably absent. A similar outcome is evident for plasma F, in which blubber P_4 and T are the only significant predictors. As with $17OHP_4$, both blubber AE and T are significant predictors of plasma AE and T. For plasma T, P_4 is also a significant predictor. Plasma E is best predicted by blubber E and blubber P_4.

4. Discussion

4.1 Examining Steroid Hormones by Demographic and Field Variables

4.1.1 Males and Sexual Maturity

To my knowledge the relationships between $17OHP_4$, AE, E, and S and physiological factors such as sexual maturity, pregnancy, and handling stress have never been studied previously in bottlenose dolphins. Thus, I examined these relationships in order to advance our understanding of general bottlenose dolphin endocrinology.

Elevated T in blood and blubber are established markers of sexual maturity in male cetaceans [101, 102, 115]. Therefore, I anticipated that adult males would exhibit elevated concentrations of T in both matrices. Furthermore, I expected that concentrations of the precursors to T (AE, $17OHP_4$, and P4) would also be elevated, in order to support increased T

production. My results partially support these hypothesis – in the first (full) PCA, T, AE, and $17OHP_4$ in both matrices tend to load together (PC1 and PC4), suggesting an association exists among these variables, and PC1 and PC4 scores separate adult males from subadult males and are correlated with age, length, and weight, indicating that the variance in these variables is likely maturity-derived. Therefore, I examined the relationship between age class and these hormone measurements (stratified by month because seasonal variation in T has been previously observed), and found that adult males have higher T, AE, and $17OHP_4$ concentrations in both plasma and blubber compared to subadults in May while only plasma T, AE, and $17OHP_4$ are significantly elevated in September [101, 115]. Notably, even though the differences by age class were not significant, the only males with quantifiable blubber T, AE, or $17OHP_4$ in September were adults. Furthermore, all three of these hormones are significantly and positively correlated with one another within each matrix. From this combined evidence, I suspect that AE and $17OHP_4$ secretion is elevated in adult males to support elevated T secretion. In other words, since AE is a substrate for T production and $17OHP_4$ is a substrate for AE production (i.e., the Δ_4 androgen pathway), the elevated demand for T secretion necessitates increased production of these two hormones as well. Some of this additional $17OHP_4$ and AE may then be secreted, leading to higher systemic $17OHP_4$ and AE measurements. In this scenario, one could also expect to observe elevated P_5 and 17-hydroxypregnenolone ($17OHP_5$) or P_4 secretion to support $17OHP_4$ production, but P_5 and $17OHP_5$ were not detected in any sample in this study, and P_4 was not detected in any male blubber; plasma P_4 was detected in four male samples, but did not exhibit any difference by maturity. Alternatively, it is possible that $17OHP_4$ and AE play some role in male physiology independent of serving as substrate for T

production – i.e. perhaps they also have direct effects on target tissues. Characterizing these potential roles is not possible with the current study design.

To examine potential AE and $17OHP_4$ signaling at target tissues, one could use transactivation reporter assays to test ability of $17OHP_4$ and AE to transactivate bottlenose dolphin hormone receptors (especially the progestogen receptor [PR] and androgen receptor [AR]); such experiments could shed light on whether these hormones are sufficient to induce receptor transactivation themselves, and thus, whether circulating concentrations of these hormones are likely to impact target tissue physiology. Definitive testing of my initial hypothesis (that $17OHP_4$ and AE are elevated to support T production) may be challenging, as it would require dosing of dolphins with exogenous steroids and/or steroidogenesis inhibitors, which may not be permitted under the MMPA. One could potentially examine expression/activity of steroidogenic enzymes in gonads collected from stranded animals to address this hypothesis. If my hypothesis holds true, one would anticipate elevated expression/activity of 17βHSD and CYP17 and/or 3βHSD (Table 1.1, Figure 1.1) in adult males compared to subadults, which could account for the increased systemic $17OHP_4$, AE, and T concentrations observed in adult males.

To determine whether blubber hormone measurements alone are capable of differentiating adult and subadult males, I performed another PCA with only blubber hormones. As in the first PCA, T, AE, and $17OHP_4$ load together to the same principle component (PC2). While separation of subadult and adult males is not absolute, there is some degree of separation of subadult and adult males, and PC2 is significantly and positively correlated with age, length, and weight in males. If investigators want to use blubber hormone measurements to define age class in males, a PC2 score of exceeding 1.0 seems to be indicative of adult males while score below 0.0 would be indicative of subadults, as no subadults or adults, respectively,

exceed these thresholds. There are subadult and adult males with PC2 scores between 0.0 and 1.0, making it impossible to differentiate between age classes from blubber steroid hormone profile alone. It is possible that this overlap is associated with the fact that differences between blubber hormone profiles are dependent upon seasons – adults and subadults do not exhibit significant differences in blubber T, AE, or $17OHP_4$ in September. Expanding sample sizes and including sampling across seasons could potentially refine this analysis and better facilitate age classification by blubber hormones alone.

Plasma F and E also varied by age class in males, with adults having higher of both, while blubber F and E did not. The difference between plasma and blubber is likely being driven by the likely lag between when changes in plasma hormone profile occur and when they are reflected in blubber; this is discussed in greater detail in Section 4.2. Similar relationships have been observed in male killer whales (*Orcinus orca*) in which younger males had lower F compared to older males [148]. Plasma T and E are positively correlated in males as well. This may suggest that sexually mature males have different adrenal function than subadults, experience greater stress compared to subadult males, and/or that there is cross talk between androgens and the adrenal axes. In other mammals, maturity-related increases in HPA activity have been linked to reduced sensitivity to negative feedback signaling within the hypothalamus with increasing age [149-152]. This may be occurring in bottlenose dolphins as well, and could be studied by examining expression of the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) in brains of stranded animals. HPA/HPG axis cross is known to occur in other mammals (Chapter 1, Section 1.3, Fig. 1.2), but these relationships are complex and bidirectional, making it difficult to predict the direction of the relationship between androgens and corticosteroids in bottlenose

dolphins. This relationship could potentially be examined by characterizing expression of the androgen receptor (AR) in the adrenal and the GR and MR in the testis of stranded animals.

4.1.2 Females and Pregnancy Status

P_4 secretion increases during pregnancy in cetaceans, and this increase can be observed in blubber [93, 94, 116-119]. Thus, I hypothesized P_4 would be elevated in both plasma and blubber in pregnant/suspected pregnant animals. As expected, plasma and blubber P_4 load strongly to the same principle component (PC2) in the full PCA, and non-pregnant and pregnant/suspected pregnant females are separated by PC2 according to scores. Examining P_4 measurements by pregnancy status indicates that pregnant and suspected pregnant females have elevated blubber and plasma P_4 measurements compared to non-pregnant females, as expected. Androgens, including T and AE, have been shown to be elevated during pregnancy in other animals, including captive bottlenose dolphins and killer whales (*Orcinus orca*), seemingly to support ovarian secretion of P_4 [5, 6, 11-23]. Thus, I also analyzed the Δ_4 androgen pathway hormones by pregnancy status. T measurements did not vary by pregnancy status, but plasma $17OHP_4$ and AE and blubber AE were elevated in pregnant animals. This could suggest that androgens also play a role in the maintenance of bottlenose dolphin pregnancy. Alternatively, this increase in AE and $17OHP_4$ may be caused by the increase of P_4 – that is, the increased availability of P_4 may lead to increased metabolism of P_4 into downstream products of the Δ_4 androgen pathway, though the absence of detectable T in pregnant females seems to refute this hypothesis. *Ex vivo* study designs utilizing gonadal tissue from stranded animals could potentially shed light on the role that $17OHP_4$ and AE may have in regulating gonadal P_4 synthesis. Determining whether increased $17OHP_4$ and AE arise due to elevated P_4 would require dosing with exogenous steroids and/or steroidogenesis inhibitors.

In the bottlenose dolphin, the killer whale, and other mammals, cortisol has been shown to increase during late pregnancy, which is thought to play a role in preparing the fetus for survival outside of the uterus and inducing parturition [1, 11, 23, 153-155]. However, Steinman et al. did not compare F between early pregnant to non-pregnant captive bottlenose dolphins, meaning it is currently unclear whether changes in corticosteroids occur during early pregnancy in *T. truncatus*. Alternatively, Robeck et al. found no difference between F measurements in non-pregnant (pre-conception) and early pregnant captive killer whales [11, 23]. The pregnant animals in this study (for which expected due date information is available) are all in the first trimester of pregnancy, thus, I predict there would be no pregnancy-related increase in corticosteroid measurements at this stage of pregnancy. I assessed variation in F and E by pregnancy status with elapsed time as a covariate and only included Barataria Bay females in this analysis because all but one pregnant females are from Barataria Bay. Barataria Bay dolphins have previously been shown to exhibit hypoadrenocorticism resulting from crude oil exposure from the Deepwater Horizon oil spill, thus I wanted to control for the potentially confounding effect of site [131]. Interestingly, plasma F and blubber E were diminished in pregnant females compared to non-pregnant animals but not suspected pregnant females. While surprising, given the pattern observed in killer whales, it is possible that a similar result would have been observed in bottlenose dolphin by Steinman et al. had they compared early pregnant and non-pregnant, which would point to a species-specific effect of pregnancy on F [11]. Alternatively, if they had studied non-pregnant and early pregnant animals and observed no difference, like in the killer whales, my result could potentially suggest a difference between captive and free-ranging animals. Given this unique result, further study is necessary to better understand how pregnancy influences adrenal function. Perhaps, as discussed in regard to

males, this suggests the occurrence of HPA/HPG axis crosstalk, resulting from increases in P_4 during pregnancy.

Being able to differentiate females by pregnancy status using blubber hormone measurements would be useful for investigations of reproductive status in remotely sampled animals. Blubber P_4 alone allows for the differentiation of non-pregnant females from pregnant or suspected pregnant females, as no non-pregnant animals had quantifiable blubber P_4. Distinguishing between suspected pregnant animals and confirmed pregnant animals is not possible with only blubber P_4. Thus, I examined whether the second PCA (blubber only) provided separation of pregnant and suspected pregnant females. Unfortunately, it does not, leading me to conclude that it is not possible to distinguish between early (first trimester) pregnant and suspected pregnant females by blubber hormones alone. Suspected pregnant and pregnant females are also indistinguishable in the full PCA, further indicating that the differences in systemic steroid hormone profiles for these groups are not sufficient to differentiate them. Nonetheless, this study suggests that $17OHP_4$ and AE could potentially be used as markers of reproductive status in addition to P_4, as these measures are also associated with pregnancy status.

I examined PC scores by days until parturition to determine whether differences existed based on stage of pregnancy. However, all pregnant animals with expected due dates in this study were in the first trimester of pregnancy, meaning there was little variation in days to parturition among pregnant females. Thus, no significant relationship exists between days to parturition and PC scores. This is not necessarily to say that endocrine profiles cannot be used to distinguish between stages of pregnancy, rather a longitudinal study examining profiles throughout pregnancy would be better required to answer this question. Indeed, previous work

has indicated there are stage-dependent differences in circulating steroid hormone profiles in bottlenose dolphins and killer whales; if these changes are reflected in blubber, it stands to reason these changes could be used to diagnose stage of pregnancy from blubber hormones alone [10, 11, 23].

4.1.3 Corticosteroids, Elapsed Time, and Site

Capture and handling is known to induce the secretion of corticosteroids in bottlenose dolphins [103, 104, 114]. Since plasma and blubber corticosteroids load to PC2 and PC3, respectively, in the full PCA, I analyzed PC2 and PC3 scores by elapsed time and found that PC2 is significantly, positively correlated with elapsed time to blood collection and PC3 is correlated with elapsed time to blubber collection. From this, I hypothesized that S (blubber only) F, E would be positively correlated with elapsed time to sample collection in each respective matrix [101, 103, 104]. S and E have been measured in the bottlenose dolphin before, but not in relation to changes in physiological state [121]. Despite this apparent lack of evidence, I hypothesized that S and E concentrations would also be significantly correlated to elapsed time and F because S is the precursor to F, which in turn is a substrate for E production. In other words, S production must increase to support elevated F production, and then more E can be produced from the increase in F. Thus, all three should rise concurrently. As expected, plasma F and E concentrations are significantly and positively correlated with elapsed time to blood collection and with one another. Furthermore, blubber S, F, and E are correlated with elapsed time to blubber collection and each other. These results support my hypothesis that stress-induced changes in systemic F cause concomitant changes in systemic S and E. As discussed in regard to androgens and progestogens, conclusively testing this hypothesis could potentially be tested by examination of adrenal tissues from stranded animals.

Since there is crosstalk between the HPA and HPG axes in other species, I suspected that the gonadal hormones could also be impacted by elapsed time mediated by the associated acute changes in corticosteroids. I did not have *a priori* hypotheses regarding the directions of these relationships given the complexity of HPA/HPG crosstalk (discussed in Chapter 1 Section 1.3, Fig 1.2). Plasma T is significantly positively correlated with elapsed time to blood collection, while blubber P_4 is negatively correlated with elapsed time to blubber collection. However, T measures are confounded by maturity in males, and P_4 by pregnancy status in females. When stratifying these correlations by these factors (as opposed to including all groups in a single analysis) these relationships are no longer statistically significant. Therefore, I conclude that acute handling stress does not appear to impact androgen or progestogen homeostasis in bottlenose dolphins.

Dolphins in Barataria Bay have exhibited hypoadrenocorticism following exposure to contaminants associated with the Deepwater Horizon oil spill [131]. Thus, I expected Barataria Bay animals to have lower corticosteroid measurements than the other sites. This is the case for plasma F but not for blubber F, as indicated by ANCOVA with elapsed time included as a covariate. This may be due to a lag between when changes in adrenal function are reflected in blood versus blubber (discussed below in Section 4.2), which would indicate that Barataria Bay animals likely do not have impaired baseline adrenal physiology (blubber measurements) but an impaired response to stress stimuli (blood measurements). E measurements by site could not be assessed by ANCOVA because these data violate the assumptions of ANCOVA.

4.2 Modeling the Relationships between Blubber and Plasma Steroid Hormones

The overall goal of this chapter is to examine the relationships between blubber and blood steroid hormone values, and thereby determine whether blubber is a suitable proxy for

blood in the assessment of systemic steroid hormone homeostasis in the bottlenose dolphin. I accomplished this by attempting to produce models that allow for the prediction of circulating steroid hormone concentrations from blubber hormone measurements. Previously, it has been demonstrated that blubber F measurements alone are insufficient to quantitatively predict circulating F concentrations [120]. We have observed intra-matrix relationships between different hormones (e.g. blubber S, F, and E are positively correlated), which leads me to hypothesize that including a wider suite of blubber steroid hormones could potentially yield quantitatively predictive models for circulating steroid hormones.

While several of the models I produced do include multiple blubber hormones as significant predictors of plasma hormones, the models all account for less than half of the variance in circulating values. This indicates a lack of a quantitative, predictive relationship between blood and blubber steroid hormones, though there is qualitative agreement between blubber and plasma measurements. This is further supported by the observation that the models for plasma 17OHP$_4$ and F do not include blubber 17OHP$_4$ and F, respectively, as significant predictors. Plasma F is best predicted by blubber T and P$_4$, which I suspect are acting as proxy variables for maturity in males (T) and pregnancy status in females (P$_4$), which are known to be associated with F, as discussed above. Blubber P$_4$ is included as a predictor variable with a negative coefficient value in the models for T, F, and E; again, this is likely functioning as a proxy for pregnancy status, for which negative relationships with these hormones have been established. In particular, the models for F and E are very poor, explaining 24.8 % and 15.5 % of the variance, respectively, which is in disagreement with the results reported by Champagne et al., who reported that blubber F measurements accounted for 57 % of the variance in circulating F [120]. However, one must note that blood and blubber were collected at different times

during the sampling process, and both plasma and blubber E and F are significantly related to their respective elapsed times. Perhaps if plasma and blubber were collected simultaneously the models would explain a greater proportion of the variance, but this assumes that blubber and blood steroid hormone concentrations change at the same rate, which may not be true.

In domesticated pigs, peak adipose P_4 concentrations exhibited a one-to-two day lag behind peak plasma P_4 concentrations and returned to baseline concentrations more gradually than plasma concentrations [156]. Therefore, it is possible that blubber steroid hormone concentrations would also change more gradually than circulating hormone concentrations. The rate of change in circulating hormone profiles depend upon the rate of synthesis in source tissues and the rate of metabolism in peripheral tissues. Conversely, assuming that blubber itself is not a significant source of steroids (which may not necessarily be true) and steroids present there are delivered by blood, the rate of change in blubber hormones will be dependent on both the rate of change in blood concentrations and the rate of hormone diffusion from blood into blubber. Thus, changes in steroid hormone synthesis and metabolism will likely be reflected in circulating hormone profiles in real-time (or close thereto), whereas changes in blubber steroid hormone measurements may lag as a function of the rate of hormone uptake by blubber. In this scenario, circulating and blubber hormone values would be qualitatively related and reflect the same chronic physiological states (i.e. maturity, pregnancy, stress) in the long term, but would not be instantaneously matched. In other words, blubber hormone values would reflect an average plasma value over a period of time. Without knowing and accounting for the differential rates of change in each matrix, it is difficult to predict circulating values from blubber measurements. This hypothesis could be conclusively tested with captive animals by dosing with exogenous labeled hormone and measuring changes in hormone concentration in each matrix

steroids over a time course, similar to what was done by Hillbrand et al. in pigs [156]. Then these rates could potentially be incorporated into these models to yield more predictive models.

The above hypothesis is predicated on the assumption that all blubber hormones are of central origins. This assumption may not be valid because adipose of other species is capable of metabolizing steroid hormones. Thus, perhaps some of the disagreement between circulating and blubber steroid hormone concentrations arises through *in situ* metabolism of steroid hormones in blubber. This hypothesis will be addressed and discussed in Chapter 5.

Overall, I conclude that quantitative prediction of circulating steroid hormone concentrations from blubber is not possible using methods and techniques presented here. Further study of steroid pharmacokinetics in cetacean blood and blubber is warranted and likely necessary to produce more quantitative predictive models. However, this does not preclude the use of blubber alone as a matrix for endocrine assessment, it simply requires blubber-specific interpretation. Blubber is still useful in the study of cetacean endocrinology as it can likely provide insight into overall endocrine status and physiological states.

Table 3.1 Sample size categorized by demographic variable

Sex	Age	Pregnancy status	Site	n=
Male	Adult	NA	Brunswick	7
			Sarasota	4
	Subadult		Barataria	6
			Sarasota	9
Female	Adult	Non-pregnant	Barataria	5
			Sarasota	8
		Pregnant	Barataria	8
			Brunswick	1
		Suspected	Barataria	3
		TBD	Sarasota	2
	Subadult	Non-pregnant	Barataria	9
			Brunswick	1
			Sarasota	4
		Pregnant	Barataria	6
		Suspected	Barataria	1
		ND	Barataria	1
			Brunswick	2
	Calf	NA	Barataria	1
	ND	ND	Brunswick	1

ND = not determined, NA = not applicable

Table 3.2 Average internal standard masses by analysis

Compound	Plasma Analysis (ng)	Blubber Analysis (ng)
P_4-$^{13}C_3$	17.652	18.474
$17OHP_4$-$^{13}C_3$	16.004	19.603
F-d_4	16.651	17.684
E-$^{13}C_3$	16.334	17.795
T-$^{13}C_3$	18.273	19.302
AE-$^{13}C_3$	18.013	19.371
E_2-$^{13}C_3$	18.364	19.260

Table 3.3 Calibration curve information for reference plasma analysis

Hormone	Mass Range (ng)	Number of Points	Curve	Intercept	Slope	r^2
P_4	113 - 0.459	6	Linear	0.000	0.0032	1.000
$17OHP_4$	10.9 - 0.114	5	Linear	-0.0029	0.4855	1.000
F	87.4 - 0.853	5	Linear	-0.1882	1.7236	0.999
E	81.0 - 0.200	5	Linear	-0.2434	4.6262	1.000
T	251 - 0.259	7	Linear	0.0007	0.1293	1.000
AE (high)	9.44 - 0.460	3	Linear	-0.2435	9.2937	0.999
AE (low)	0.929 - 0.0545	4	Linear	-0.0035	3.0277	0.999

Table 3.4 Calibration curve information for reference blubber analysis

Hormone	Mass Range (ng)	Number of Points	Curve	Intercept	Slope or a, b coefficients	r^2
P_4	113 - 5.62	3	Quadratic	-0.0003	0.0003; 0.0031	1.00
$17OHP_4$	5.51 - 0.535	4	Quadratic	-0.0043	-0.2406; 0.7394	0.998
S	1.54 – 0.0779	3	Linear	-0.0127	4.3052	0.997
F	41.5 - 0.856	3	Quadratic	-0.0295	-0.215, 1.0981	1.000
E	1.66 - 0.0838	3	Linear	-0.0127	4.3052	0.997
T	12.5 - 0.260	4	Linear	-0.0032	0.2488	0.999
AE	22.6 - 0.193	4	Quadratic	0.0857	1.445; 1.4197	1.00

Table 3.5 Limits of quantification (ng). Observed limits of quantification (LOQ_{obs}) are determined by the lowest calibration standard used in the calculation of the standard curve. Calculated limits of quantification (LOQ_{calc}) are calculated as three times the standard deviation of the blank measurements plus the mean of blank measurements

Hormone	Plasma Analysis		Blubber Analysis	
	LOQ_{obs}	LOQ_{calc}	LOQ_{obs}	LOQ_{calc}
P_4	0.459	0.353	5.62	1.85
$17OHP_4$	0.114	0.107	0.535	0.112
S	NQ	NQ	0.0779	-
F	0.853	1.87[a]	0.856	0.474
E	0.200	0.845[a]	0.0838	0.0521
T	0.259	-	0.260	0.244
AE	0.0545	0.0215	0.193	-

- = Negative value

NQ = analyte not detected in experiment

[a] One blank had a peak while the remaining blanks had none, indicating potential contamination of that individual blank

Table 3.6 PCA rotated component matrix. Bolded values highlight variable loading greater (in magnitude) than 0.3, indicating significant loading to the component

	PCA 1				PCA 2		
	PC1	**PC2**	**PC3**	**PC4**	**PC1**	**PC2**	**PC3**
Blubber 17OHP4	0.139	-0.058	0.114	**0.885**	0.053	**0.797**	0.061
Blubber T	**0.474**	0.222	0.053	**0.656**	0.068	**0.874**	0.034
Blubber AE	**0.79**	-0.161	0.117	0.045	0.135	**0.371**	**0.736**
Blubber P4	0.274	**-0.794**	-0.086	-0.115	-0.235	-0.157	**0.824**
Blubber E	-0.042	0.186	**0.946**	0.087	**0.970**	0.070	-0.087
Blubber F	-0.03	0.137	**0.959**	0.081	**0.972**	0.070	-0.040
Plasma 17OHP4	**0.794**	-0.052	-0.075	0.205	---	---	---
Plasma T	**0.73**	**0.421**	0.012	0.291	---	---	---
Plasma AE	**0.85**	0.053	-0.101	0.085	---	---	---
Plasma P4	0.111	**-0.839**	0.01	-0.095	---	---	---
Plasma E	**0.32**	**0.692**	**0.326**	-0.158	---	---	---
Plasma F	0.289	**0.788**	0.251	-0.031	---	---	---

Table 3.7 Summaries of final models

Hormone	Model	Adj. R^2	Critical Value
P_4	$\log_{10}(Plas\_P_4) = b_0 + b_1 \cdot \log_{10}(Blub\_P_4)$	0.455	F (1, 74) = 63.733
$17OHP_4$	$\log_{10}(Plas\_17OHP_4) = b_0 + b_1 \cdot \log_{10}(Blub\_AE) + b_2 \cdot \log_{10}(Blub\_T)$	0.299	F (2, 73) = 17.026
AE	$\log_{10}(Plas\_AE) = b_0 + b_1 \cdot \log_{10}(Blub\_AE) + b_2 \cdot \log_{10}(Blub\_T)$	0.486	F (2, 73) = 36.523
T	$\log_{10}(Plas\_T) = b_0 + b_1 \cdot \log_{10}(Blub\_T) + b_2 \cdot \log_{10}(Blub\_AE) + b_3 \cdot \log_{10}(Blub\_P_4)$	0.440	F (3, 72) = 20.3616
F	$\log_{10}(Plas\_F) = b_0 + b_1 \cdot \log_{10}(Blub\_P_4) + b_2 \cdot \log_{10}(Blub\_T)$	0.248	F (2, 73) = 13.647
E	$\log_{10}(Plas\_E) = b_0 + b_1 \cdot \log_{10}(Blub\_E) + b_2 \cdot \log_{10}(Blub\_P_4)$	0.155	F (2, 73) = 7.870

Table 3.8 Summary of model parameters

	Parameter	Value	95% C.I.	Standard Error
P_4	b_0	-1.392	-1.695, -1.09	0.152
	b_1	0.938	0.704, 1.172	0.117
$17OHP_4$	b_0	-0.463	-0.635, -0.291	0.086
	b_1	0.233	0.101, 0.365	0.066
	b_2	0.312	0.117, 0.506	0.098
AE	b_0	-0.855	-1.052, -0.659	0.098
	b_1	0.499	0.348, 0.650	0.076
	b_2	0.322	0.100, 0.544	0.111
T	b_0	0.257	-0.047, 0.562	0.153
	b_1	0.543	0.297, 0.790	0.124
	b_2	0.356	0.184, 0.528	0.086
	b_3	-0.264	-0.479, -0.048	0.108
F	b_0	0.484	0.396, 0.573	0.044
	b_1	-0.128	-0.189, -0.068	0.030
	b_2	0.086	0.019, 0.153	0.034
E	b_0	1.101	1.016, 1.186	0.043
	b_1	0.106	0.023, 0.188	0.041
	b_2	-0.075	-0.144, -0.006	0.034

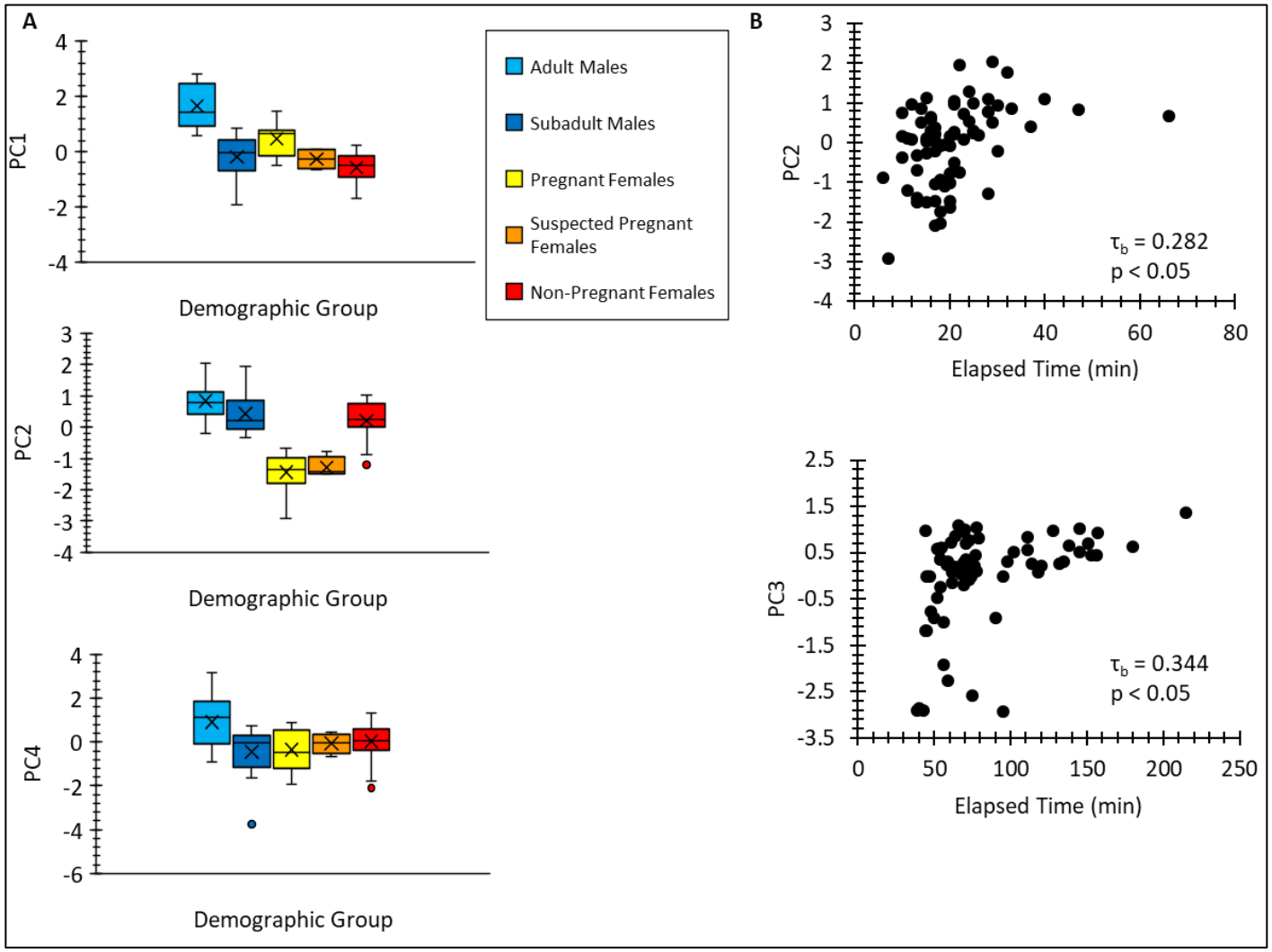

Figure 3.1 PCA 1 (plasma and blubber) Principle component scores by demographic and sampling variables. A) PC1, PC2, and PC4 scores by sex,

maturity (males), and pregnancy status (females), B) PC2 and PC3 scores by elapsed time to blood and blubber collection, respectively. For A, box lower bound indicates the first quartile, the upper bound indicates the third quartile, and the horizontal line indicates the median. Whiskers are 1.5 times the interquartile range plus or minus the upper or lower bound, respectively. Values external to this range are included as individual points. X markers indicate mean.

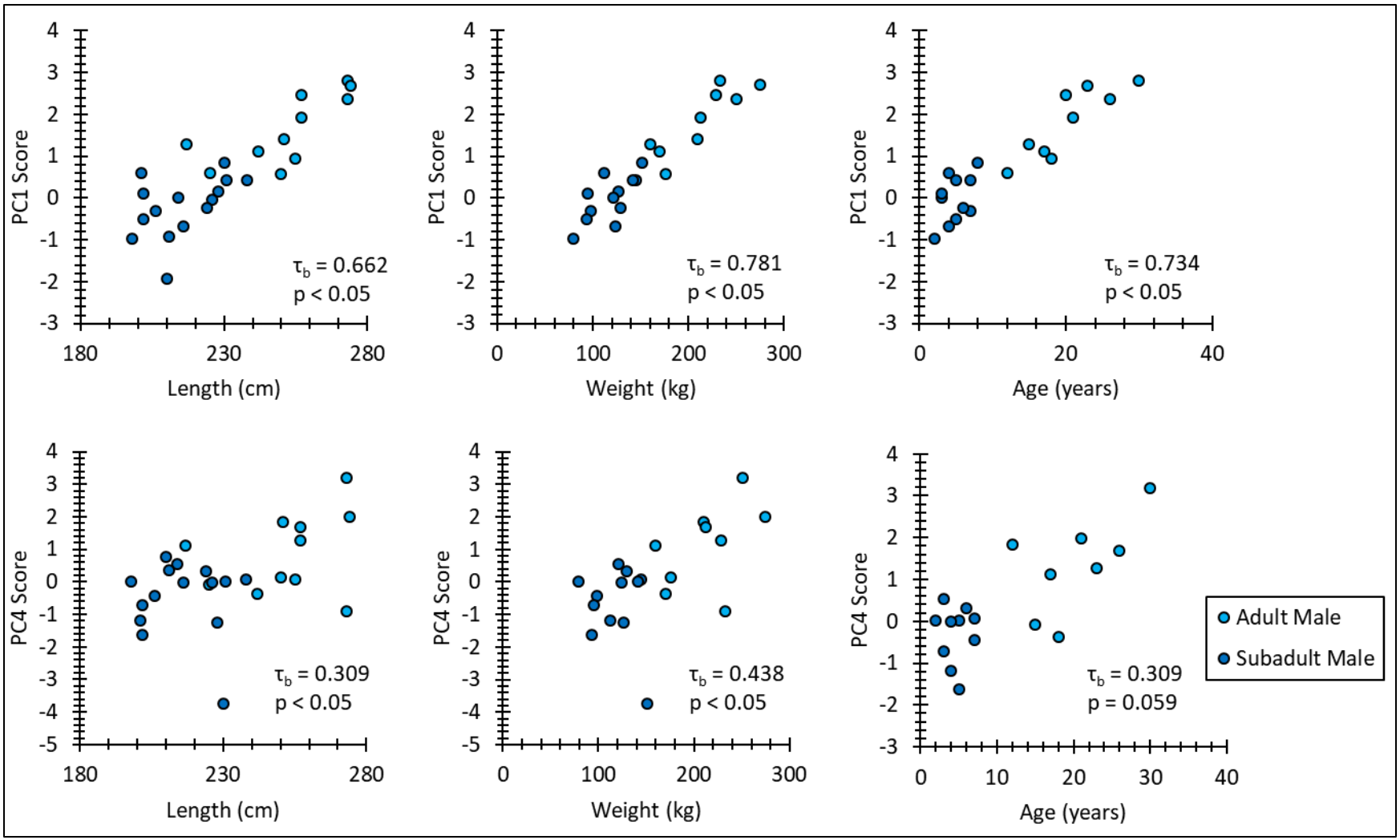

Fig 3.2 Correlations (Kendall's tau-b) between full PCA PC2 and PC4 scores and length, weight, and age in males.

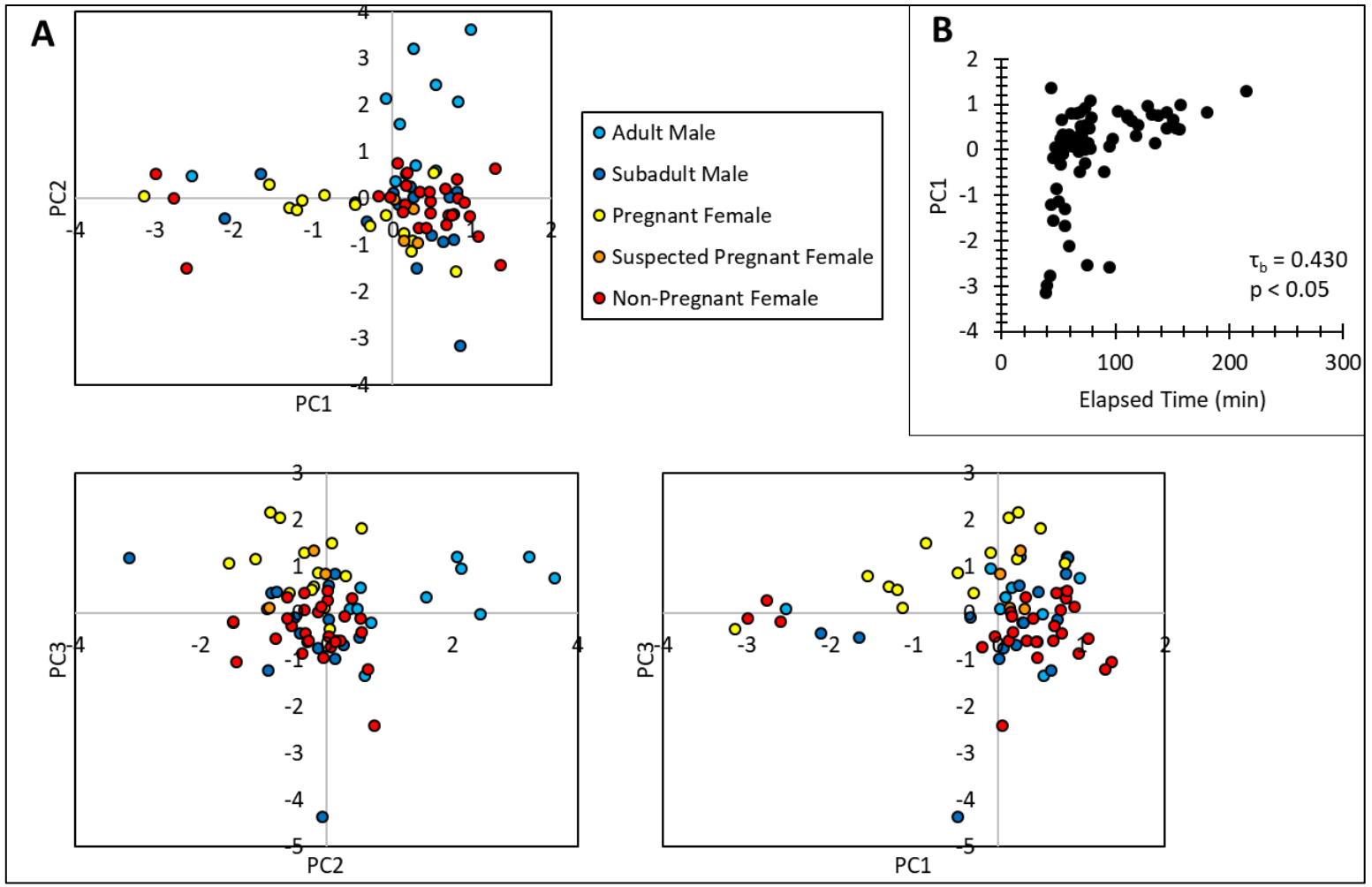

Fig 3.3 PCA 2 (blubber only). A) Principle component score plots with demographic groupings. B) Relationship between PC1 scores and elapsed time to blubber sample collection

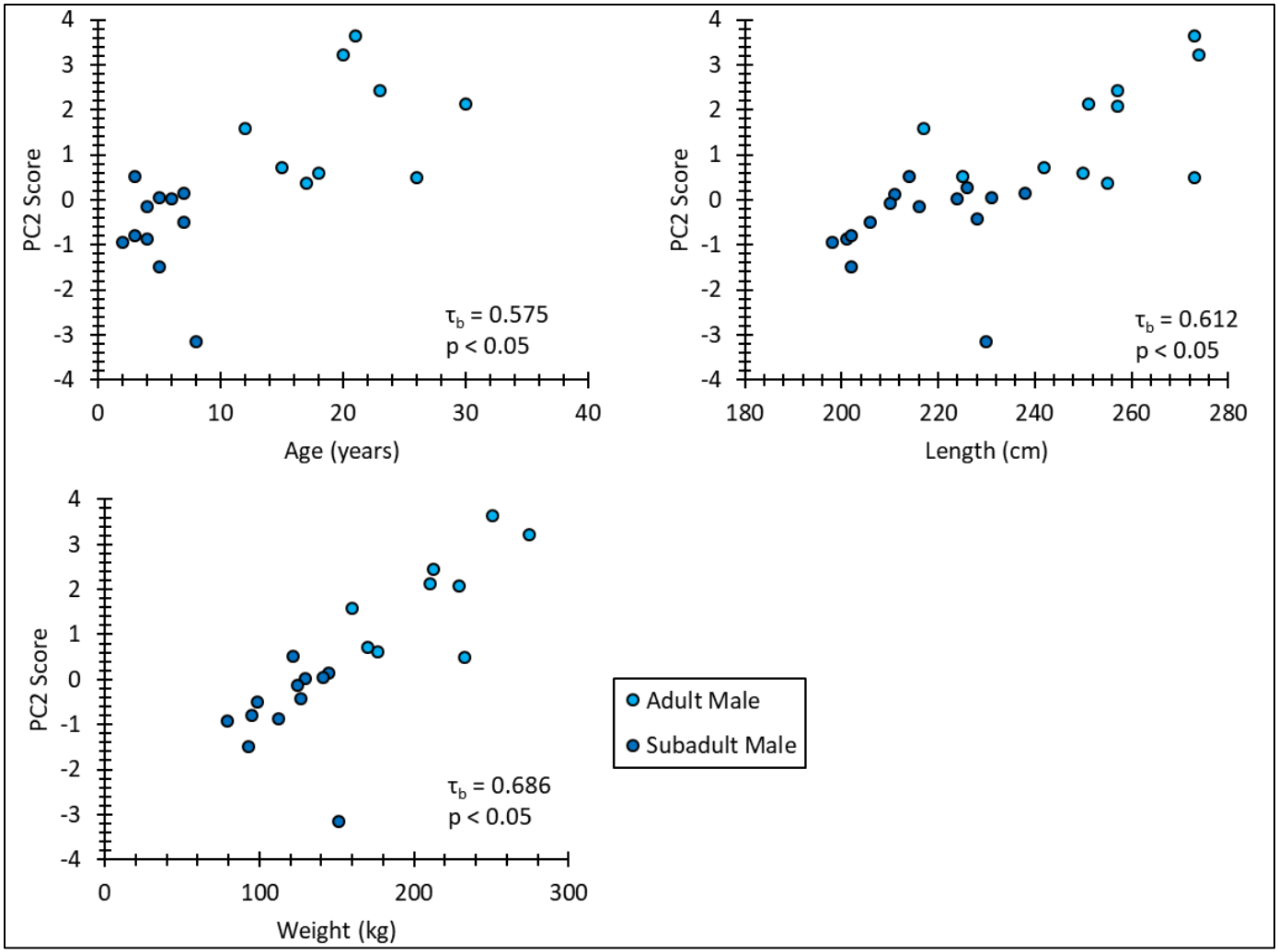

Fig 3.4 Correlations (Kendall's tau-b) between blubber only PCA PC2 scores and age, length, and weight in males

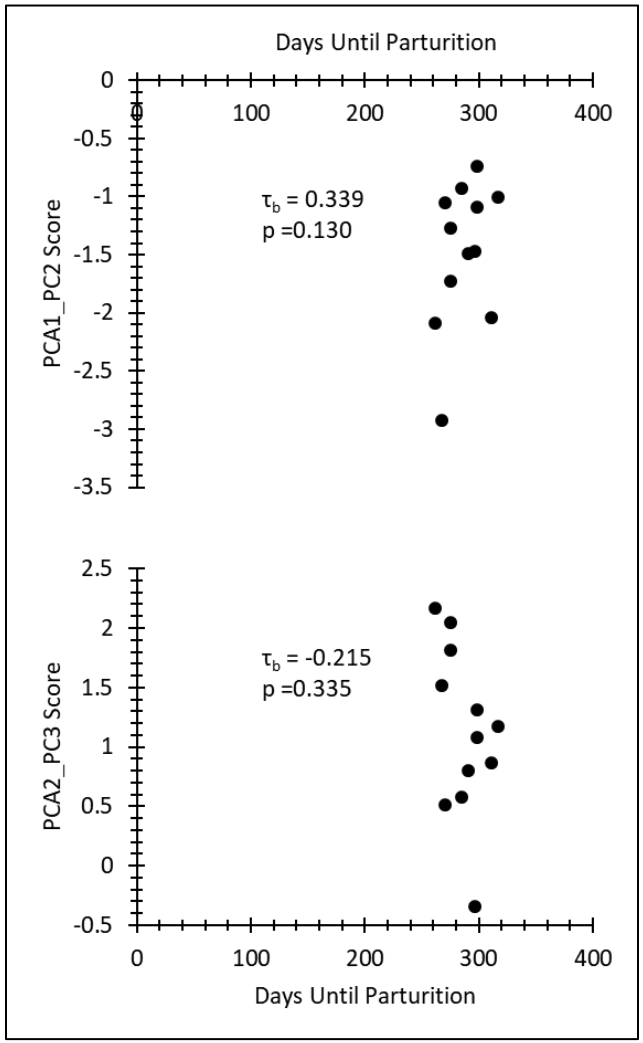

Fig 3.5 Correlations between due date and full PCA PC2 (PCA1_PC2) scores and blubber PCA PC3 (PCA2_PC3) scores.

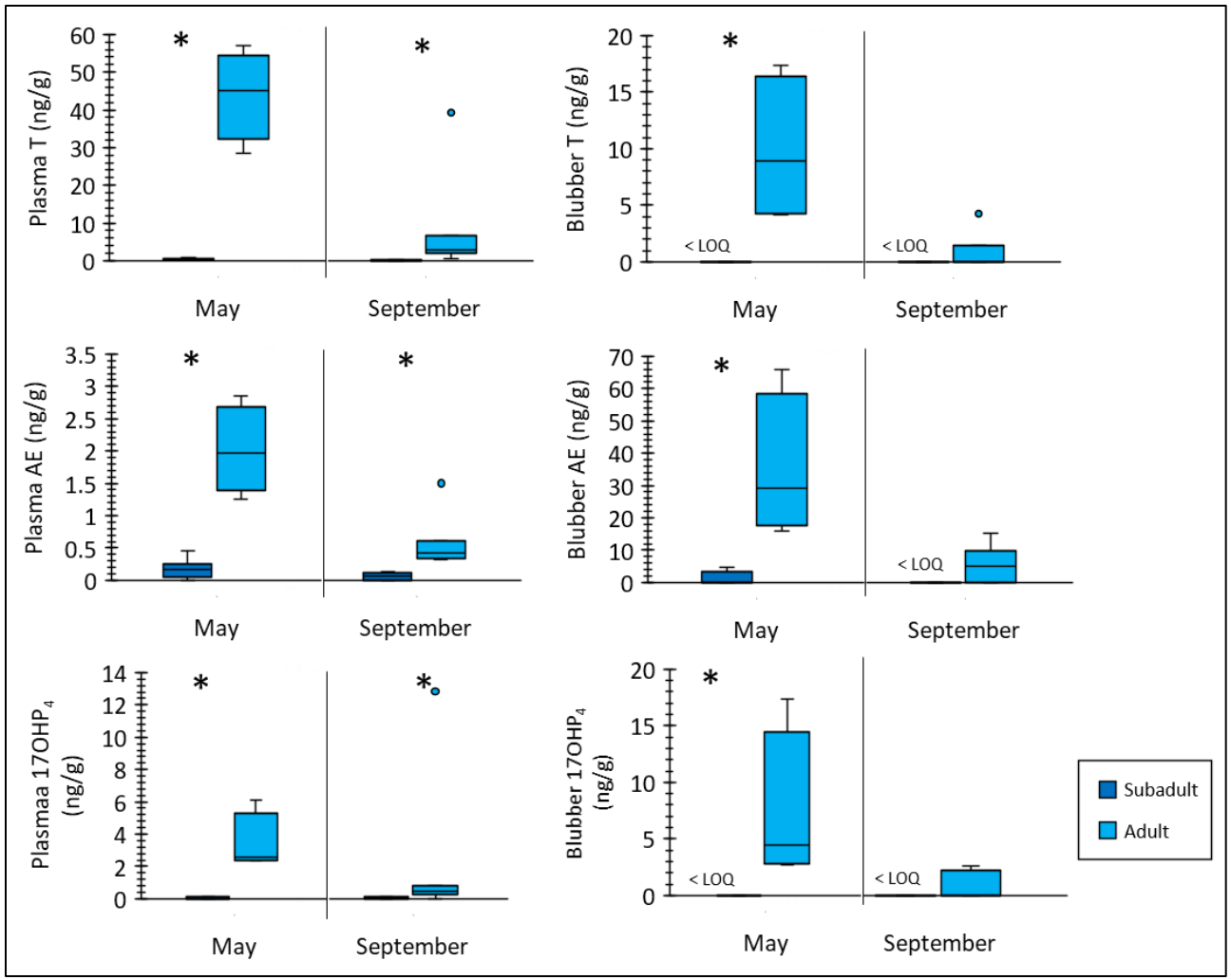

Figure 3.6 Blubber and plasma steroid hormone measurement distrubtion by age class in male bottlenose dolphins separated by month. Box

lower bound indicates the first quartile, the upper bound indicates the third quartile, and the horizontal line indicates the median. Whiskers are 1.5 times the interquartile range plus or minus the upper or lower bound, respectively. Values external to this range are included as individual points. Panels marked with an asterisk (*) indicate that differences between age class are significant ($p < 0.05$) per Mann-Whitney U test.

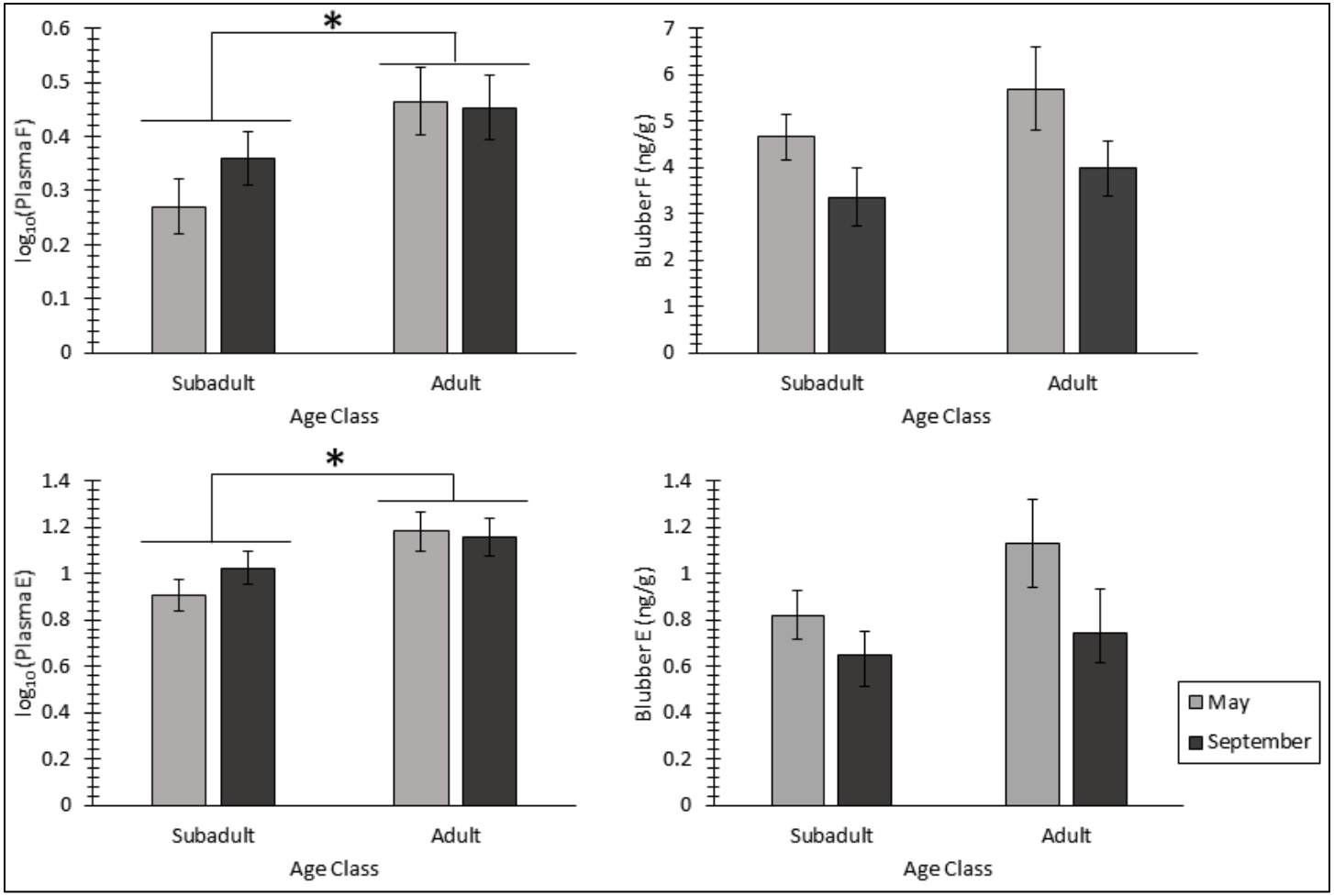

Fig 3.7 Estimated marginal means for cortisol and cortisone in plasma (log_{10} transformed) and blubber by age class and month in males when corrected for differences in elapsed time. Brackets with asterisks indicate statistically significantly differences ($p < 0.05$) per two-way analysis of

covariance (ANCOVA). Error bars indicate standard error of the marginal mean.

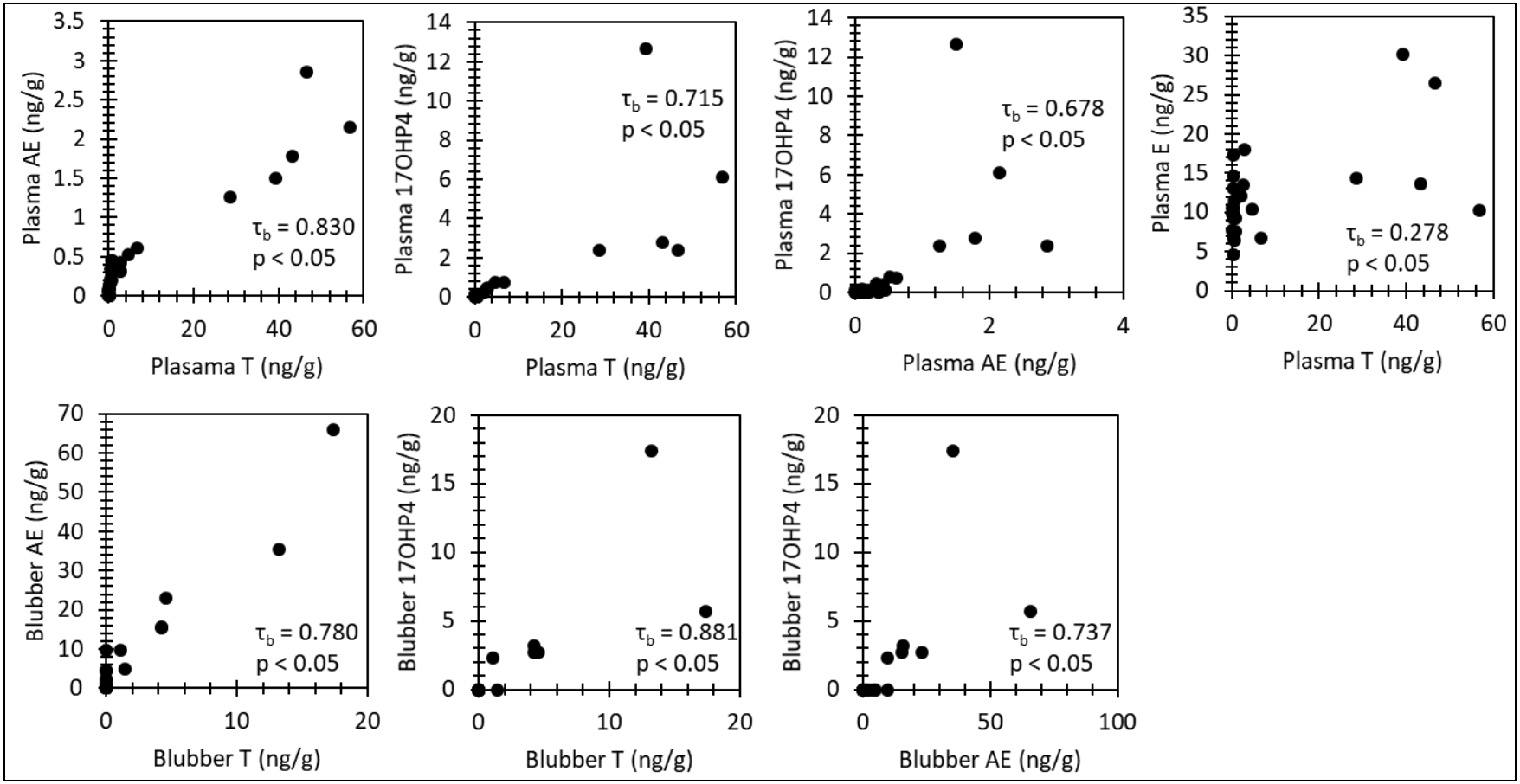

Figure 3.8 Relationships among steroid hormones within each biological matrix in male bottlenose dolphins

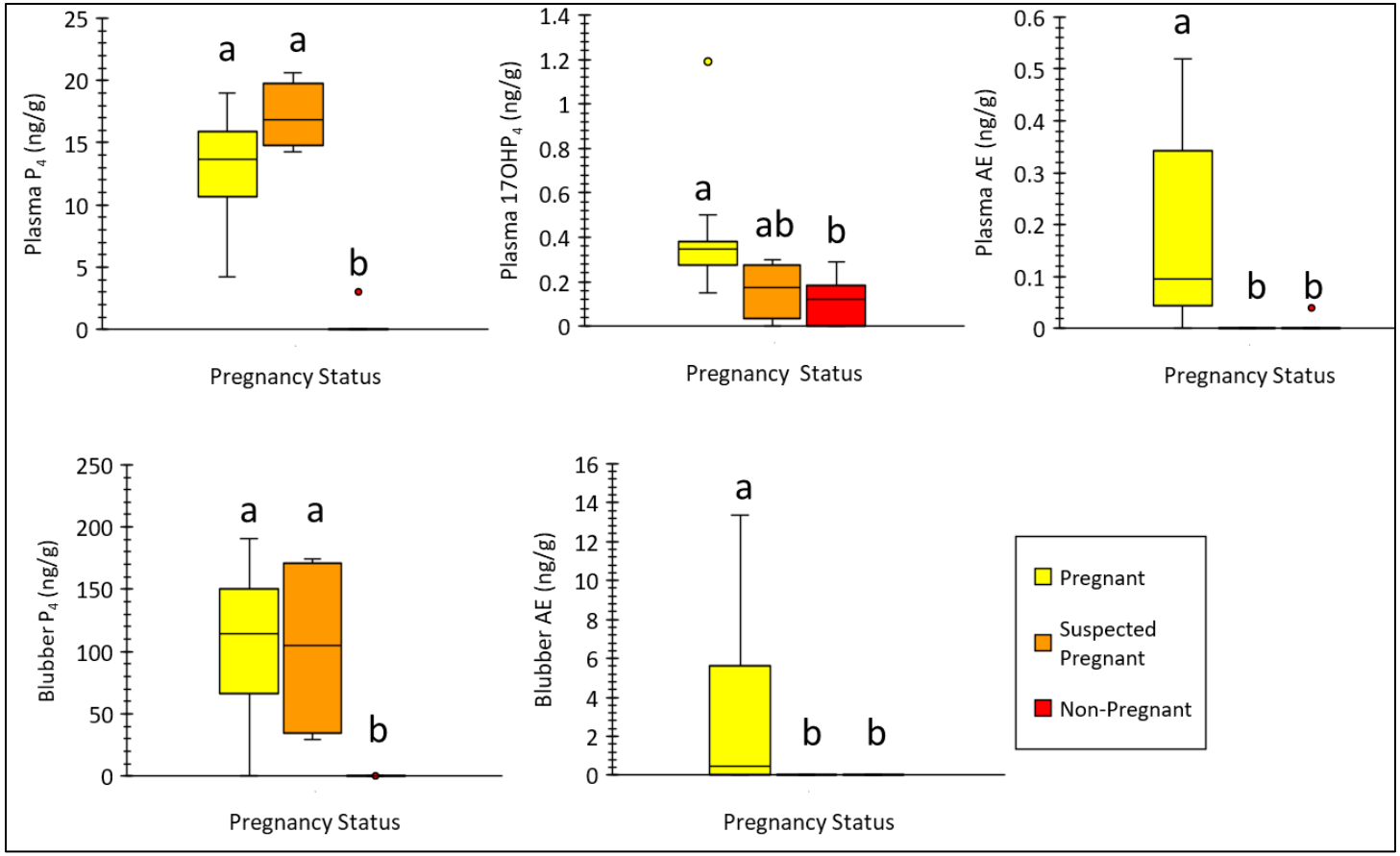

Figure 3.9 Plasma and blubber gonadal steroid hormone measurement distributions in female bottlenose dolphins by pregnancy status. Box lower bound indicates the first quartile, the upper bound indicates the third quartile, and the horizontal line indicates the median. Whiskers are

1.5 times the interquartile range plus or minus the upper or lower bound, respectively. Values external to this range are included as individual points. Letters over boxes indicate statistical groupings – those with different letters are significantly ($p < 0.05$) different per Kruskal-Wallis test with Bonferroni post-hoc test.

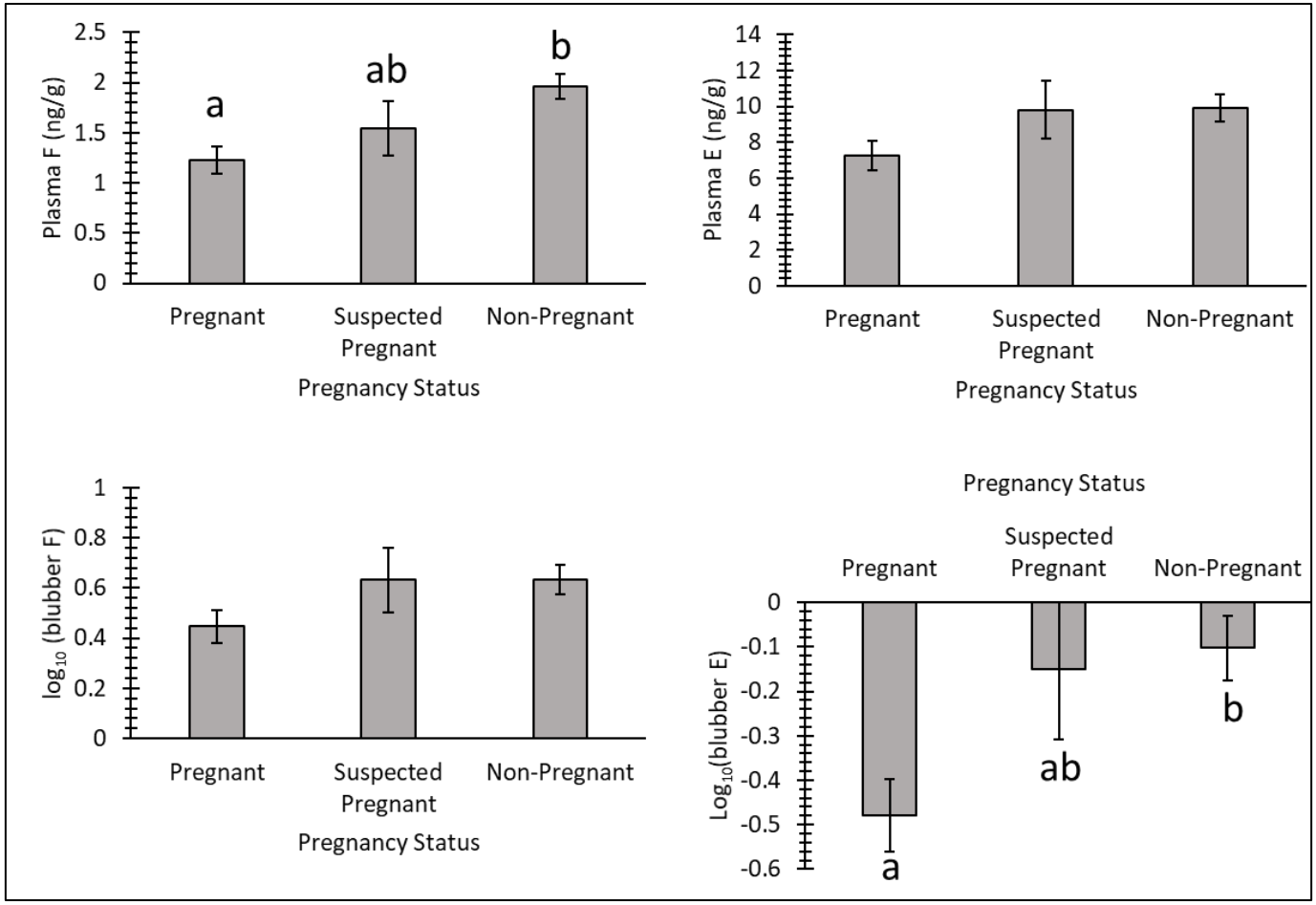

Figure 3.10 Estimated marginal means for plasma and blubber (log_{10} transformed) cortisol and cortisone by pregnancy status in Barataria Bay

females when corrected for in elapsed time. Letters over boxes indicate statistical groupings – those with different letters are significantly ($p < 0.05$) different per one-way analysis of covariance (ANCOVA). Error bars indicate standard error of the marginal mean

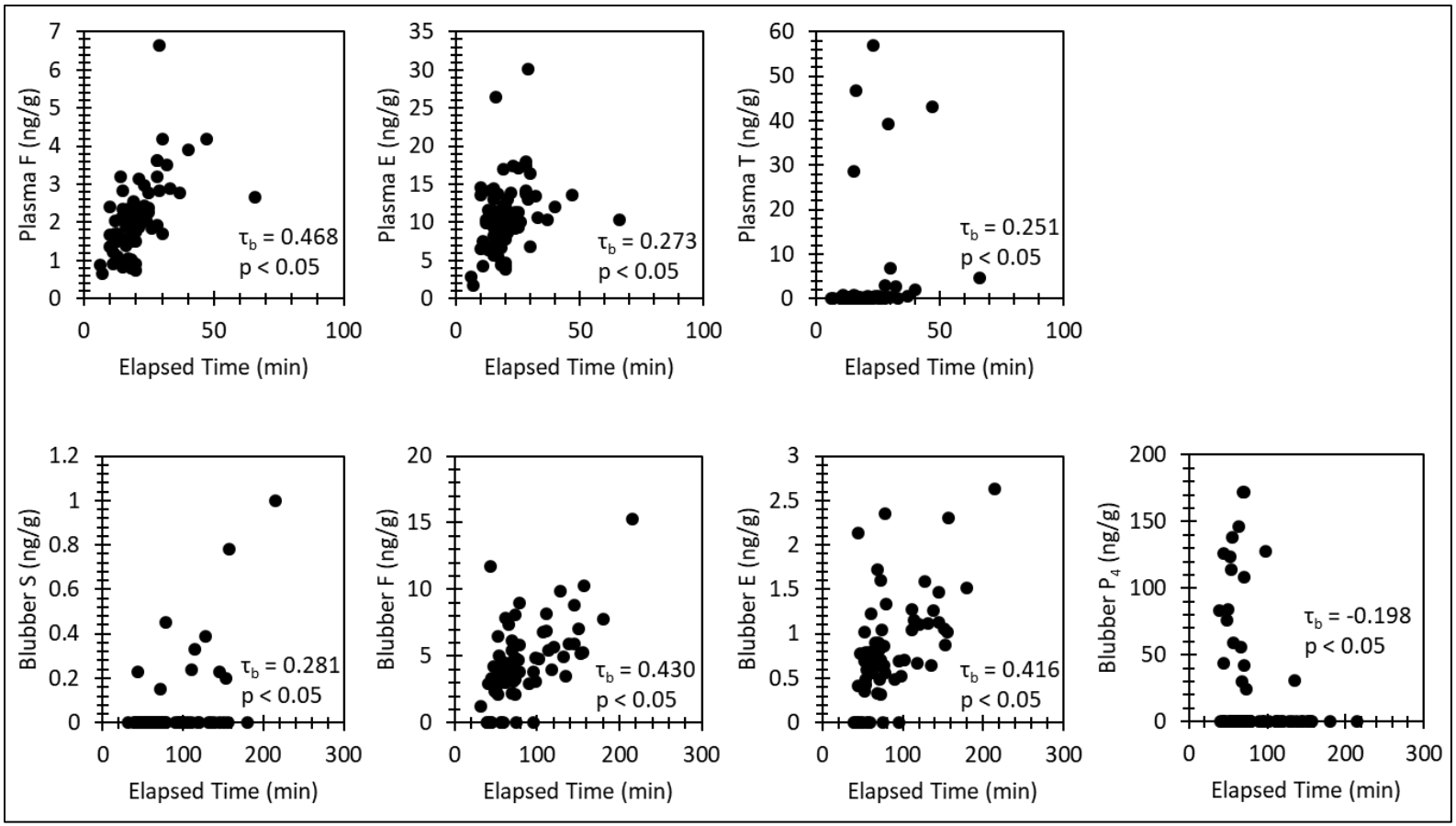

Figure 3.11 Relationships between plasma and blubber hormone concentrations by elapsed time to blood and blubber collection, respectively. All relationships are significant ($p < 0.05$) per Kendall's tau-b correlation.

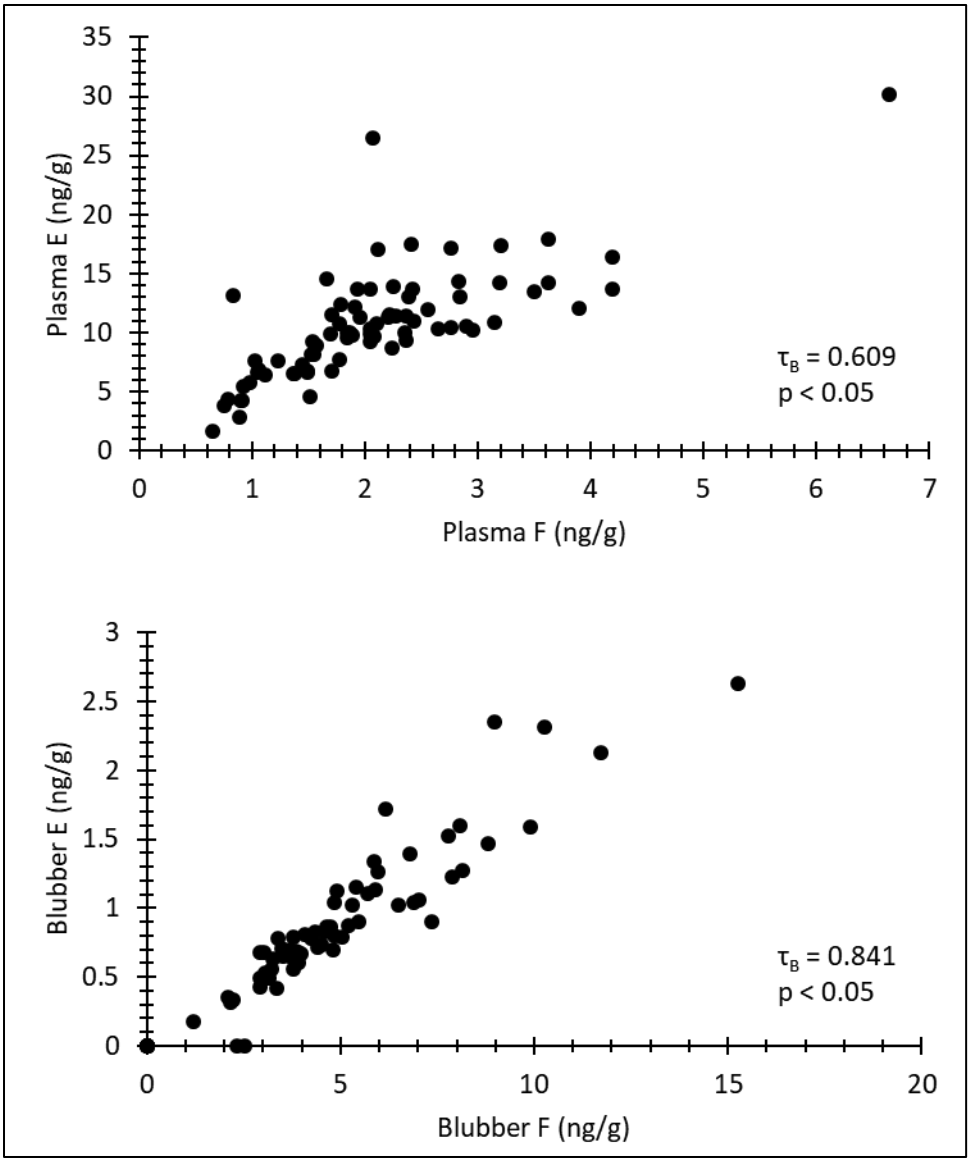

Figure 3.12 Relationship between cortisol and cortisone within each matrix. Each relationship is significant ($p < 0.05$) per Kendall's tau-b.

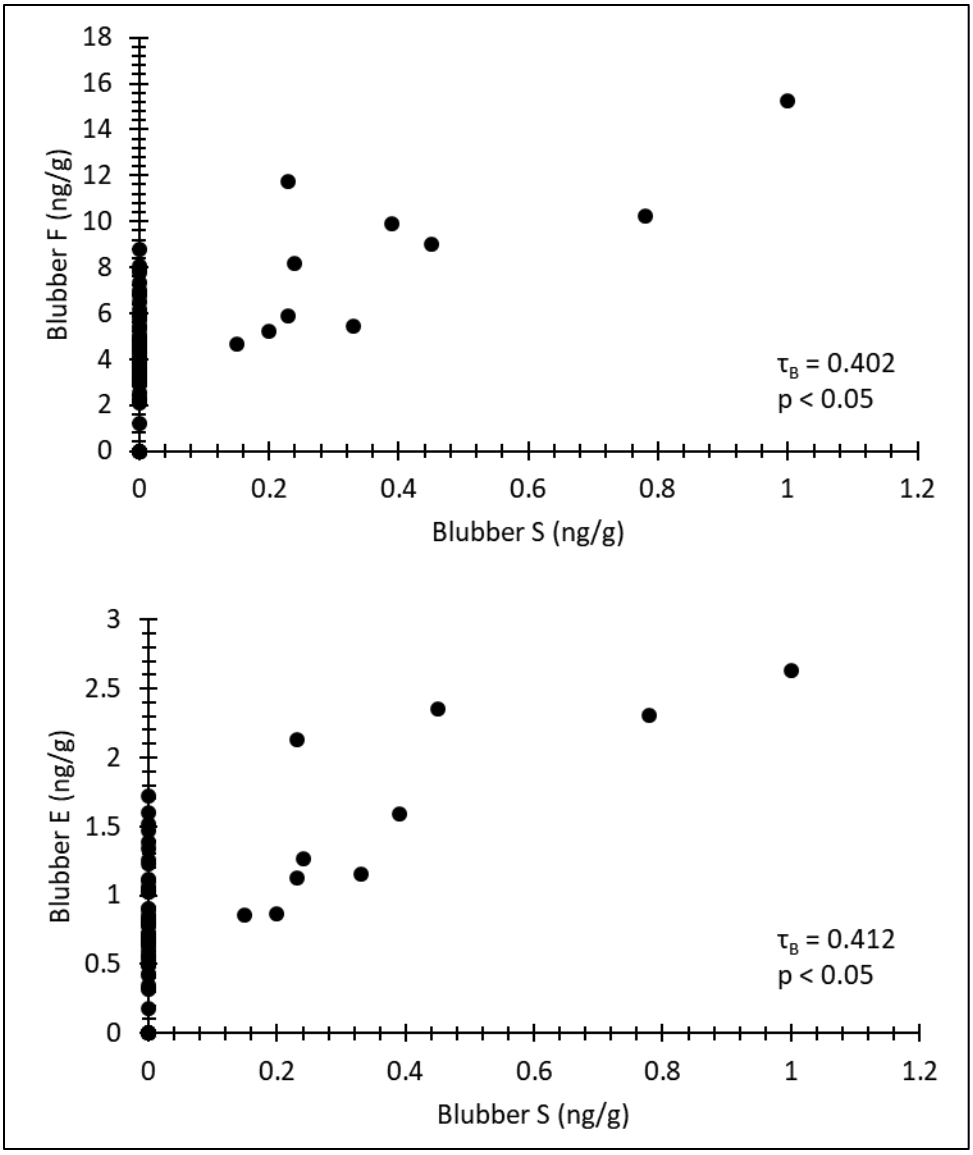

Figure 3.13 Relationship between blubber 11-deoxycortisol and the other blubber corticosteroids. Both relationships are significant ($p < 0.05$) per Kendall's tau-b.

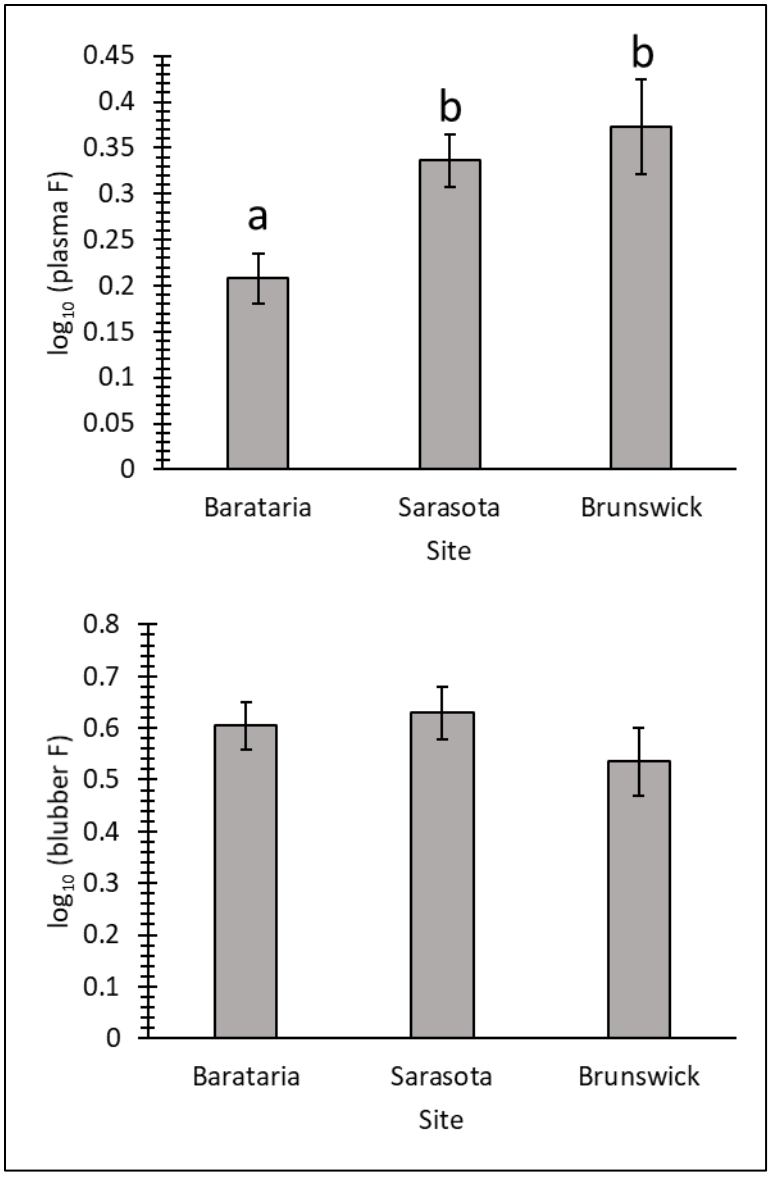

Fig 3.14 Estimated marginal means for blubber and plasma cortisol (log_{10} transformed) by site after correcting for elapsed time. Letters over boxes indicate statistical groupings – those with different letters are significantly ($p < 0.05$) different per one-way analysis of covariance (ANCOVA). Error bars indicate standard error of the estimated marginal mean

CHAPTER 4: Examining the Relationships between DDx Burden and Blubber Steroid Hormones in *T. truncatus*

1. Introduction

Odontocetes (toothed whales), such as bottlenose dolphins, are apex predators in the marine environment. This trophic position combined with their large adipose (blubber) reserves make them prone to accumulating high burdens of lipophilic persistent pollutants like DDTs through biomagnification [79]. DDTs disrupt vertebrate steroid hormone homeostasis through a variety of mechanisms, including impacts on steroid hormone synthesis, metabolism/clearance, signaling (through mimicry), and transport (Chapter 1, Section 2; Table 1.4). These effects have been demonstrated in a variety of vertebrates, but only two studies have examined the potential impacts of DDx exposure on cetacean endocrine function: DDE and circulating testosterone (T) concentrations were negatively correlated in males Dall's porpoise, while aldosterone was not significantly correlated with DDE; plasma estrone (E_1) was positively correlated with plasma *p,p'*-DDE in female pilot whales, while several other steroid hormones exhibited relationships with other organochlorine contaminants [108, 109]. Given that cetaceans experience such significant DDx exposure, it is imperative that we further examine the potential impacts of these exposures on their endocrine function to effectively manage and conserve these species.

To study contaminant-mediated endocrine disruption in cetaceans, in which lethal sampling and dosing with contaminants is impossible, investigators must rely on non-lethal sample matrices and incidental exposures. Thus, the population of bottlenose dolphin inhabiting St. Andrews Bay, FL, USA was identified as a potential population for the examination of DDx-mediated disruption of steroid hormone axes in bottlenose dolphins due to the fact that these

animals exhibit elevated DDx burdens compared to other populations in the southeastern United States (Table 1.3) [129, 130] (Balmer et al. unpublished). Importantly, only remotely collected blubber/skin biopsies are available from this population, meaning any assessment of endocrine disruption in these animals must be performed with one of these two matrices. Blubber is already established as a matrix for POP analysis in cetaceans, and skin can be used to determine genetic sex. In Chapter 3 of this dissertation, I explored the utility of blubber as a matrix for the assessment of steroid hormone homeostasis, and concluded that, while blubber is not quantitative proxy for blood, it is useful for the study of cetacean endocrinology, as blubber hormone measurements likely reflect chronic endocrine status. Thus, using blubber and skin alone, I have the ability to study DDx-mediated changes in systemic steroid hormone homeostasis in bottlenose dolphins.

From the two studies that have previously studied the relationships between DDx-burden and steroid hormones in cetaceans, I hypothesize that blubber T will be negatively correlated with DDE in male bottlenose dolphins, and E_1 will be positively correlated in females. I also predict that DDTs will exhibit significant relationships with the other steroid hormones detected in dolphin blubber. However, I do not have *a priori* hypotheses about the direction of these relationships due to the challenges associated with studying endocrine disruption in free-ranging wildlife. Considering that these animals have likely experienced chronic exposure to a varied mixture of DDTs and other POPs that, individually, have the capacity to mediate endocrine disruption at multiple levels in the endocrine system, it is difficult to predict how such complex exposure scenarios will affect systemic steroid hormone measurements at any individual point in time. I predict there will be differences between males and females because the different sexes experience very different exposures; males continuously accumulate POPs

throughout their entire lives, while females accumulate up until their first lactation, during which time they offload significant portions of their POP burden to offspring via milk [81]. Thus, POP concentrations in the blubber of males reflects lifetime exposure while, in females, it does not.

2. Methods

2.1 Animals and Sample Collection

Blubber samples were collected from free-ranging bottlenose dolphins in St. Andrews Bay, FL, USA by remote dart biopsy using a modified rifle or crossbow, as described by Balmer et al. [129]. Upon retrieval of darts, skin was removed from the biopsy and the blubber (approximately 0.8 g) was split in half longitudinally. Skin was stored in 20% DMSO saturated with sodium chloride at room temperature. Each sub-sample of blubber was frozen in liquid nitrogen dry shippers. One subsample of each biopsy was sent to the National Institute of Standards and Technology (NIST) Environmental Specimen Bank at Hollings Marine Laboratory, Charleston, SC, where it was stored at -80 °C until use in hormone analyses. The other subsample was sent to the National Oceanic and Atmospheric Administration (NOAA) Northwest Fisheries Science Center, Seattle, WA, USA where it was stored at -80 °C until use in contaminant analysis (see Section 2.4). Sex was determined by genotypic analysis of skin by polymerase chain reaction (PCR), performed by collaborators at the NOAA Southeast Fisheries Science Center, Lafayette, LA USA, as described previously [157]. Due to the remote nature of these sampling efforts, no additional demographic or field data (e.g., age, length, pregnancy status) were collected.

2.2 Calibration and Internal Standards

The same calibration and isotopically-labeled internal standards from the previous chapter were used here (Table 2.1). Calibration (Cal) and internal standard (IS) mixture solutions were diluted in methanol, with the concentration of each compound in the final mixture calculated gravimetrically (ng compound/g mixture). Average masses of IS compounds amended to each tube are reported in Table 4.1.; Cal mass ranges used for quantification are reported in Table 4.2.

2.3 Blubber Hormone Extraction and Analysis

Blubber hormone extraction and measurement was completed according to the methods described in Chapter 3 Section 2.3 of this dissertation. Limits of quantification are reported in Table 4.3.

2.4 Blubber Contaminant Extraction and Analysis

POP analysis was performed by collaborators at the National Oceanic and Atmospheric Administration (NOAA) Northwest Fisheries Science Center, Seattle, WA, USA using methods for have been described previously [129]. Contaminants were extracted by accelerated solvent extraction (ASE) after blubber was dried with magnesium or sodium chloride. Polar compounds and lipids were removed from the extracts using a gravity flow column and size-exclusion chromatography, respectively. Total lipid content was assessed by thin-layer chromatography with flame ionization detection. POPs were measured by gas chromatography-mass spectrometry (GC/MS) – included in this assay were six DDTs (*p,p'*- and *o,p*- DDT, DDE, and DDD), 45 polychlorinated biphenyls (PCBs), 15 brominated diphenyl ethers (BDEs), 8 chlordanes (CHLs), hexachlorobenzene (HCB), dieldrin, and mirex. In the coming analyses and discussion, ΣDDT, ΣPCB, ΣBDE, and ΣCHL will refer to the summation of POP values within these classes.

Contaminant values are reported as concentrations (ng of contaminant / gram of lipid); ranges of POPs measured are reported in Table 4.4.

2.5 Statistics

A principle components analysis (PCA) was utilized to explore patterns among contaminant variables. Values below the limit of quantification (LOQ) were assigned a random value between zero and LOQ. Data were $\log_{10}$ transformed before being mean centered and unit scaled. Suitability for PCA was examined – any variable without at least one correlation with $r^2 > 0.3$ was removed from the analysis; Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy for the whole set and for each individual variable was >0.5 (variables with KMO measures <0.5 were removed); and Bartlett's test of sphericity was significant ($p < 0.05$) [147]. Factors with eigenvalues greater than 1 were extracted. Varimax rotation was utilized to simplify interpretability. Inclusion of each individual contaminant in the analysis precluded the PCA because many of the contaminants are very highly correlated, leading to a non-positive definite relationship. Thus, included in the PCA were the six individual DDTs, mirex, dieldrin, and hexachlorobenzene, ∑PCBs, ∑CHLs, and ∑BDEs. Relationships among PC scores and hormones were analyzed by Kendall's tau-b.

Remaining, analyses were stratified by sex due to the fact that POP exposure and hormone profiles should vary by sex. Samples were collected in two months – July and October – thus differences in hormone measurements by month were examined by Mann-Whitney U test in which values below LOQ were censored to the same value below LOQ to ensure all such values would be tied in this rank-based statistical test. For males, T and AE concentrations varied significantly by month, thus male analyses were further stratified by month to control for the potentially confounding effect of season. In females, animals with blubber progesterone values

in or exceeding the range for pregnant animals published by Perez et al. were classified as likely pregnant [118]. None of the remaining hormones differed by pregnancy status, thus remaining analyses of females were not stratified by pregnancy status. Correlations between hormones and contaminants were analyzed by Kendall's tau-b, for which values below LOQ were censored to the same value below LOQ.

3. Results

3.1 Principle Component Analysis

The DDTs load together to the same component (PC1), and cluster somewhat together separated from the other POPs (Fig 4.1 A). PC1 scores are negatively correlated with T (in males) and F (in females), but not the other hormones, while none of the hormones are significantly correlated with PC2 (Fig 4.1B).

3.2 Correlations

In males sampled in October, T exhibits significant negative correlations with *o,p*-DDD, *o,p*-DDE, and *p,p*-DDD (Fig 4.2). In males sampled in July, there are no significant correlations between any hormones and the DDTs. In females (inclusive of both months), F is negatively correlated with each of the individual DDTs (Fig 4.5) and summations of each POP class (Fig. 4.6). T in males and F in females are also significantly correlated with other individual POPs, and the POPs themselves are all very strongly positively correlated with one another (not shown).

4. Discussion

Male and female bottlenose dolphins exhibit differential POP exposure profiles because females offload significant portions of their POP burden by mobilizing lipid reserves (and thus,

the contaminants stored within) during lactation, while males have no such opportunity to offload POPs [81]. Therefore, POP measures in males are an estimate of their lifetime exposure to contaminants, whereas in females they are not. Without life history data (such as the number of lactations), it is impossible to estimate lifetime exposure in females. As such, studying the impacts of exposure will require different interpretations for each sex. For this reason, the analyses (except the PCA) are stratified by sex.

I predicted that T and DDE would be negatively correlated in males given the pattern observed in Dall's porpoise [108]. Notably, only three of 16 males sampled in July had quantifiable T whereas 10 out of 17 males sampled in October had quantifiable blubber T. Without age/age class information, I cannot ascertain whether this is caused by this population having a late breeding season or if a disproportionate amount of subadults were sampled in July while more adults were sampled in October. Nonetheless, this ultimately led to significant differences in T measurements by month, which prompted me to stratify analyses by month. If methods to estimate age class remotely (without hormonal data) become available, it would be beneficial to repeat this analysis with age class included as a variable. This apparent variation by month – regardless of whether it is a function of physiology or sampling bias – complicates the analysis and limits my conclusions. In males sampled in October, T is negatively correlated with *o,p*-DDE as well as both isomers of DDD, but similar patterns do not emerge in July samples (likely because only three males had quantifiable T). These results could indicate that exposure to DDD and/or *o,p*-DDE negatively impacts T homeostasis in male bottlenose dolphins. If DDx exposure has no impact on T homeostasis, one would expect a positive correlation between T and DDTs in males because T is linked to sexual maturity – that is, males with higher T would likely be older, and should therefore exhibit higher DDx measurements compared to males with

lower T. The negative correlations here, though, may indicate that males with higher *o,p*-DDE and/or DDD burdens experience impaired T secretion and/or elevated T metabolism. However, these findings are difficult to interpret, and ultimately it is impossible to ascribe any mechanism for the observed relationships because these DDTs and T are also significantly correlated with a multitude of other POPs. Therefore, it could be that one of the other POPs (or a mixture of them) is responsible for disrupting T homeostasis, while these DDTs are only positively correlated with the causative POPs, thus spuriously appearing related to T. However, the DDTs load together onto the PC1 in the PCA, while the other POPs load stronger to PC3, and T is negatively correlated with PC1 scores but not PC2 score, which may support the conclusion that this effect is DDx-specific.

Without performing controlled dosing studies it is impossible to conclusively determine whether DDx/POP exposure impacts T homeostasis in male bottlenose dolphins. But these results warrant further investigation, especially considering that this is the second cetacean species in which a negative correlation between T and DDE has been observed. Because T plays an important role in regulating male reproductive physiology, resource managers should consider exploring reproductive physiology in males from St. Andrew's Bay, particularly in relation to other populations with lower DDx exposure – perhaps by using capture-release studies to collect semen samples to examine sperm count and quality, or examining testicular anatomy with ultrasound. Studying male reproductive behavior could also be useful, but may prove difficult unfeasible in this free-ranging population.

A positive correlation between *p,p'*-DDE and plasma E_1 was observed in female pilot whales; thus, I hypothesized that similar relationships would be evident in bottlenose dolphins [109]. However, E_1 was not detected in any samples in this dissertation. Instead, I observed

significant negative correlations between F and the DDTs in females. Similar relationships have been observed in male polar bears, and *p,p'*-DDT, DDE, And DDD and *o,p*-DDD have been shown to disrupt adrenal steroidogenesis in a multitude of species (Table 1.4) [75]. As in males, these results are difficult to interpret given that F and the DDTs are also correlated with other POPs. Interpretation is furthered hindered by the occurrence of lactational offloading in females and our lack of life history data. Once again, PC1 scores, but not PC2 scores, from the PCA are negatively correlated with F, supporting the conclusion that this relationship is DDx-specific. While not conclusive, these results should prompt further investigation of adrenal health in this population; considering the variety of important roles adrenal hormones play in vertebrate stress physiology and development, POP-mediated adrenal disruption could have serious implications for health and fitness.

Overall, I conclude that DDTs and POPs could be negatively impacting steroid hormone homeostasis in the population of bottlenose dolphins inhabiting St. Andrews Bay. More conclusive mechanistic studies are impossible given the legal and logistical difficulties associated with performing toxicological studies in marine mammals, but surveys of individual health (i.e., reproductive, immunological, and developmental endpoints) and population health could shed further light on this matter.

Table 4.1 Average internal standard mass

Compound	**Average Mass** (ng)
P_4-$^{13}C_3$	18.624
17OHP_4-$^{13}C_3$	19.761
AE-$^{13}C_3$	19.529
T-$^{13}C_3$	19.459
F-d_4	17.828
E-$^{13}C_3$	17.939

Table 4.2 Calibration curve information for St. Andrews Bay blubber hormone analysis

Hormone	**Mass Range** (ng)	**Number of Points**	**Curve**	**Intercept**	**Slope**	**R^2**
P_4	2.73 – 5.70	4	Linear (forced)	0.000	0.0038	0.998
17OHP_4	5.584 – 0.5392	3	Quadratic	-0.0324	-2.5671; 1.3795	1.000
AE	12.462 - 0.2573	3	Quadratic	0.0259	5.892; -0.4187	1.000
T	16.52 - 0.7743	3	Linear	0.0023	0.2121	1.000
F	2.384 - 0.1197	3	Quadratic	0.0037	27.051; -0.5483	1.000
E	2.208 - 0.1109	3	Linear	-0.0355	7.8055	0.999

Table 4.3 Limits of quantification for St. Andrews Bay blubber hormone analysis. Observed limits of quantification (LOQ_{obs}) are defined as the lowest calibration standard used in the calculation of the standard curve. Calculated limits of quantification (LOQ_{calc}) are calculated as three times the standard deviation of the blank measurements plus the mean of the blank measurements

Hormone	**LOQ_{obs}**	**LOQ_{calc}**
P_4	5.70	0.00551
17OHP_4	0.5392	0.517
AE	0.2573	0
T	0.7743	-
F	0.1197	0
E	0.1109	0.092961

- = Negative value

Table 4.4 Summary of persistent organic pollutant (POP) measurements in St. Andrews Bay bottlenose dolphins

POP	**Min** (ng/g)	**Max** (ng/g)
p,p'-DDT	< 1.8	2300
p,p'-DDE	8.5	52000
p,p'-DDD	< 1.8	5700
o,p-DDT	< 1.6	940
o,p-DDE	< 1.8	410
o,p-DDD	< 1.4	640
∑DDTs	8.5	62000
∑CHLs	2.7	2200
∑PCBs	57	46000
∑PBDEs	1.7	800
Mirex	< 1.2	170
Dieldrin	< 2.0	130
HCB	< 1.2	33

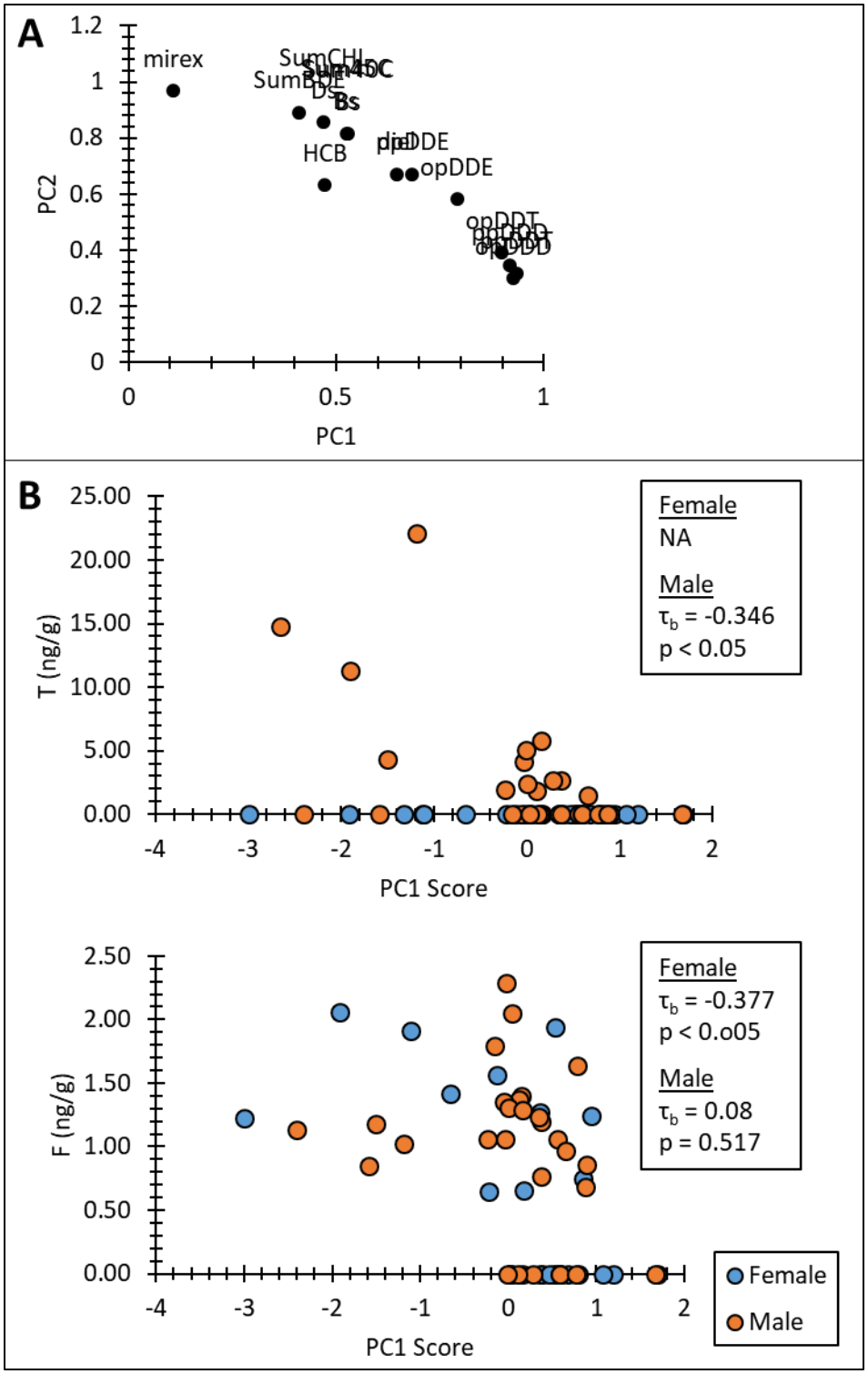

Fig. 4.1 PCA on contaminants. A) Variable loading plot, B) correlations between PC1 and T and E

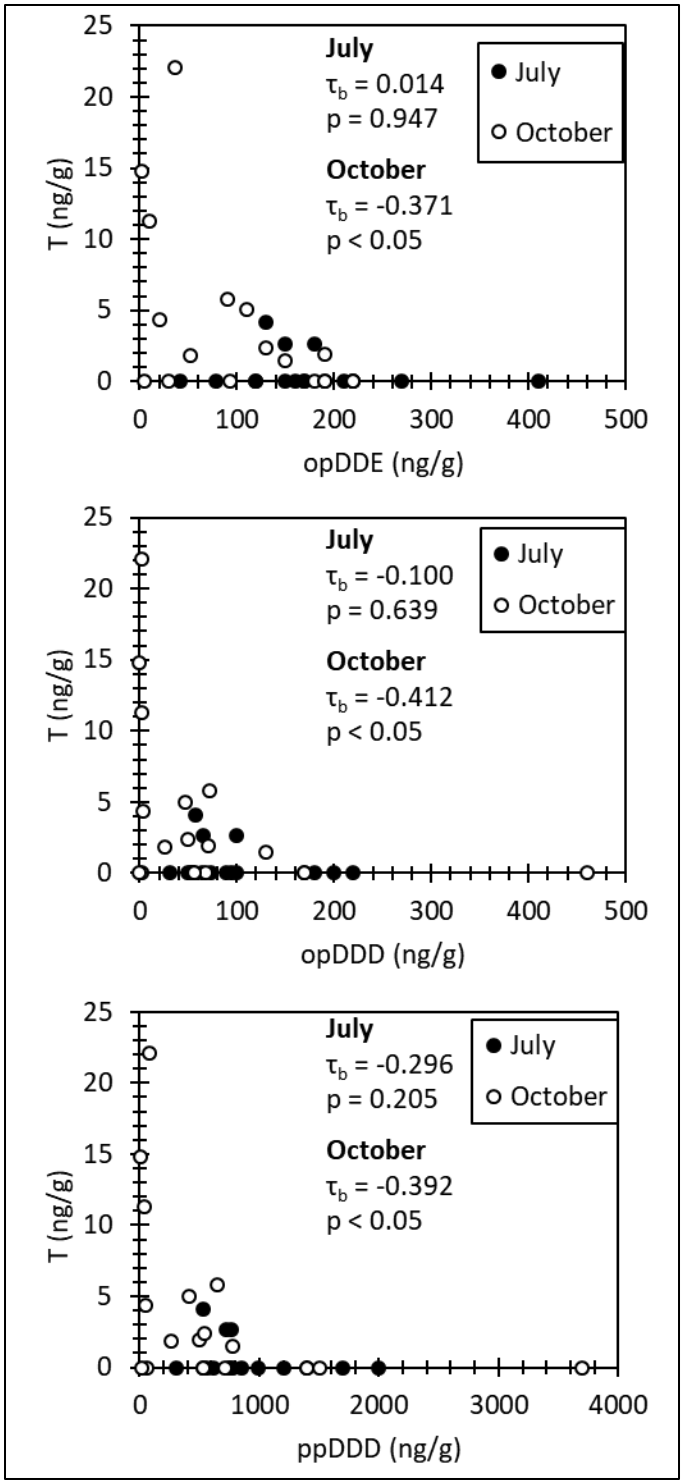

Fig 4.2 Correlations between T and *o,p*-DDE, *o,p*-DDD, and *p,p'*-DDD in males by month.

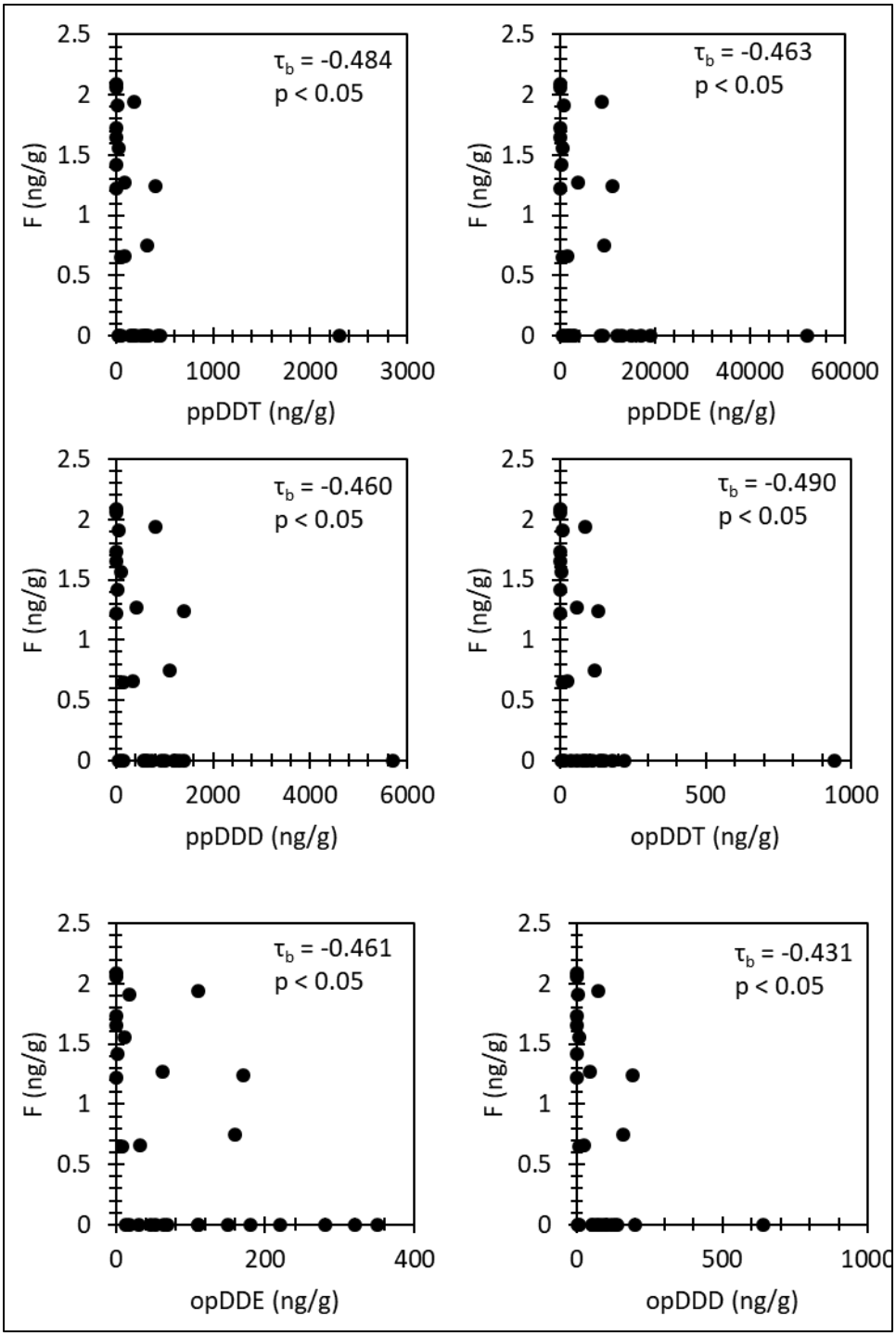

Fig 4.3 Correlations between F and individual DDTs in females

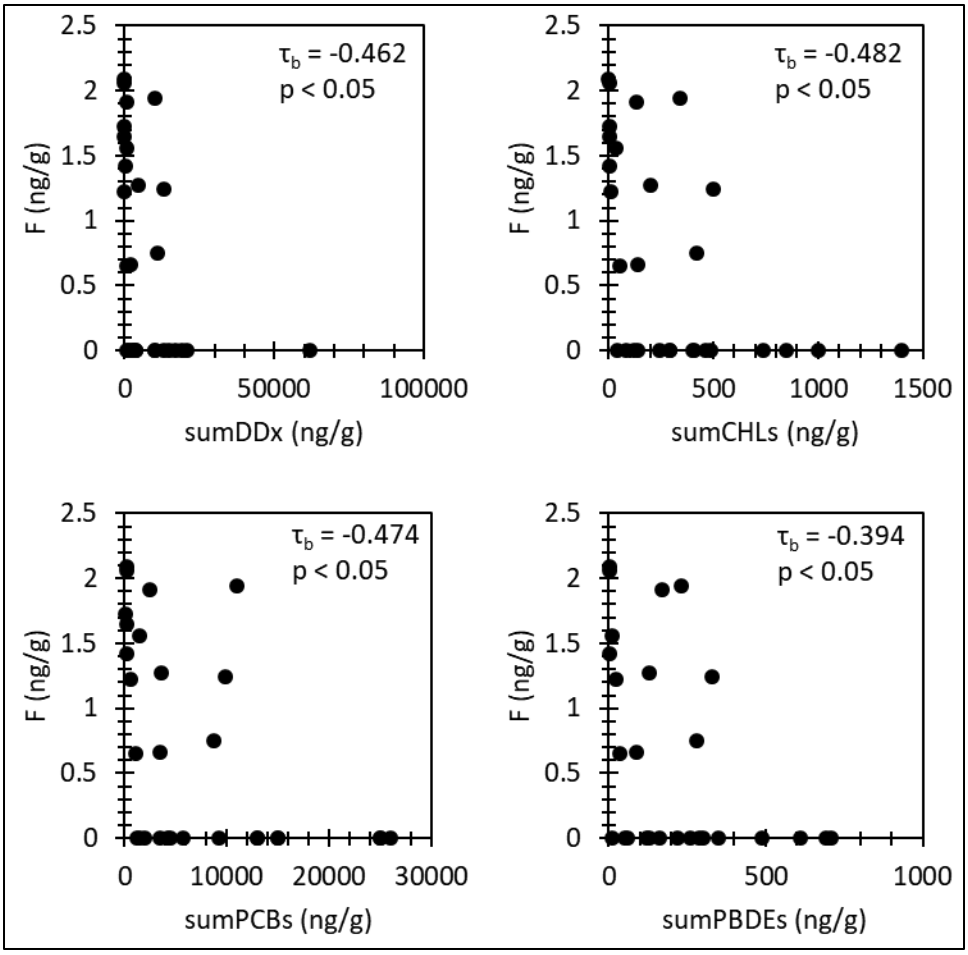

Fig 4.4 Correlations between F and contaminant classes in females

CHAPTER 5: Metabolism of Cortisol and Cortisone by Microsomes from *T. truncatus* Blubber

1. Introduction

Blubber steroid hormones seem to qualitatively reflect systemic endocrine status in cetaceans, as physiological conditions that cause changes in circulating steroid hormone profiles produce similar changes in blubber steroid hormones – i.e., progesterone (P_4), testosterone (T), and cortisol (F) are elevated in blubber during pregnancy, the breeding season/sexual maturity, and stress, respectively [114-120]. However, as demonstrated in Chapter 3, blubber steroid measurements are not ideal quantitative predictors of circulating steroid hormone concentrations. This may be due in part to a temporal lag between when changes in circulating hormone concentrations occur are reflected in blubber. Additionally or alternatively, the apparent disagreement between blood and blubber hormone measurements could indicate that the hormones detected in blubber are not entirely of central origin – that is, perhaps blubber metabolizes or synthesizes steroid hormones itself.

Metabolism of steroid hormones in peripheral tissues is well established in other mammals, and is thought to occur in order to control local concentrations of hormones [122-124, 158-164] (reviews: [165-167]). Human and rodent adipose tissues express genes involved in steroid hormone metabolism, and both have exhibited the ability to metabolize steroid hormones, including corticosteroids, estrogens, and androgens [3, 122-124, 158, 161, 162, 164, 168, 169]. From this work and the observation that blubber F and cortisone (E) are significantly and positively correlated in bottlenose dolphin blubber (discussed in Chapter 3, section 4.1; Fig. 3.9), I hypothesize that blubber metabolizes corticosteroids. I specifically expect to observe the conversion of F to E (dehydrogenase reaction) by blubber microsomes *in vitro*, as seen in human and rodent adipose [122, 123, 158]. The enzyme that catalyzes F to E, 11β-

hydroxysteroid dehydrogenase (11βHSD), exhibits reticular localization in human embryonic kidney (HEK) and Chinese hamster ovary (CHO) cell lines, hence the use of microsomes, which are fragments of the endoplasmic reticulum produced by cellular fractionation [170, 171]. 11βHSD also catalyzes the reverse (reductase) reaction, converting E to F (Table 1.2). Thus, one might expect to observe the conversion of E to F in blubber. However, the previous studies in human and rodent tissues did not explore the reductase reaction, claiming that that the dehydrogenase direction (F to E) predominates in the *in vitro* environment, thus making it difficult to study the reductase reaction [122, 123, 158]. Therefore, given my use of an *in vitro* design, I only expect to observe enzymatic conversion of F to E in *T. truncatus* blubber in this study.

2. Methods

2.1 Sample Collection and Microsome Preparation

Full-depth blubber samples were collected from three male stranded (code 1) bottlenose dolphins (one juvenile, one subadult, and one adult) from the southeastern United States, and were stored at -80 °C until analysis. Blubber microsomes were prepared via methods adapted from Huderson et al. [172]. Skin was removed, and approximately 4 g of blubber were minced in glass beakers on dry ice using razors and forceps (prior to mincing all glass, razors, and forceps were rinsed three times with acetone then hexane). Minced tissue was homogenized in 4 to 5 mL of sucrose-TKM buffer (sucrose 0.25 M, Tris 80 mM, KCl 25 mM, $MgCl_2$ 5 mM, pH 7.4) on ice with a polytron homogenizer. Homogenates were centrifuged at 10,000 x g for 10 min at 4 °C, then the supernatant was transferred to a new tube and centrifuged again at 15,000 x g for 15 min at 4 °C. Supernatant was transferred to a new tube and centrifuged at 100,000 x g for 60

min at 4 °C. The microsomal pellet was washed three times with sucrose-TKM buffer before being reconstituted in 1 mL of the same buffer. Protein concentrations in the microsomal preparations were measured with a microplate-based Bradford assay, using a kit and following manufacturer instructions (GeneCopoeia, Rockville, MD, USA).

2.2 Corticosteroid Metabolism Assays

Corticosteroid metabolism was assayed using methods adapted from Livingstone et al. [122]. F or E standards in methanol were gravimetrically added to clean borosilicate culture tubes, and solvent was brought to dryness under N_2 (100-130 kPa) in a water bath at 40 °C. Krebs-Ringer buffer (118 mM NaCl, 3.8 mM KCl, 1.19 mM KH_2PO_4, 2.54 mM $CaCl_2$, 1.19 mM $MgSO_4$, and 25 mm $NaHCO_3$, pH 7.4), cofactor (2 mM; NADP+ or NADPH, for dehydrogenase and reductase reactions, respectively; Sigma-Aldrich, St. Louis, MO, USA), and microsomes (60 µg/mL protein) were added to the dried hormone, bringing the total reaction volume to 250 µL. The final concentration of substrate (F or E) in the reaction mixture was approximately 1 µM. Reaction mixtures were briefly vortexed before being incubated at 37 °C for 90 minutes. Negative controls included reaction mixtures containing only hormone, cofactor, and buffer (hereafter referred to as "No Protein Controls" or NPCs), and reaction mixtures containing only microsomes, cofactor, and buffer (hereafter referred to as "No Hormone Control" or NHC). An NHC was utilized for each individual sample. Mouse liver microsomes (male CD-1 mice; Sigma-Aldrich, St Louis, MO, USA) were utilized as a preliminary positive control; this control experiment was performed before proceeding with blubber microsomes to ensure that assay conditions were acceptable. All treatments, except mouse liver positive controls, were performed in at least triplicate.

Following the 90 min incubation, 150 µL of internal standard mixture (containing cortisol-d_4 and cortisone-$^{13}C_3$) was gravimetrically amended to each reaction mixture (Table 5.1). Ethyl-acetate liquid:liquid extraction was used to extract hormones. Ethyl acetate (2 mL, which is equal to five times the volume of reaction mixture plus internal standard) was added to each tube. These mixtures were vortexed for 2 min, then allowed to rest undisturbed for 5 min to allow phase separation. The upper ethyl acetate layer was transferred to a clean borosilicate tube, and was dried under N_2 (100-130 kPa) in a water bath at 40 °C. Dried extracts were reconstituted in 50:50 methanol:water (volume fraction), and transferred into amber autosampler vials containing glass inserts. F and E were measured and quantified by LC-MS/MS using the C18 separation and quantification methods described in Chapter 2 Sections 2.5 and 2.6. Standard curve parameters and limits of quantification (LOQ) are reported in Table 5.2.

My hypothesis is binary (positive/negative) – thus, for both assays (dehydrogenase and reductase) I established a threshold above which a result would be classified as positive. For the dehydrogenase reaction, the NPCs contained quantifiable E, therefore the threshold for this experiment was calculated as three times the standard deviation of the E concentration in NPCs plus the mean E concentration in the NPCs. For the reductase reaction, neither negative controls (NPC or NHC) exhibited F peaks, thus the presence of any F signal indicated a positive result. Because the hypothesis being tested is dichotomous (positive/negative) and I expect all sample treated with hormone to produce a positive signal, sample size power analysis indicates that three samples is sufficient to resolve such a result with 80% power and alpha equal to 0.05. If a positive result was detected, the experiment was repeated with microsomes that had been denatured by boiling for 20 min, and additional NHCs were performed with denatured

microsomes to ensure that denaturing did not produce additional/different interferences compared to normal microsomes.

2.3 Statistics

Statistical analyses were performed with IBM SPSS Statistics 23 or 24 (IBM, North Castle, NY, USA). A two-way analysis of variance (ANOVA) utilizing treatment (endogenous or denatured) and individual as fixed factors was used to determine whether denaturation of microsomes significantly reduced relative hormone signal compared to endogenous microsomes.

3. Results

3.1 Blubber 11βHSD Dehydrogenase Activity Assay: Metabolism of Cortisol to Cortisone

No E was detected in any NHCs, but NPCs had detectable E, which was caused by contamination of the neat F standard that was used for dosing (Fig 5.1A). Mouse liver microsomes treated with F produced an E signal several orders of magnitude greater than baseline E contamination observed in NPCs, indicating assay conditions were acceptable (Fig 5.1B). Blanks run after positive results exhibit a lack of E, indicating that positive results are not a result of E carry-over (Fig 5.1C). Microsomal preparations from each blubber sample exhibited E signals greater than the threshold (three times the standard deviation of NPCs plus mean of NPCs), while denatured microsomes failed to exceed the threshold (with the exception of one replicate from one sample); mean relative E signal is significantly reduced in denatured compared to endogenous microsomes as indicated by two-way ANOVA (Fig 5.2).

3.2 Blubber 11βHSD Reductase Activity Assay: Metabolism of Cortisone to Cortisol

No F was detected in neither NHCs nor NPCs in the reductase experiment (Fig 5.3A). F was detected both in mouse liver microsomes (Fig 5.3B) and one blubber sample (mean: $1.365 \bullet 10^{-4} \pm 1.034 \bullet 10^{-4}$ ng F per ng E dosed) (Fig 5.3C). No F was detected in denatured microsomes from this sample.

4. Discussion

The experimental design for this study was based on that used in Livingstone et al., in which the ability of rat adipose to metabolize corticosteroids was demonstrated. However, whereas Livingstone and colleagues used whole adipose tissue homogenate in their study, I isolated and used blubber microsomes, which provides an important improvement over the use of whole tissue homogenate. As demonstrated in Chapters 3 and 4, blubber contains endogenous F and E, which would make it very difficult to interpret results from this experiment if whole blubber homogenate were used. Conversely, blubber microsomal isolates do not contain endogenous F or E, as demonstrated by the lack of hormone in the NHCs, which simplifies interpretation compared to whole blubber homogenate.

Whereas NHCs did not contain F or E, the NPCs for the dehydrogenase experiment did contain E. Thus, in the dehydrogenase (F to E) direction, the threshold for a positive result was based on the baseline contamination of the NPCs (three times the standard deviation of the NPCs plus the mean of the NPCs). Since there is E contamination in the F standard, I must control for the quantity of F standard used in each replicate; while each replicate should have received the same dose, slight variation could potentially impact the results. Therefore, the F dose was calculated gravimetrically and E values for are reported relative to the mass of F

dosed. Furthermore, I also ensured that there was no E carry-over, leading to false positives, by confirming that no E was evident in blanks that were run immediately after positive samples.

The positive control in both experiments (mouse liver microsomes) exhibit production of E and F in the dehydrogenase and reductase reactions, respectively. Notably, I did not expect to observe a positive result in the reductase direction (E to F) because previous studies state (but do not demonstrate) that the reductase reaction is not observable *in vitro* [122, 123, 158]. I do not report the quantity of E or F produced by mouse liver microsomes because this was simply used as a preliminary qualitative positive control before proceeding with the blubber microsome experiments.

In the blubber dehydrogenase activity experiment, the relative E measured in each of the treated blubber microsomes exceeded the threshold for a positive result. Thus, the amount of E measured in these assays cannot be attributed to contamination, and I conclude that *T. truncatus* blubber microsomes metabolize F to E *in vitro*, as I hypothesized. This is further supported by the fact that denaturing these microsomal preparations significantly reduced mean relative E signal compared to endogenous microsomes. Notably, while all three blubber samples produced E, one sample (the subadult) exhibited greater production than the other two. This may be driven by differences in 11βHSD expression between the samples, though without protein or gene expression data, this is speculation. Furthermore, without knowing the endogenous factors that influence 11βHSD expression/activity in bottlenose dolphin blubber, it is impossible to establish any sort of causal mechanism for this apparent variation by individual. Future studies should repeat this experiment with a larger sample size and include measures of 11βHSD gene/protein expression in the analysis.

In the blubber 11βHSD reductase experiment, only one blubber sample ("Blubber 3") exhibited detectable F in all three of its replicates, while the other two did not have detectable F in any replicates. Furthermore, denatured Blubber 3 microsomal preparations did not have quantifiable F, suggesting that the F detected in the normal microsomes was produced enzymatically. Notably, this is the same sample that seemed to have a higher signal in the dehydrogenase direction, which further supports the hypothesis that 11βHSD expression and/or activity is higher in this individual. With a positive result in only one of three individuals, I very cautiously conclude that blubber microsomes can potentially metabolize E to F *in vitro*. Future studies should repeat this experiment with expanded sample size.

These conclusions provide preliminary evidence that blubber possesses the ability to metabolize steroid hormones, in keeping with what has previously been observed in other mammals. However, there are several important limitations to this study design that need to be addressed. First, with only three samples, all of which came from stranded (i.e., stressed) animals, the generalization of these results to other individuals, populations, the species, or other marine mammals is precluded. Second, because these experiments were performed with isolated microsomes, this study should be repeated with blubber explants *ex vivo*. I utilized microsomes instead of explants to simplify the interpretation, but *ex vivo* experiments would be more conclusive in indicating whether corticosteroid metabolism occurs in living blubber. Despite these caveats, the results presented here are compelling and should prompt broader investigation of blubber as a site of steroid hormone metabolism, as this could dramatically impact the interpretation of blubber steroid hormone measurements. Future studies should also test whether blubber metabolizes other steroids in addition to F and E, especially the

androstenedione (AE) to T because human adipose performs this conversions and blubber T and AE are significantly and positively correlated (Fig 3.3) [161].

Table 5.1 Average internal standard masses

Compound	**Average Mass** (ng)
F-d_4	17.69357
E-$^{13}C_3$	17.00714

Table 5.2 Calibration curve parameters and limits of quantification (ng). Observed limit of quantification (LOQ_{obs}) is defined as the lowest calibration standard used in the calculation of the standard curve. Calculated limits of quantification (LOQ) are calculated as three times the standard deviation of the NHC measurements plus the mean of NHC measurements

Hormone	**Range** (ng)	**Number of Points**	**Curve**	**Intercept**	**Slope**	**R^2**	**LOQ_{obs}**	**LOQ_{calc}**
F	0.05269 - $7.879 \bullet 10^{-5}$	3	Linear	0.0007	1.9757	0.9999	$7.879 \bullet 10^{-5}$	-
E	1.185 - 0.05455	4	Linear	-0.0037	3.7352	0.9995	0.05455	0.02107

- = negative value

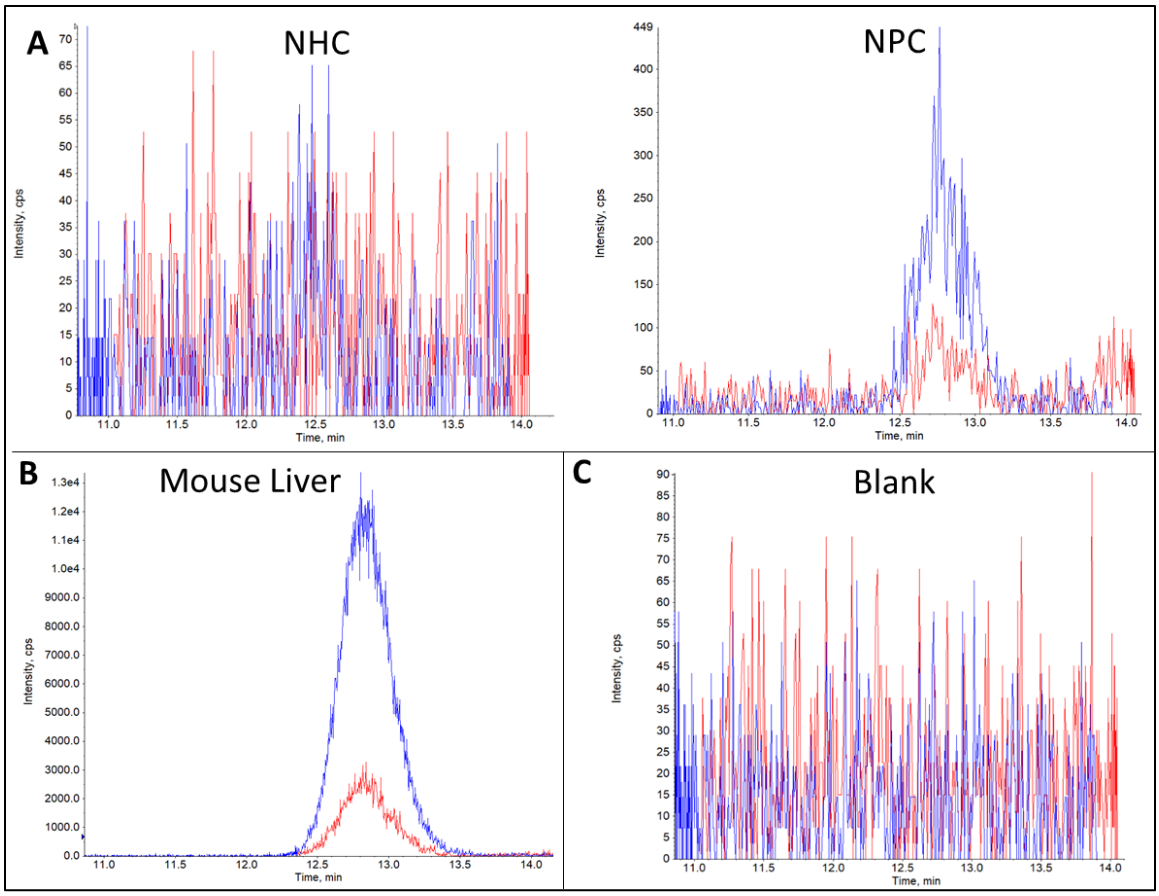

Fig 5.1 Dehydrogenase reaction. A) Representative no hormone control (NHC) and no protein control (NPC) chromatograms where blue and red chromatograms are the primary and secondary transitions of cortisone (E), and intensity (y-axes) units are counts per second (CPS), B) chromatogram of mouse liver microsomes, C) representative chromatogram of blank run immediately after a positive sample indicating lack of carry-over

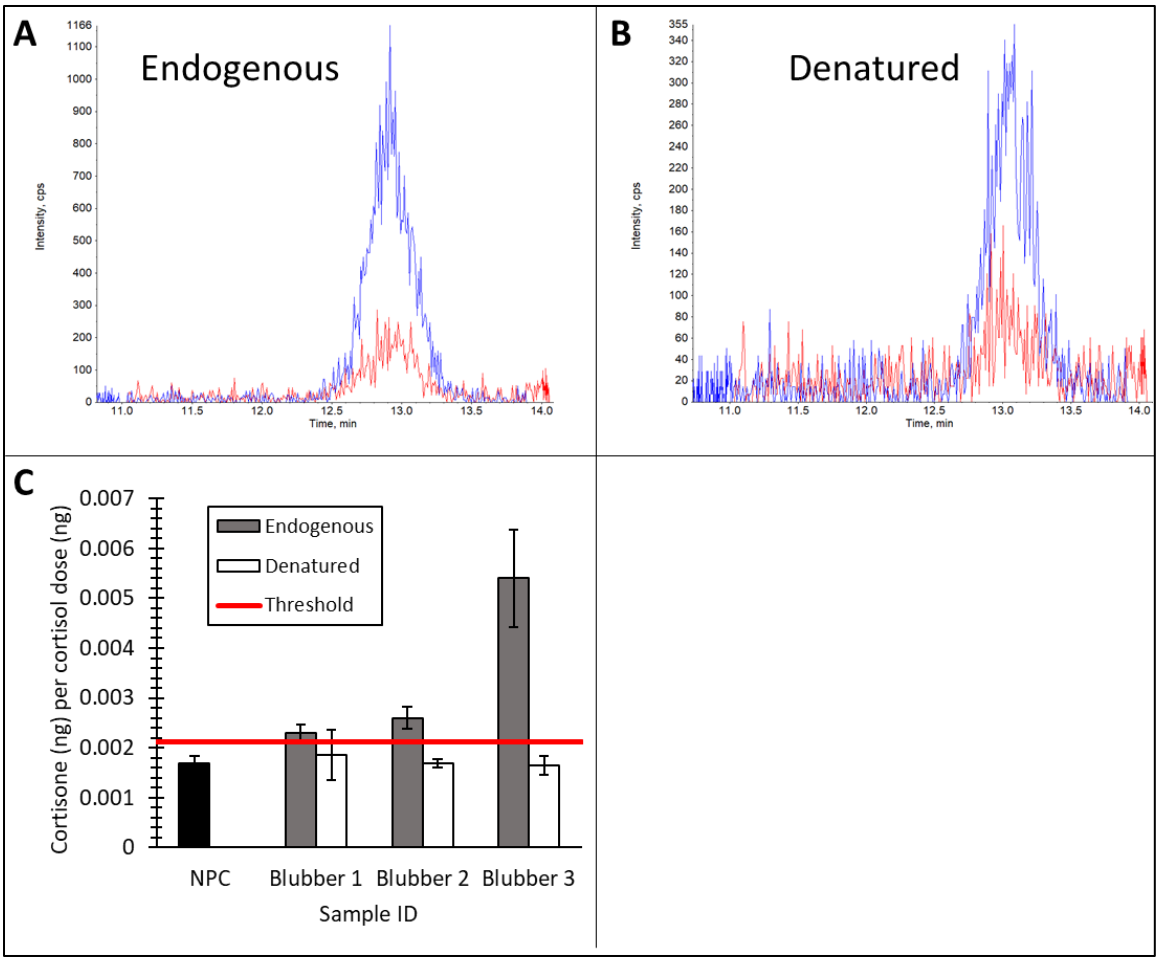

Fig 5.2 Dehydrogenase Reaction. A-B) Representative cortisone (E) chromatograms for Blubber-3 endogenous (non-denatured) (A) and denatured microsomes (B) where blue and red chromatograms are the primary and secondary transitions of E, and intensity (y-axes) units are counts per second (CPS); C) mean measured E mass relative to the dose of F in endogenous and denatured blubber microsomes – horizontal red line indicates threshold for positive result and error bars are standard deviations. The main effect of denaturation is significant ($p < 0.05$) per two-way ANOVA

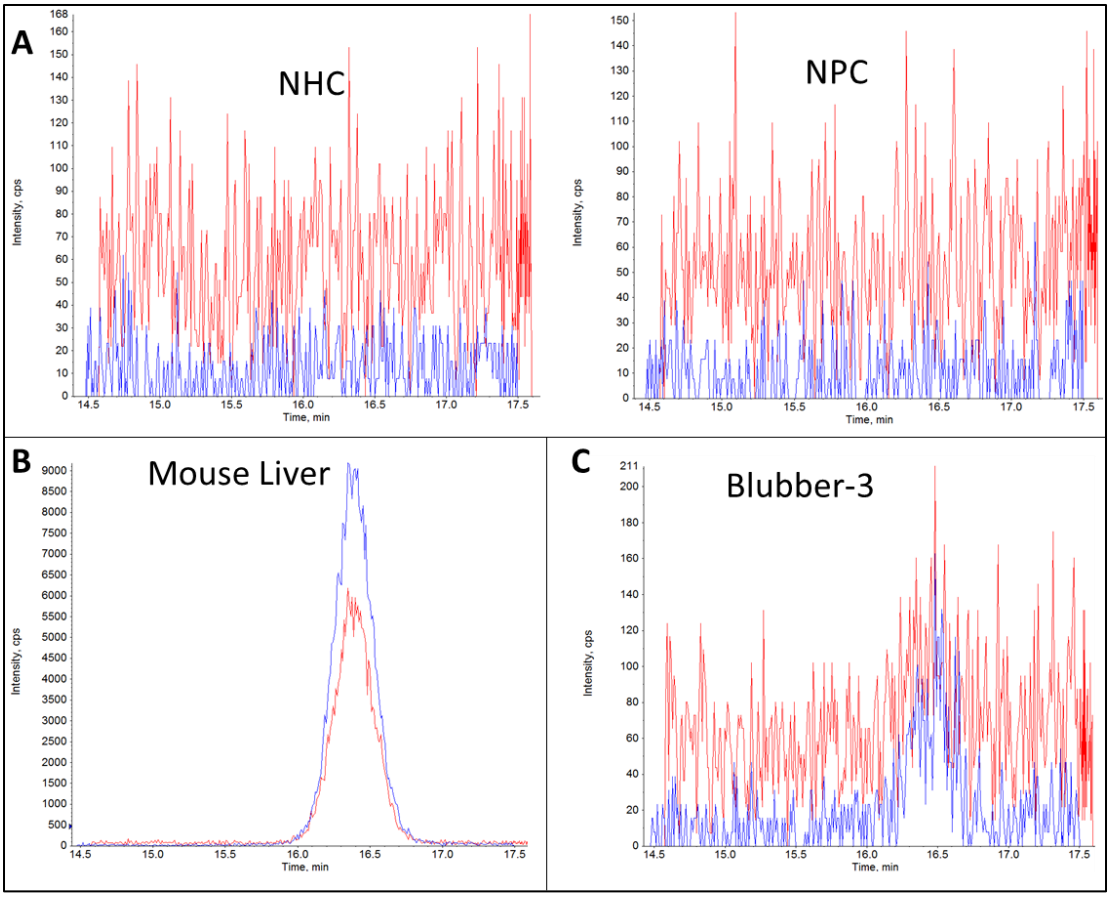

Fig 5.3 Reductase reaction. Representative chromatograms for no hormone control (NHC) (A), no protein control (A), mouse liver microsomes (B), and blubber 3 (C), where the blue and red lines are the primary and secondary transition for cortisol

CHAPTER 6: Summary, Future Directions, & Overall Conclusions

1. Summary

The legal, ethical, and logistical limitations impeding the study of marine mammal physiology required a creative approach to the study of POP-mediated endocrine disruption in cetaceans. Being unable to dose animals with DDTs meant that I had to rely on animals that were incidentally exposed to DDTs through "natural" means. The population of bottlenose dolphins inhabiting St. Andrews Bay, FL, USA met this requirement, as they exhibit DDx burdens significantly higher than other populations in the southeastern United States due to high local DDx contamination (Table 1.3) [129, 130] (Balmer et al. unpublished). Unfortunately, sample availability from this population is limited, owing to the cost and logistical concerns associated with permitting and sampling. Ultimately, this meant that in order to study DDx-mediated endocrine disruption, I would need to exclusively use remotely collected blubber and skin biopsies. Blubber is already an established matrix for measuring POP exposure in marine mammals. Conversely, it was unclear at the onset of this work whether blubber was a suitable matrix for systemic endocrine assessment in bottlenose dolphins. There is a body of evidence indicating that blubber steroid hormone measurements can be used to differentiate qualitative physiological states (such as pregnancy, male sexual maturity, and stress), but there had been minimal work comparing blubber hormones to circulating hormones [114-119]. Considering that blood is the preferred matrix for endocrine assessment, it was critical that the relationship between blood and blubber hormones be better elucidated before attempting to use blubber to study endocrine disruption. This was the first aim of this dissertation.

Recently published work in human blood and bottlenose dolphin blubber demonstrated the utility of LC-MS/MS techniques in the study of endocrinology [121, 128]. Whereas traditional

methods (immunoassays) have limited specificity and can only be used to measure a single analyte at a time, LC-MS/MS provides improved specificity and allows multiple analytes to be measured simultaneously. As such, using LC-MS/MS, investigators are able to quantify a broad suite of steroid hormones – including several that are already commonly studied due to their prevalence in circulation (progesterone [P_4], testosterone [T], estradiol [E_2], and cortisol [F]) and others that are precursors to these, minor products of the adrenal and gonads, and/or considered less potent/inactive – thereby enabling the assessment of potentially interesting and physiologically relevant relationships among hormones. Therefore, I wanted to use LC-MS/MS for all steroid hormone measurements in my dissertation. While a method had already been developed and published for bottlenose dolphin blubber and human blood, no such method existed for dolphin blood matrices. Due to the possibility for matrix-dependent interferences, I needed to validate a method for dolphin blood before proceeding with its use in comparing blood and blubber steroid hormone measurements.

Thus, in Chapter 2, I describe the validation of solid phase extraction (SPE) to LC-MS/MS techniques for the measurement of steroid hormones in bottlenose dolphin blood matrices (plasma and serum). Using a spike recovery experiment, I determined that up to eight hormones can simultaneously be measured accurately and precisely, including P_4, 17-hydroxyprogesterone ($17OHP_4$), androstenedione (AE), T, E_2, estrone (E_1), F, and cortisone (E) in both matrices. 11-deoxycortisol (S), 11-deoxycorticosterone (DOC), and corticosterone (B) were not reliably quantifiable, as indicated by poor and highly variable percent recoveries. I propose that this may be due to a lack of matched internal standards for these compounds. Should isotopically labeled internal standards become available for S, DOC, and/or B, these experiments should be repeated. After the spike retrieval, I demonstrated that five of these hormones could be

precisely measured endogenously in plasma, while serum was less precise; endogenous P_4 and the estrogens were not detected in the samples used for this analysis. This led me to conclude that plasma is the preferred blood matrix for use in this paradigm. I go on to compare plasma and serum, and concluded that hormones in each matrix are significantly and positively correlated. This is important because previous studies often utilized serum, thus, in showing that serum and plasma are correlated, I provide assurance that measurements made in plasma are reasonably comparable to those in serum. Overall, this experiment led me to conclude that SPE to LC-MS/MS is valid for the measurement of steroid hormones in bottlenose dolphin blood, and allowed me to proceed with the comparison of blood and blubber steroid hormones.

In Chapter 3, I assessed the relationship between plasma and blubber steroid hormone measurements from matched samples (measured by LC-MS/MS) to determine whether blubber is a usable proxy for blood in bottlenose dolphin endocrine assessment. These samples were collected from three populations of free-ranging bottlenose dolphins that are being used as reference populations due to their low DDx exposure compared to St. Andrews Bay animals. Ultimately, I determined that blubber hormones are not quantitatively predictive of circulating steroid hormone concentrations, and I suggest three potential explanations for the poor agreement. First, while blood and blubber samples were individual-matched, they were not time-matched – i.e., blood was collected first during sampling and blubber was collected some time later (sometimes over two hours after blood). Considering that capture and handling induced the HPA stress response, modeling the relationship between blood and blubber corticosteroids using mismatched samples is likely responsible for the poor models for plasma F and E. This could also impact the other steroids, due to HPA/HPG cross talk, though none of the other hormones indicated appreciable relationships with elapsed time to sample collection.

Second, steroid hormone concentrations in blood and blubber could potentially change at different rates, which would produce qualitative agreement between the two matrices in the long term but poor instantaneous quantitative agreement. This hypothesis is derived from evidence in domesticated pigs indicating that peak steroid hormone concentrations in adipose tissue occur one-to-two days after peak circulating concentrations and return to baseline much more gradually [156]. If this is true in bottlenose dolphins, then circulating hormone concentrations provide insight into instantaneous steroid hormone status, whereas blubber hormones perhaps indicate chronic endocrine status. Third, the observed poor agreement between circulating and blubber steroid hormone concentration could potentially be caused by metabolism of steroid hormones in blubber. Adipose tissue in other mammals has been demonstrated to express steroidogenic enzymes and metabolize steroid hormones [3, 122-124, 158, 161, 162, 164, 168, 169]. If blubber has the same capacity and hormones delivered by blood to blubber are metabolized there, then blubber and blood hormones may not be well aligned. This question is the subject of my third aim.

Importantly, Chapter 3 is the first time that 17OHP$_4$, AE, S, and E have been examined in the context of bottlenose dolphin physiology. The sample set utilized in Chapter 3 includes subadult and adult males; pregnant, non-pregnant, and suspected pregnant females; and animals experiencing varying degrees of capture and handling stress. Therefore, I characterized hormone profiles within these groups, with particular attention paid to these four hormones that have not been examined with physiological context in dolphins before. I found that adult males tend to exhibit higher concentrations of T and its precursors AE and 17OHP$_4$, and that these three hormones are positively correlated within each matrix. I take this to suggest that the entire androgen biosynthesis (Δ^4) pathway is upregulated in sexually mature males, which is

expected considering the role that T plays in regulating male reproduction. In pregnant females, P_4, $17OHP_4$, and AE are elevated compared to non-pregnant females, while suspected pregnant females only have elevated P_4. The elevated P_4 is expected as it plays an important role in the maintenance of pregnancy and, thus, is known to be increased during pregnancy. AE may be elevated to support increased secretion of P_4 as in other species, or this could be a byproduct of increased P_4 secretion [5, 6, 12-22]. $17OHP_4$ may be elevated for similar reasons. There are maturity- (male) and pregnancy- (female) related differences in corticosteroid concentrations, which may point to the occurrence of HPA/HPG cross talk in the bottlenose dolphin. As expected, F and E are positively related to elapsed time to sample collection. The corticosteroids are all correlated with one another within each matrix, suggesting that the corticosteroid pathway is upregulated during the stress response. F is also lower in Barataria Bay animals, which is expected because this population is known to exhibit hypoadrenocorticism due to crude oil exposure stemming from the Deepwater Horizon oil spill [131]. Finally, I explored the ability of blubber steroid hormones alone to distinguish between different physiological states. It appears that blubber T, AE, and $17OHP_4$ can be used to somewhat differentiate subadult and adult males, with adults tending to have higher concentrations of these three hormones; though, this may only be possible during the breeding season, as these patterns become less prominent during non-breeding season. Alternatively, while blubber P_4 is sufficient to distinguish non-pregnant and pregnant/suspected pregnant females, neither blubber nor plasma hormone profiles fully distinguish between pregnant and suspected pregnant females; but the inclusion of $17OHP_4$ and AE could potentially be used to better ascertain pregnancy status. These results support the conclusion that blubber hormone measurements can be used to assess overall endocrine status in bottlenose dolphins despite the fact that they are not able to quantitatively

predict circulating hormone concentrations (i.e., chronic changes in systemic steroid hormone homeostasis are reflected in blubber steroid hormone measurements).

Having reached the conclusion that blubber is a usable matrix for endocrine assessment in Chapter 4, I address my primary research question: does DDx exposure impact steroid hormone homeostasis in bottlenose dolphins? As discussed above, this study utilized remotely collected blubber and skin biopsies from bottlenose dolphins from St. Andrews Bay, FL, USA which have been shown to experience high DDx exposure compare to other free-ranging populations in the southeastern U.S. Due to the prohibition of lethal sampling and contaminant dosing in marine mammals, I did not have the ability to characterize molecular mechanisms of disruption. Rather I was limited to examining the relationships between contaminants and hormones in blubber. I stratified my analyses in this chapter by sex (determined by genotypic analysis of skin), due to the differential exposure paradigms that male and female bottlenose dolphins experience – i.e., females offload lipophilic POPs during lactation, while males do not have opportunities to offload [81, 82]. I observed negative correlations between the DDTs and T in males and F in females. Only two studies examining steroid hormones in relation to DDTs in cetaceans have been published. In the first, a negative correlation between DDE and T was observed; therefore, the negative relationship between T and several DDTs in males observed here is in agreement with that work. Neither of the previous studies included F in their analyses, so the negative relationship between DDTs and F in females is unique. These results suggest that DDx-exposure may be impacting steroid hormone homeostasis in this population. However, the DDTs are strongly positively correlated with the other classes of POPs (PCBs, PBDEs, chlordanes), and T (males) and F (females) are also negatively correlated with these POPs. Therefore, it is impossible to determine whether these impacts are mediated by DDTs, one of the other POPs,

or a mixture. Without controlled dosing studies, which will not be legally permitted, this cannot be resolved. Nonetheless, it does appear that endocrine disruption is occurring in some capacity in St. Andrews Bay dolphins.

The final aim of this dissertation (Chapter 5) was to test the hypothesis that blubber can metabolize steroid hormones (discussed above in regard to Chapter 3). I specifically tested whether blubber microsomes could interconvert F and E. I selected this particular reaction because it has been well characterized in the adipose of other mammals, and blubber F and E are very tightly, positively correlated [123, 158, 173]. In treating blubber microsomes with F, I observed E signals in excess of what was observed in negative controls, suggesting that E was produced by blubber microsomes. This was observed for all three blubber samples that I tested. When treating blubber microsomes with E, I observed F signal greater than negative control in only one of three samples. From this in conclude that blubber can convert F to E and can potentially convert E to F.

2. Future Directions

The work herein has generated a number of additional research questions that need to be addressed to better understand cetacean endocrinology and endocrine disruption, primarily related to the function of blubber in the cetacean endocrine system.

The findings in Chapter 3 demonstrate that blubber hormone profiles generally reflect those seen in blood and can be used to define various steroid-linked physiological states, but there is still a great deal of variation in circulating steroid hormone measurements that are not accounted for by blubber measurements. I suspect that this most likely related to differences in the rate at which blubber and blood hormone values change. Dosing captive animals with

labeled hormones and monitoring the uptake into blubber over time would address this question and could potentially yield rates that could be incorporated into the models discussed in Chapter 3 to produce more predictive models. If such studies are not permitted, perhaps similar experiments could be performed *ex vivo* using blubber explants.

Chapter 3 also uncovers a number of questions about basic cetacean endocrinology and the interrelationships between steroid hormones. The positive correlations between T, AE, and $17OHP_4$ in males, the upregulation of AE and $17OHP_4$ during pregnancy, and the positive correlations between S, F, and E are all interesting and warrant further investigation. I suspect that the correlation between T, AE, and $17OHP_4$ in males is related to the upregulation of T at sexual maturity in males – in order for T secretion to increase, additional precursors (AE and $17OHP_4$) must also be produced. This hypothesis could be tested by dosing captive males with $17OHP_4$ and AE could shed light on this issue – if my hypothesis is true, after dosing with $17OHP_4$ one should see dose-dependent increases in AE and T, and after dosing with AE one should see increased T. If my hypothesis is incorrect, there will be no effect of treatment of T concentration. Alternatively, treating males with inhibitors of CYP17 could test this hypothesis. If my hypothesis is correct, a CYP17 inhibitor should significantly reduce T, AE, and $17OH_4$, while, if my hypothesis is incorrect, $17OHP_4$ would be reduced, but T would not be impacted. If dosing with steroids or steroidogenesis inhibitors is prohibited, one could potentially examine expression/activity of 17βHSD and CYP17 and/or 3βHSD in the gonads of stranded males, and, if my hypothesis is correct, expression/activity of these steroidogenic enzymes should be positively correlated with one another and age/length/weight in males.

Alternatively, the increase in AE and $17OHP_4$ observed in adult males compared to subadult males may be occurring independently of the increased demand for T. Perhaps AE and

17OHP$_4$ are acting directly on target tissues themselves. This hypothesis could be addressed by using transactivation assays to characterize the potency with which AE and 17OHP$_4$ transactivate the dolphin androgen receptor (AR) and progestogen receptor (PR). Then, if they are capable of transactivating the receptors, one could characterize tissue distribution of AR and PR expression from stranded tissues to determine which tissues they are likely to impact. Furthermore, one could also characterize potential changes in receptor expression with age in order to study changes in sensitivity related to maturity.

Similar studies could be used to study the relationship between P$_4$, 17OHP$_4$, and AE in pregnant animals. If AE and/or 17OHP$_4$ plays a role in stimulating P$_4$ secretion, then dosing pregnant females (or ovary explants from pregnant females) with AE or 17OHP$_4$ should lead to elevated P$_4$ secretion and expression/activity of the steroidogenic enzymes involved in P$_4$ biosynthesis. If this does not occur then likely they are elevated as a byproduct of increased substrate (P$_4$) availability, or, as discussed in males, to directly impact target tissues expressing the AR and PR. Once again this could be tested by examining potency in transactivation assays and characterizing receptor expression by tissue and pregnancy status.

I hypothesize that systemic S, F, and E are correlated for the same reason that T, AE, and 17OHP$_4$ are regulated. In order to secrete more F, more substrate (S) must be produced, and then, the increased availability of F leads to an increase in E. If this is true, dosing adrenal explants with ACTH should lead to an increase in the expression/activity of CYP21, CYP11B1, and 11βHSD. Furthermore, coadministering ACTH with 17OHP$_4$ (substrate for S production) to adrenal explants should lead to increased S, F, and E secretion. If S secretion is unrelated to the stress response, then administering ACTH would not impact expression/activity of CYP21 and coadministration of 17OHP$_4$ would not lead to increased S production; thus there would be no

impact on F or E production either. Alternatively, as before, it is possible that systemic S and E are directly mediating an effect on target tissues, and examining their abilities to transactivate the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) would address that hypothesis.

Ex vivo studies utilizing explants from stranded animals and transactivation assays could also help address the questions remaining from Chapters 4. For example, treating gonadal or adrenal tissues with DDTs and steroid precursors could shed light on whether the negative correlations observed between DDTs and T (males) and F (females) are related to impaired gonadal/adrenal steroidogenesis. If DDTs impact steroidogenesis in the expected manner, DDx-treated explants should exhibit lower T and F secretion compared to control-treated explants. To examine impacts on steroid metabolism, one could treat liver explants with DDTs and would expect to observe elevated clearance of T and F. One could potentially gain insight into DDx-mediated effects on hormone metabolism *in vivo* using urine samples. If DDTs increase T and F metabolism, one should observe a positive correlation between DDx burden and urinary polar metabolites of T and F. Finally, one could test the ability of DDTs to disrupt hormone signaling at target tissues using transactivation assays – DDx-mediated induction of bottlenose dolphin steroid hormone receptors in the absence of hormone and/or the inhibition of transactivation in the presence of hormone would suggest DDx can directly impact signaling in dolphins as in other species.

Finally, the question discussed in chapter 5 regarding the ability of blubber to metabolize hormones is probably better addressed *ex vivo* than with the design I utilized. My experiment was intended to provide preliminary evidence for the metabolism of corticosteroids in blubber; it is not intended as a conclusive test. An *ex vivo* design utilizing more animals would

provide far more conclusive evidence that living blubber tissue is capable of metabolizing F and E. Future studies should also consider whether blubber metabolizes other hormones, particularly AE.

3. Conclusions

The overall goal of this dissertation project was to examine the potential impacts of DDx exposure on steroid hormone homeostasis in bottlenose dolphins, which I accomplished despite a multitude of legal, logistical, and ethical impediments. In the process of pursuing this goal, I reached a number of other useful conclusions such that the impact of this work extends beyond the field of cetacean ecotoxicology. These additional impacts include the development of novel analytical methods, better understanding of basic cetacean endocrinology, and the most thorough assessment of blubber as a matrix for endocrine assessment that has been performed to date, all of which should facilitate better, more thorough examinations of cetacean endocrinology in the future. In conclusion, this dissertation advances the fields of cetacean endocrinology and toxicology, and, thereby, promotes better cetacean conservation.

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