PHYSIOLOGICAL ASSESSMENT OF COMMON BOTTLENOSE DOLPHINS (*TURSIOPS TRUNCATUS*) ACROSS A SALINITY GRADIENT

A Thesis

by

MAKAYLA GUINN

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This thesis meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

Dara Orbach, PhD
Chair

Frauke Seemann, PhD
Committee Member

Jennifer Pollack, PhD
Committee Member

December 2023

ABSTRACT

Common bottlenose dolphins (*Tursiops truncatus*) are important bioindicators of ecosystem welfare and can inhabit environments with variable natural salinities. Anthropogenically-induced climate change exacerbates natural fluctuations in salinity and magnifies physiological imbalances in marine species. Bottlenose dolphins are well-suited model organisms to study the effects of environmental disturbances because they accumulate indices of stress in their blubber. Prolonged low salinity (< 10 ppt) exposure in dolphins elevates adrenal steroid hormones (i.e., aldosterone, cortisol) and promotes lesion development. However, the tolerances of and consequences for dolphins in hypersaline systems remain unknown. I assessed the physiological condition of three dolphin stocks in the Gulf of Mexico inhabiting areas of different natural salinities: Mississippi Sound, MS (0 – 30 ppt), Redfish Bay, TX (22 – 35 ppt), and Upper Laguna Madre, TX (37 + ppt). Steroid hormones were measured in remotely biopsied dolphin blubber using high-resolution liquid chromatography-mass spectrometry. Skin lesions were assessed using images of the dorsal fins and bodies of dolphins photographed from a research boat. There is a positive relationship between cortisol and salinity, indicating high salinity may impose physiological stress in dolphins. Testosterone concentrations in males are seasonal, with peaks in the fall and winter months. Progesterone levels in females were highest in the spring and summer and were indicative of gestation events. Skin lesions are most prominent on dolphins in the fall and winter, and a negative correlation between lesion prevalence and water temperature suggests cold water has a strong effect on epidermal integrity and lesion susceptibility in dolphins. I present the first physiological assessment of free-ranging dolphins in a natural hypersaline bay. The dolphin health data collected from this research can help fill

national data gaps for GoM dolphin stocks outlined by the National Oceanic and Atmospheric Administration, fill local data gaps for RB and ULM dolphin stocks, inform coastal communities of local marine ecosystem health and potential impact on human health by utilizing dolphins as bioindicators, and contribute to the understanding of how global climate change impacts the ability of marine organisms to adapt to highly variable environmental conditions. Additionally, this research will contribute to the planning of environmentally sustainable infrastructure (e.g., desalination plants) and promote environmental stewardship, ecotourism, and appreciation for natural resources.

DEDICATION

To Dani, for being a friend, mentor, and inspiration in my career.

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1. INTRODUCTION

Free-ranging common bottlenose dolphins (*Tursiops truncatus*; hereafter 'dolphins') generally spend their entire lives in marine waters with an average salinity of 35 parts per thousand (ppt; Takeshita et al., 2021). Coastal stocks typically inhabit transition zones between fresh (< 1 ppt) and marine (35 ppt) environments and reside in bay, sound, and estuarine systems (BSE; Ewing et al., 2017; Hornsby et al., 2017; USGS, 2018). Dolphins maintain a physiological tolerance for polyhaline salinity zones (18 – 30 ppt) but actively avoid extreme mesohaline conditions (5 – 8 ppt; McClain et al., 2020; Booth & Thomas, 2021). There are only six coastal hypersaline (≥ 36 ppt) lagoons in the world that receive freshwater inflow and maintain tidal interaction with the ocean (Javor, 1989; Tunnell & Judd, 2002; Vega-Cendejas & Hernández de Santillana, 2004). Dolphins inhabit five of these six hypersaline systems; however, no physiological studies have been performed and the effects of hypersalinity on dolphins are poorly understood. Elucidating the consequences of variable salinity exposure on dolphins is a current management priority of the National Oceanic and Atmospheric Administration (NOAA) (Phillips & Rosel, 2014); this is particularly relevant for the Texas region of the northern Gulf of Mexico (GoM), where all seven BSE stocks of bottlenose dolphins are identified as imperiled (Phillips & Rosel, 2014) and one stock inhabits a coastal hypersaline lagoon. By assessing the physiological response of dolphins exposed to a large salinity gradient (0-36+ ppt), it is possible to begin filling data gaps of importance to NOAA and proactively monitor the health and sustainability of marine species in habitats where salinity is highly variable.

1.1. Salinity as a Driver of Physiological Stress

Salinity stress in an organism is the rapid or gradual change in salt concentration in the environment that prompts osmotic imbalance and reduces an animal’s fitness (Evans & Kültz,

2020). The temporal and spatial distribution of organisms in a coastal environment is strongly influenced by variations along the salinity gradient between BSEs and the offshore system (Vega-Cendejas & Hernández de Santillana, 2004; Pollack et al., 2009). Natural fluctuations in salinity are often exacerbated by anthropogenically induced stressors that alter global precipitation and the hydrological cycle (Trenberth, 2011; Olson, 2018). In the southwestern United States (US), increasingly hot and dry climates raise salinity levels, reduce precipitation and streamflow, and increase evaporation (Seager et al., 2007; Miller et al., 2021). Other topics of growing concern are reduced freshwater inflow from estuary dewatering and upstream diversions (Asquith, 1997), and industrial brine discharge from oil, gas, and desalination practices (Montagna et al., 2021). In contrast, frequent tropical storms and hurricanes driven by climate change rapidly introduce substantial freshwater to coastal environments that can persist for months and disrupt the ecology of local ecosystems (Greening et al., 2006). For example, the inundation of rain from Hurricane Harvey reduced salinity levels in Galveston Bay, Texas, to 0 ppt in 3.6 days and did not return to pre-storm levels of 14 ppt for two months (Fazioli & Mintzer, 2020).

Due to the increasing intensity and frequency of tropical weather (Karl et al., 2009), dolphin behavior and movement responses have been well documented. Following two major hurricanes in the Bahamas, approximately 30% of the dolphin population left the study area and did not return for almost two years (Elliser & Herzing, 2011). Heavy flooding from hurricanes may displace and strand dolphins in inland areas outside of their usual habitats (Rosel & Watts, 2008; Schumann et al., 2013; Mullin et al., 2015). Unusual mortality events (UME; i.e., unexpected and large die-offs of a marine mammal population) are often linked to intense tropical systems (McClain et al., 2020). However, decreased anthropogenic activity after

Hurricane Katrina may have increased calf encounter rates and foraging activity in Mississippi Sound (MS) (Miller et al., 2010).

The GoM experiences an annual storm season from June through November (National Hurricane Center, 2023) and is the main drainage for approximately two-thirds of the US mainland (Davis et al., 2002). In addition to terrestrial water, sediments, nutrients, and pollutants drain into the GoM and can release and concentrate salt in a variety of forms (e.g., calcium, sulfate, chloride; Nijssen et al., 2001; Curtis, 2008; Olson, 2018). Thus, the salinity gradient of the coastal GoM is complex and may pose challenges to near-shore marine fauna.

1.2. Maintaining Osmotic Balance in the Marine Environment

Dolphins are capable of occupying habitats across a broad salinity gradient due to adapted homeostatic and osmoregulatory mechanisms necessary for conserving water and avoiding dehydration. The specialized reniculate (multilobed) kidneys of dolphins are structured to concentrate urine and mediate water-electrolyte imbalances under variable salinity conditions (Ortiz, 2001). Mediation of acute differences between the internal and ambient environment of dolphins involves the renin-angiotensin-aldosterone system (RAAS; Atkinson et al., 2015; Rash & Lillywhite, 2019). Renin secretion from the kidney during periods of acute sodium or water imbalance stimulates the downstream release of aldosterone through a series of enzymatic reactions (Ortiz, 2001). Aldosterone is an adrenal mineralocorticoid hormone responsible for solute and fluid homeostasis in mammals (Cole et al., 2019); in dolphins, aldosterone serves an important role in regulating sodium levels in response to incidental seawater ingestion, water retention during extended natural fasts, and increased skin permeability during exposure to low saline conditions (Ortiz et al., 2001; Phillips & Rosel, 2014; Atkinson et al., 2015).

Aldosterone production can also be regulated by activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to severe or chronic stressors (Houser et al., 2011). During periods of perceived or physiological stress, the adrenocorticotropic hormone (ACTH) stimulates the anterior pituitary gland to release cortisol, the main stress hormone in mammals, and aldosterone (Houser et al., 2011; Peterson et al., 2023). Circulating aldosterone levels are low in cetaceans (whales, dolphins, and porpoises), however, increased secretion of both aldosterone and cortisol has been reported in dolphins during exposure to low salinity conditions (Ewing et al., 2017; McClain et al., 2020), cold water (Houser et al., 2011), and handling by humans (Thomson & Geraci, 1986; St. Aubin et al., 1996). Elevated aldosterone has also been reported during routine physical assessment in captive beluga whales (*Delphinapterus leucas*; Schmitt et al., 2010), intense underwater sound exposure in bottlenose dolphins (Romano et al., 2004), and lactation in female gray whales (*Eschrichtius robustus*; C. Wittmaack, personal communication).

The release of aldosterone and cortisol into circulation is highly context-dependent and occurs only when required (Cole et al., 2019); upon cessation of the stressor, circulating aldosterone and cortisol levels in the bottlenose dolphin resume baseline concentrations within 24 hours (Champagne et al., 2018). When not in circulation, > 90% of cortisol and < 20% of aldosterone remain bound by cortisol-binding globulins (CBGs) and are biologically inactive (Perogamvros et al., 2012; Champagne et al., 2018). In human plasma, the majority of aldosterone appears to be bound to albumin (Katayama & Yamaji, 1982; Jensen et al., 2015), although no specific binding proteins have been identified for aldosterone in marine mammals (Champagne et al., 2018). Aldosterone thus serves a primary role in cetacean stress response and mediating physiological imbalances that are secondary to acute or chronic stressors (Thomson & Geraci, 1986; St. Aubin & Geraci, 1989).

1.3. Proxies for Dolphin Health

1.3.1 Steroid Hormones

Steroid hormones like aldosterone and cortisol are lipophilic and regularly sequester from the blood to the lipid-rich blubber of dolphins through diffusion (Deslypere et al., 1985). As apex predators that integrate broadly across the marine ecosystem, dolphins are frequently exposed to a wide range of environmental and anthropogenic stressors, which they accumulate evidence of in their tissues (Wells et al., 2004). Marine mammal blubber is highly stratified and concentrates steroid hormones within the proximate layer while the distal layer may be representative of full-depth values indicative of overall condition (Trana et al., 2015; Kershaw et al., 2017). While steroid concentrations in blubber reflect circulating levels at the scale of several hours to days, blood concentrations reflect rapid changes within 45 minutes and may potentially include stress induced by sample collection (Champagne et al., 2018). Since blubber exhibits a delayed stress response compared to circulating matrices, monitoring the direct effects of any given stressor using blubber can be challenging (Teerlink et al., 2018). However, because several adrenal steroids are released during periods of physiological stress, identifying correlations between adrenal hormones may help delineate specific stressors (Ortiz & Worthy, 2000). For example, circulating cortisol and aldosterone were used to assess salinity stress in bottlenose dolphins exposed to freshwater conditions (McClain et al., 2020). A significant correlation was identified between serum corticosterone and cortisol as well as corticosterone and aldosterone in free-ranging Atlantic bottlenose dolphins that were temporarily restrained in nets (Ortiz & Worthy, 2000). Similarly, a correlation between cortisol and cortisone as well as cortisol and corticosterone was found in free-ranging bottlenose dolphin skin after temporary capture and

release (Galligan et al., 2020). Evaluation of several steroid hormones is important for interpreting overall physiological condition.

Steroid hormones can be divided into four classes: estrogens (female reproductive hormones, e.g., estradiol), androgens (male reproductive hormones, e.g., testosterone), progestogens (pregnancy hormones, e.g., progesterone), and corticosteroids (stress hormones, e.g., aldosterone, corticosterone, cortisol, cortisone). Biological tissues commonly used for steroid hormone analyses in dolphins include blubber (Kellar et al., 2015; Champagne et al., 2016, 2018; Boggs et al., 2017, 2019; Galligan et al., 2020; Sherman et al., 2021), blood/serum/plasma (Houser et al., 2011; Fair et al., 2014; Boggs et al., 2016; Sherman et al., 2021), and skin (Champagne et al., 2018; Bechshoft et al., 2020). Remote biopsy sampling is a less invasive method to collect biological tissue from free-ranging dolphins than direct handling. Skin and blubber samples collected from free-ranging cetaceans can be used for subsequent hormone (Kellar et al., 2006; Sinclair et al., 2015; Boggs et al., 2019; Graham et al., 2021), contaminant (Hansen et al., 2004; Balmer et al., 2015; Méndez-Fernandez et al., 2016; Galligan et al., 2019), genetic (Rosel, 2003; Dolah et al., 2015; Neely et al., 2018; Vollmer et al., 2021), stable isotope (Browning et al., 2014; McCormack et al., 2020), metabolomic (Misra et al., 2019), and proteomic analyses (Kershaw et al., 2018). Remote biopsy sampling of dolphins elicits a minimal behavioral response and there is strong evidence for rapid epidermal regeneration and wound healing without physiological complications (Weller et al., 1997; Krützen et al., 2002; Gorgone et al., 2008; Tezanos-Pinto & Baker, 2012; Fruet et al., 2016). The use of remote biopsy in endocrine research has enabled the proactive monitoring of steroid hormones and effective health assessments of free-ranging dolphins, advancing insights about

the regulation of critical physiological processes such as development, reproduction, and metabolism (Boggs et al., 2016).

Traditional methods to measure steroid hormones include radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs), which bind hormones and amplify their signals through a radioactive signal or enzymatic change, respectively (Boggs et al., 2016). While extremely sensitive and capable of high throughput, RIAs and ELISAs are often less reliable than direct quantification methods due to antibody selectivity, cross-reactivity, poor analyte detection, and difficult reproducibility (Cross & Hornshaw, 2016; Boggs et al., 2019). Immunoassays often require high sample volumes and are explicitly designed to measure a single hormone per assay, limiting the ability and cost-effectiveness of measuring several hormones simultaneously. The lack of standardization of high-quality steroid hormone assays hinders the ability to draw definitive and consistent quantitative conclusions (Stanczyk et al., 2007; Boggs et al., 2016). However, advancements in liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) provide higher resolution, sensitivity, and specificity than RIAs and ELISAs (Stanczyk et al., 2007; Boggs et al., 2016, 2019). LC-MS/MS entails ionization of the analyte (e.g., hormone), followed by separation and detection in the mass analyzer depicted as a mass spectrum, where ion abundance is a function of the mass-to-charge ratio from which the analyte concentration is derived (Stanczyk et al., 2007). Because steroid hormones have similar structures, fragmentation patterns, and identical masses in some cases, steroid separation can be difficult (Boggs et al., 2016). The capacity for LC-MS/MS to separate and fragment multiple steroids necessary for accurate identification and quantification makes it the preferred method in endocrine studies (Boggs et al., 2016, 2019).

1.3.2 Skin Disorders

While steroid hormones provide useful information regarding the metabolic response of an individual, external indicators of physiological health can provide insights into local environmental conditions. Skin disorders in dolphins are largely associated with frequent tropical storms (Rosel & Watts, 2008), which may lead to prolonged out-of-habitat displacements (Ewing et al., 2017) and UMEs (Mullin et al., 2015). Coastal dolphins are particularly susceptible to epidermal disease and large proportions of coastal populations are often impacted because they inhabit regions where near-shore pollution is abundant (Wilson et al., 1999; Van Bressem et al., 2003; Bearzi et al., 2009; Toms et al., 2020). Low salinity increases the permeability of dolphin skin and fluid accumulation in the superficial epithelial layer, resulting in lesions (synonymously referred to as 'skin disorders' hereafter) that vary in color, shape, and prevalence (Phillips & Rosel, 2014; Ewing et al., 2017). Exposure to water < 10 ppt for several days or weeks generally induces skin pallor and varying degrees of proliferative, erosive, or ulcerative lesions (Mullin et al., 2015; Takeshita et al., 2021). Despite the rapid and continual sloughing of skin, which enhances barrier properties and limits microbial attachment (Hicks et al.,1985; Geraci et al., 1979), overgrown mats of fungi, algae, and/or bacteria may persist and penetrate the skin as epidermal integrity decreases, thereby increasing the extent and severity of lesions (Takeshita et al., 2021). Once compromised, the dolphin risks secondary infection and an influx of freshwater into the body, which alters internal salt-water homeostasis (Duignan et al., 2020; Takeshita et al., 2021). Electrolyte imbalances including hyponatremia (low blood sodium), hypochloremia (low blood chloride), and low osmolarity may ensue within a day of exposure to waters < 20 ppt (Deming et al., 2020; Takeshita et al., 2021). Dolphins exposed to hypersaline (> 36 ppt) conditions, however, show dramatically higher rates of wound healing compared to populations in polyhaline (18 – 30 ppt) environments (Hurst & Orbach, 2022).

Regulation of the immune response to inflammatory agents such as skin disorders is dependent on the intensity of the stressor (Liu et al., 2017). In humans, chronic stressors can over-activate the immune system and suppress immune function, leading to pro-inflammation (Miller et al., 2009; Morey et al., 2015; Liu et al., 2017). Immunosuppression has also been linked to chronic stress, contaminant exposure, and illness in horses (*Equus caballus*; Mills et al. 1997), mice (*Mus musculus*; Stark et al., 2001), striped dolphins (*Stenella coeruleoalba*; Aguilar & Borrell, 1994), harbor seals (*Phoca vitulina*; Hutchinson & Simmonds, 1994; de Swart et al., 1996; Ross et al., 1996), and northern elephant seals (*Mirounga angustirostris*; Deyarmin et al., 2019). Two distinct UME reports of bottlenose dolphins noted skin lesions associated with morbillivirus, in addition to immunosuppression from secondary infection and chronic stress (Geraci, 1989; Kuehl & Haebler, 1995). Persistent skin disorders in dolphins from compromised immune function and chronically circulating levels of stress may have fatal physiological consequences.

The extent and severity of skin disorders in dolphins are often estimated from visual assessment using photo identification of dorsal fins (Bearzi et al., 2009; Toms et al., 2020). The dorsal fin is the most visible part of the dolphin during surfacing and has unique individually identifiable features that enable comparisons of animals across time, space, and populations. Using dorsal fins as a whole-body proxy, however, underestimates the extent and severity of lesions, degree of stress in the animal, and other underlying health concerns (Hart et al., 2012); visual assessment thus has limitations in determining the cause(s) of lesions. Specific visual characteristics have been linked to etiologies of some lesion types and increased confidence in the identification and estimation of varying skin conditions in dolphins, although the categorization process remains subjective (Toms et al., 2020). Most skin disorders have

unknown etiologies and cannot be definitively assigned to a category (Toms et al., 2020). Measurements of lesion prevalence (i.e., presence or absence) are recommended in lieu of lesion classification and do not depend on lesion etiology (Toms et al., 2020).

1.4. Physiological Condition Across a Salinity Gradient

Large deviations in salinity from average seawater conditions directly influence the regulation of fluid and electrolyte homeostasis in marine organisms (McClain et al., 2020). Physiological imbalance, especially resulting from extreme salinity fluctuation(s), is an innate stressor that leaves biological traces of increased endocrine activity and epidermal modification. Knowledge of the physiological responses of dolphins to variable salinity conditions is limited to low-saline exposure, highlighting the need for research on small cetaceans that include high salinity. The Texas Coastal Bend (TCB) region of South Texas along the GoM includes both the Upper Laguna Madre (ULM), a naturally occurring hypersaline (≥ 36 ppt) bay, and Redfish Bay (RB), a separate system of geographically distinct dolphins exposed to polyhaline (22 – 30 ppt) salinity conditions (Phillips & Rosel, 2014). While the average home range for coastal dolphins varies by population, it is estimated to be 50 – 80 km^2 for bottlenose dolphins in the GoM (Shane et al., 1986; Mazzoil et al. 2017). Routinely photographed dolphins found in ULM and RB maintain observed ranges ~100 km apart (connected by a single channel) and only nine unique individuals (approximately 0.5% of the total combined population) have been documented to travel between systems (unpublished data), suggesting strong site fidelity consistent with distinct populations. While western Mississippi Sound (MS) is generally a polyhaline system (23 ppt average; Miller et al. 2013), frequent extreme fluctuations in salinity expose resident dolphins to variable conditions (0 – 30 ppt), providing an excellent contrast to the ULM and RB stocks.

By combining high-resolution LC-MS/MS steroid hormone analysis with epidermal assessments of three distinct dolphin stocks, this research is the first to measure the physiological health of dolphins across a broad salinity gradient that includes exposure to a natural hypersaline system. This study contributes information to fill data gaps outlined by NOAA, enables comparative quantification of salinity stress in dolphins across a salinity gradient, and enhances the current knowledge of cetacean life history strategies associated with extreme environmental perturbations often faced by dolphins in the GoM.

2. RESEARCH OBJECTIVES

No physiological study of bottlenose dolphins has used a salinity gradient that includes hypersaline conditions. The objectives of this research were to: (1) comparatively assess steroid hormone levels and epidermal health of dolphins in MS (0-30 ppt), RB (22-30 ppt), and ULM (≥ 36 ppt); (2) compare current (2022) steroid hormone levels and epidermal health of RB dolphins to archived data from RB (2012-2014); (3) use steroid hormone levels and epidermal health data from MS, RB, and ULM dolphin stocks to expand our understanding of dolphin physiology across a large salinity gradient (0-36+ ppt).

2.1. Hypotheses

I hypothesized that there will be differences in:

H1) steroid hormone concentrations between dolphins inhabiting MS, RB, and ULM

H2) the proportion of skin disorders between dolphins inhabiting MS, RB, and ULM

H3) the physiological condition of RB dolphins sampled in 2012-2014 or 2022

2.2. Predictions

I predicted that:

P1) aldosterone and cortisol will be positively correlated with high (ULM) and low (MS) salinity levels due to osmotic imbalance (McClain et al., 2020)

P2) there will be a negative association between the proportion of skin disorders and salinity levels since high salinity promotes wound healing (Hurst & Orbach, 2022)

P3) the concentrations of corticosterone, cortisone, and/or cortisol measured in 2022 and the proportion of dolphins with skin disorders for RB dolphins will be higher than previous measures of the same stock due to increased localized environmental degradation and associated elevated stress events (Phillips & Rosel, 2014).

3. METHODS

3.1. Field Sites

3.1.1 Western Mississippi Sound

Mississippi Sound (MS), Mississippi, is approximately 4792 km^2 with an average depth of 4 m and an average salinity of 23 ppt (USFW, 1982; Miller et al., 2013). MS is located along the northeastern GoM and extends west from Dauphin Island in Alabama to the coastline bordering Louisiana (Miller et al., 2013; Figure 1C). Five islands (Cat, Ship, Horn, Petit Bois, and Dauphin Island) separate MS from the GoM, with dolphin distribution along these islands varying by season (Pitchford et al., 2016); dolphins inhabiting MS tend to utilize the coastal region during the summer and the offshore region during the winter, likely due to seasonal variation in prey availability (Miller et al., 2013). MS is a relatively shallow basin that experiences large fluctuations in both sea surface temperature (9 – 33 °C) and salinity (0 – 30 ppt), which are compounded by intense freshwater input from river discharges (Mississippi, Pearl, Pascagoula, and Mobile River) and frequent tropical weather (Vollmer et al., 2021). Despite relatively persistent mesohaline (5 – 18 ppt) conditions, estuarine habitats ≥11 ppt along the northern GoM and southeastern Atlantic are suitable for bottlenose dolphins (Ewing et al., 2017; Hornsby et al., 2017; Fazioli & Mintzer, 2020). Bottlenose dolphins are the only cetacean species routinely present in MS and this population is one of the most consistently monitored populations in the northern GoM (Mullin et al., 2017). MS dolphins are divided into a relatively stable inshore MS stock and an adjacent Northern coastal stock; these two combined stocks are comprised of resident (26.5%) and transient (73.5%) dolphins (Mattson et al., 2006; Pitchford et al., 2016).

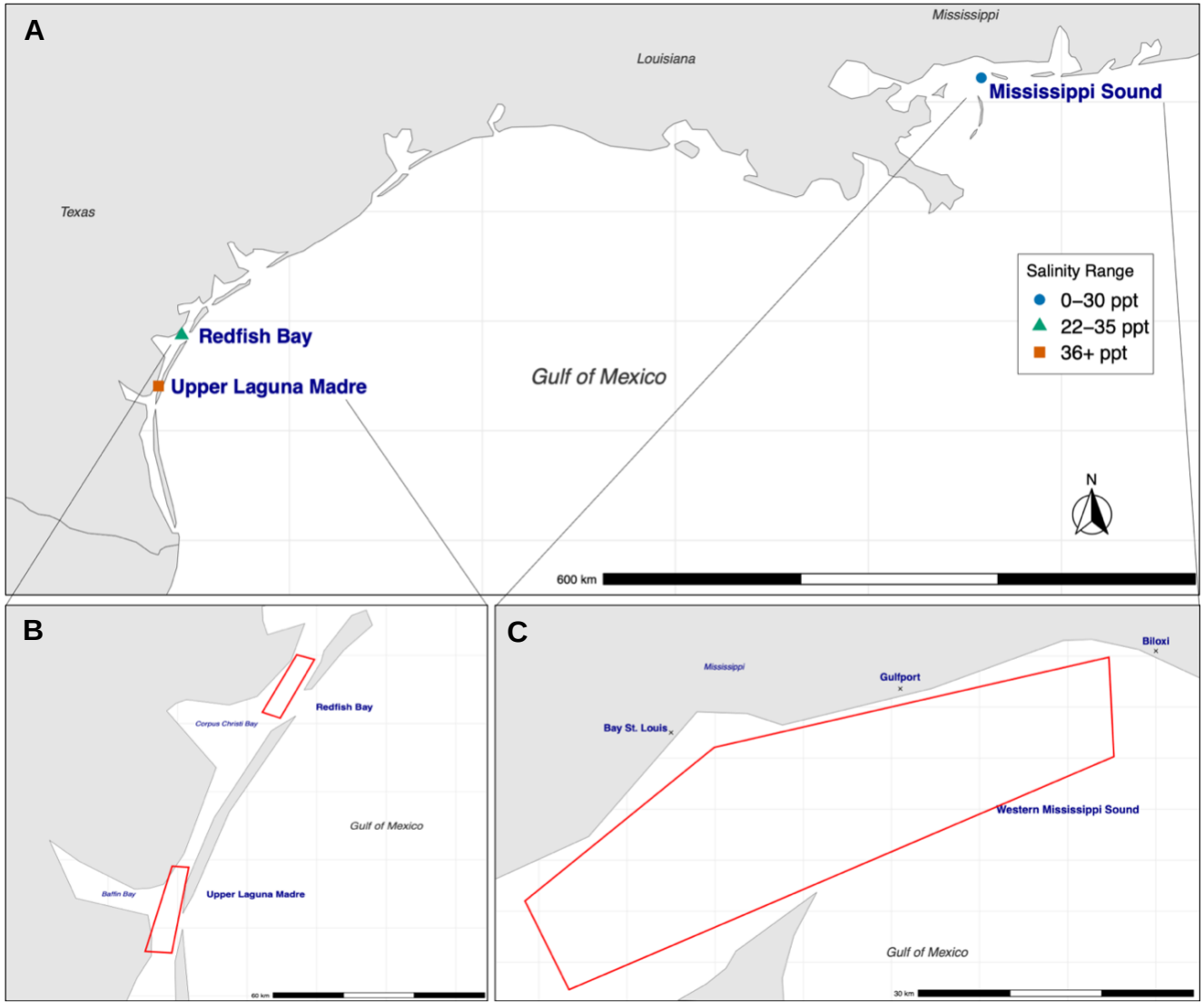


Figure 1. Field sites within the Gulf of Mexico. A) Mississippi Sound (blue), Redfish Bay (green), and Upper Laguna Madre (orange). Colors indicate the average salinity levels of each site. B) Survey boundaries (red) where samples were collected in RB, ULM, and C) MS. The map was produced in R statistical software.

3.1.2 Redfish Bay

Port Aransas (PA), Texas, is one of five major ports along the Texas coastline that connects BSE waters with the GoM. Several waterways merge in PA to form Redfish Bay (RB) (Figure 1A), including Aransas Channel, Lydia Ann Channel, Intracoastal Waterway, and Corpus Christi Ship Channel. The boundaries of RB start at the convergence point of these channels, extend northeast via the Lydia Ann Channel towards Aransas Bay, and extend southwest via the Corpus Christi Ship Channel towards Corpus Christi Bay. RB is enclosed by the Intracoastal Waterway that connects the two bays on either side. Within these boundaries, RB is approximately 56 km^2 and on average 4.3 m deep, while dredged channels extend to 12 m deep (Phillips & Rosel, 2014; NOAA Office of Coast Survey, 2022). Seasonal changes in water temperature (15 – 30 °C) and salinity (22 – 30 ppt; average 27.99 ppt) make the coastal waters of RB very dynamic (Phillips & Rosel, 2014; Montagna et al., 2021). The environmental conditions of RB are overall poor based on water and sediment quality, benthic indices, and contaminants (Phillips & Rosel, 2014). Dolphin abundance in RB varies by season and is characterized by fluctuating water temperatures and prey availability, with historically low site fidelity in the summer (Shane, 1980; Leatherwood & Reeves, 1983; Weller, 1998; Lynn & Würsig, 2002). Dolphin abundance remains high despite large oil tankers and personal boats heavily utilizing the area. Dredging, recreational and commercial fishing, chemical pollution, and oil and gas pollution are all high-level threats to dolphins in RB, making the population a high priority for proactive monitoring (Phillips & Rosel, 2014).

3.1.3 Upper Laguna Madre

The Laguna Madre (LM), Texas, is a 185 km long, 3 – 6 km wide, and 1.1 – 4 m deep hypersaline lagoon that is separated from the GoM by Padre Island, an extensive barrier island

(Phillips & Rosel, 2014). LM is divided into an upper (76 km) and lower (91 km) region (Tunnell & Judd, 2002) and extends south from Corpus Christi Bay to South Bay on the U.S.-Mexico border (Figure 1B). LM is one of six naturally occurring coastal hypersaline lagoons in the world (Javor, 1989; Tunnell & Judd, 2002; Vega-Cendejas & Hernández de Santillana, 2004; Phillips & Rosel, 2014) with an average salinity of 36 ppt. However, exceptionally high salinities regularly exceed 45 ppt and in some cases even triple that of the GoM due to low circulation and little freshwater inflow (Tunnell & Judd, 2002; Onuf, 2007; Olsen, 2014; Phillips & Rosel, 2014). LM contains vast amounts of undeveloped shoreline and is a nursery for many fish and invertebrate species (Phillips & Rosel, 2014). The species richness of LM was one-third of BSE systems to the north (CCBNEP, 1996). Bottlenose dolphins were not reported to be present in LM prior to 1979 (Leatherwood & Reeves, 1983; Mullin et al. 1990; CCBNEP, 1996). Of the 528 km^2 of habitat surveyed in the LM proper (Upper LM and Lower LM), ~37.6% was identified as 'acceptable' dolphin habitat (water depth > 0.2 m at mean tide; Leatherwood & Reeves, 1983). Recent ecological surveys of the LM revealed increasing fish diversity, likely due to habitat expansion of tropical species, suggesting a link between current dolphin presence and prey availability (Fujiwara et al., 2019).

3.2. Data Collection

3.2.1 Equipment Preparation

Biopsy instruments were sanitized following the protocol outlined by Sinclair et al. (2015). Tools were scrubbed in warm antibacterial soap, rinsed, and soaked in a 10% bleach solution for 10 minutes. Tools were then rinsed with warm water, deionized water, and 200 proof ethanol. Processing instruments and sampling tips were air-dried and wrapped in sterilized aluminum foil.

3.2.2 Remote Biopsy and Photography

Remote biopsy surveys in Texas waters were conducted in May 2022 and November 2022. Blubber samples and photographs were opportunistically collected from dolphins in RB and ULM under NOAA permit number 21938 and Texas A&M University-Corpus Christi IACUC permit number 2021-10-031. RB and ULM were traversed by a research boat (6 m Inmar 550R, 90 HP outboard Suzuki engine). Dolphin groups (individuals within 10 m of one another engaged in the same predominant behavioral state; Smolker et al., 1992) were assessed prior to each approach to determine their suitability for sampling and ensure safe boating conditions. If a neonate or calf < 1 year was present, biopsy sampling was not attempted. Groups exhibiting evasive behaviors (e.g., deep dives, frequent change in direction) or largely consisting of previously sampled individuals were also avoided. Once a group was deemed approachable, initial GPS coordinates were recorded and the group was followed for no more than 30 minutes per permit rules to reduce stress induced by prolonged encounters. Photographs of the dorsal fin (for subsequent identification), the visible body of all dolphins in each group, and each attempted biopsy were collected using a Sony Cyber-Shot RX10 IV camera. The photographer was positioned perpendicular to the animals in optimal lighting when possible and attempts were made to photograph both the left and right side of each dolphin.

A modified crossbow device (Barnett Panzer V, 68 kg draw weight, Barnett Outdoors, LLC, Tarpon Springs, FL USA) was used to collect dolphin blubber. Darts fitted with either a 7x25 mm or 10x25 mm stainless-steel sampling tip (Ceta-Dart, Copenhagen, Denmark) were aimed 0.07 – 0.1 m below the dorsal fin, above the midline of the dolphin, and fired at a distance between 3 – 7 m. Externally beveled edges and internal angled prongs in the sampling tip held

the tissue. The foam affixed to the anterior end of the dart adjacent to the tip enabled the dart to immediately eject upon penetration and the sample to float above the water.

Upon biopsy collection, the tissue was immediately retrieved from the dart and processed on ice using a cutting board covered with a sterile Teflon sheet. The skin layer was removed from the blubber using a sterilized scalpel and forceps, and stored separately for genetic sexing and as a precaution if hormones could not be detected in the blubber. Tissue samples were kept in labeled vials on dry ice in a vapor shipper until returned to laboratory facilities where they were transferred to a -80 °C freezer. Data on water parameters (temperature, salinity, dissolved oxygen (all measured using a YSI Pro Solo), pH), environmental conditions (wind speed and air temperature (measured using an anemometer), depth, Beaufort Sea State), group composition (number of dolphins, age classes, predominant behavioral states), and biopsy sample metadata (photograph frames, time of sample collection, number of tissue subsamples, location of dart contact with dolphin, individual and group reaction to biopsy, individual and group post-biopsy behavior) were recorded as soon as the group follow was completed. Each group was monitored for strong behavioral responses to the biopsy dart deployment (e.g., breach, tail slap) and suitability for a second biopsy attempt. Data were recorded including when biopsy attempts were unsuccessful (miss or hit with no sample collected). A maximum of three attempts were made to biopsy any group. Archived blubber samples from dolphins in RB (2012 – 2014) and MS (2013) were provided by NOAA (samples were selected based on salinities) from previous biopsy sampling (NMFS permit numbers 779-1633 and 14450) that used similar techniques.

3.3. Hormone Analyses

3.3.1 Equipment Preparation

Glassware, metal, and plasticware were immersed in a 2% Alcanox (powdered detergent) solution for at least 6 hours. Tools were then rinsed three times with tap water followed by three rinses with deionized water. Glassware and plasticware were then immersed in a 5% hydrochloric acid (HCL) solution for at least 6 hours to further remove any trace contaminants. Metal tools were not cleansed in HCL to avoid rusting. All glassware, metal, and plasticware were then rinsed three times with deionized water and three times with Milli-Q water (< 5 ppb organic carbon), wrapped in aluminum foil, and dried in an oven (75 °C). Metal and glassware were then combusted in a muffle furnace for 6 hours at 450 °C to complete sterilization. Plasticware was not combusted to prevent melting and was considered completely sanitized upon drying in the oven.

3.3.2 Standard Solution Preparation

In the quantitative mass spectrometer approach, isotopically labeled standards are used as quality control checks to reduce instrument noise (the standard deviation of the background signal) and minimize variabilities from sample preparation and analyte extraction (Wieling, 2002). Isotopically labeled standards behave chemically very similarly to the endogenous analyte, which allows for improved selectivity and sensitivity by a mass detector (Chen et al., 2009). The non-labeled version of a standard is the native state of an analyte (e.g., hormone) as found in an organism and is necessary for the mass spectrometer to detect a match. All isotopically labeled (aldosterone-$^{13}C_3$, cortisol-9,11,12, 12-D_4, 17β-estradiol-2,3,4-$^{13}C_3$, progesterone-2,3,4-$^{13}C_3$, testosterone-2,3,4-$^{13}C_3$; Certilliant, Round Rock, TX) and non-labeled standards (aldosterone, corticosterone, cortisol, cortisone, 17β-estradiol, progesterone, testosterone; Certilliant, Round Rock, TX) (Appendix A) were prepared prior to sample analyses. Since labeled standards are not manufactured for cortisone and corticosterone,

aldosterone and cortisol, respectively, were used to infer their behavior in the instrument due to structural similarities. Cortisol, cortisone, and corticosterone were selected as proxies of overall stress in dolphins, while aldosterone was selected as a proxy of stress related to osmoregulation. Estradiol, progesterone, and testosterone were used to interpret potential confounding agents of stress, such as individual sex and reproductive status.

Because steroids are structurally similar (Boggs et al., 2016), two working stocks containing all non-labeled standards were prepared to ensure the separation of isomers (i.e., aldosterone and cortisone) and to create a distinct MS/MS mass fragmentation database for each individually targeted steroid (Figure 2). A stock standard solution (1,000 ppm concentration equivalent to 1 mg/mL) was prepared for each hormone. A 10 µL aliquot of each stock hormone solution was diluted to 10 mL with acetonitrile (ACN; LC/MS optima grade) to create a 1 ppm stock solution. Serial dilutions were performed using 1 ppm working stock solutions to create a standard curve of 0.001 ppb, 0.01 ppb, 0.025 ppb, 0.05 ppb, 0.1 ppb, 1 ppb, 5 ppb, 10 ppb, 50 ppb, 100 ppb, 250 ppb, and 500 ppb. The standard curve was used to identify the chromatographic retention time of each hormone, identify unique fragmentation patterns, and verify hormone separation and subsequent detection. Labeled standards were prepared in the same way to create a 20 ppb and 10 ppb solution for spiking the test samples.

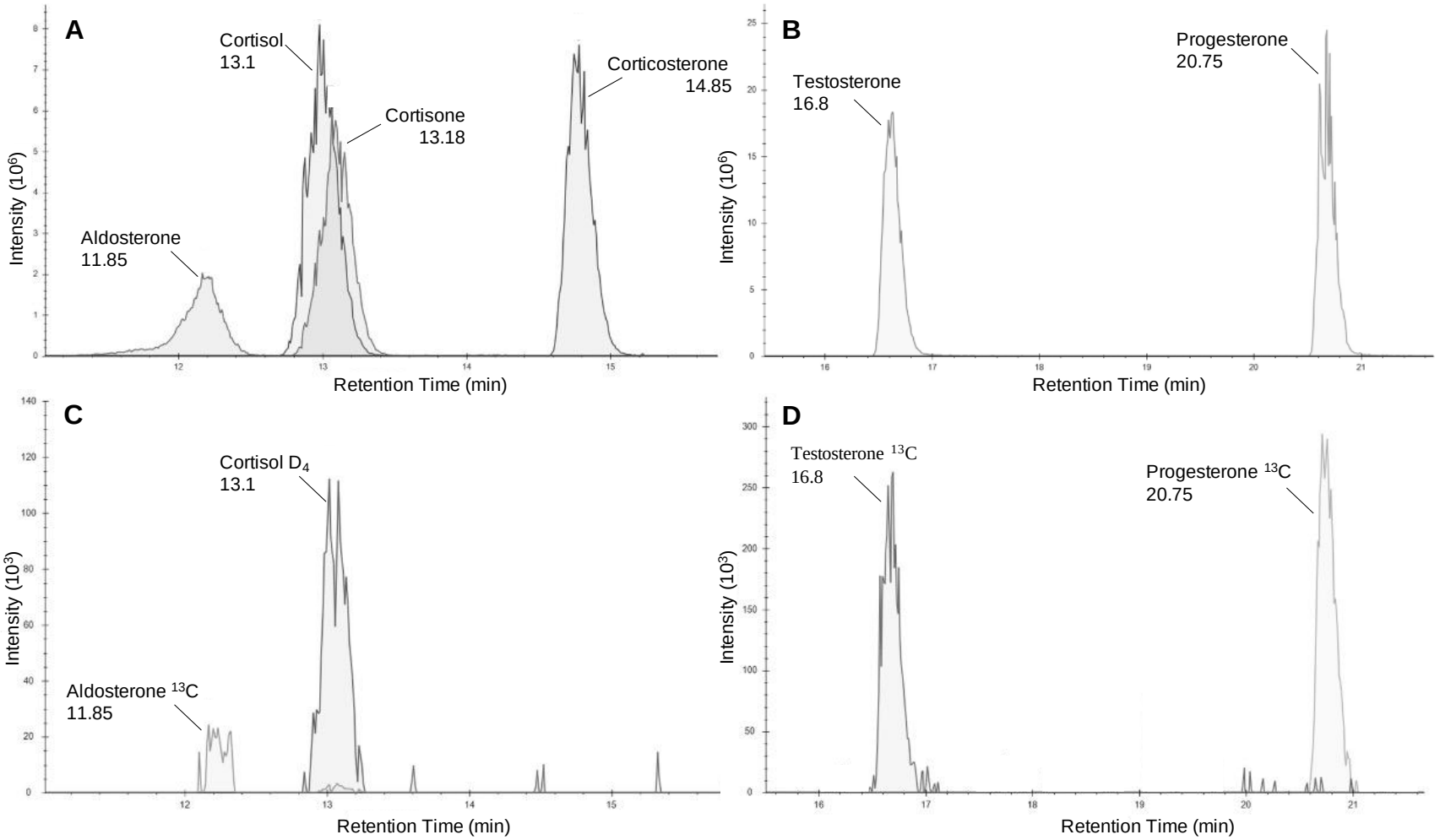


Figure 2. Chromatographic separation of hormone standards. Non-labeled (A-B) and labeled (C-D) internal standards with steroid names and retention times (number listed below hormone name) are included for each peak.

3.3.3 Recovery and Precision Assessment

A stranded code 2 (fresh dead) bottlenose dolphin was used to obtain large homogenous sections of blubber needed for replicate analyses. The average lipid concentration of code 2 stranded dolphins is 44.58%, which is comparable to live free-ranging dolphins (McFee unpublished, mentioned in Boggs et al., 2019). Thus, using blubber from a stranded dolphin for validation analyses likely evaluates the effects of the sample matrix and extraction efficiency.

Using combined techniques from and Boggs et al. (2017) and Wittmaack et al. (2021), two extraction solvents (ACN and Milli-Q water) and three sample masses (50 mg, 150 mg, and 400 mg) were tested to develop the protocol for this study. Two replicates of five were prepared for each sample mass using Milli-Q water, and two replicates of three for ACN. The first set of replicates for each sample mass was spiked with 20 ppb of labeled standard solution prior to homogenization (equal to 10 ppb in the final solution). The second set of replicates was spiked with 10 ppb in the final reconstitution step. All replicates were run alongside the standard calibration curve to obtain recovery (% analyte extracted; $\frac{expected\ concentration}{actual\ concentration}$ *100) and precision (consistency of extraction; % relative standard deviation, RSD) values, which were computed in Microsoft Excel.

3.3.4 Blubber Sample Preparation

Based on the recovery and precision of hormone extraction (section 3.3.3), biopsied blubber samples were homogenized and steroid hormones (i.e., aldosterone, corticosterone, cortisol, cortisone, estradiol, progesterone, and testosterone) were extracted using the following protocol. Frozen blubber samples were removed from the –80 °C freezer and 150 mg of tissue was excised using a feathered scalpel blade. The blubber was minced on a sterilized glass petri dish on dry ice and added to a homogenization tube pre-filled with 1.4 mm zirconium beads

(OPS Diagnostics; PFMB 1400-100-32). An aliquot of 1,200 µL ACN (LC/MS optima grade), a solvent that is partial to extracting organic matter, was added to the sample tube. Samples were homogenized (Bertin Precellys Evolution) three times for 30 seconds at 6,500 RPM with a 5-minute interval on ice in between homogenizations to maintain a stable temperature. Samples were then centrifuged (VWR 2405-37) at 4 °C for 5 minutes at 7,300 RPM. The remaining tissue was incubated in the extraction solvent overnight at –20 °C.

After at least 12 hours, a pipette was used to transfer the supernatant into a labeled glass test tube. An aliquot of 800 µL ACN was added to the original homogenization tube that was vortexed for 30 seconds and centrifuged for 5 minutes at 4 °C and 7,300 RPM. The supernatant was extracted and added to the glass test tube, and the original homogenization tube was rinsed twice more for three full extractions. The supernatants collected in the glass test tube were vortexed for 30 seconds. An aliquot of 2.75 mL hexane was then added to remove the fat (but not the hormones) from the ACN layer. After vortexing the solution for 30 seconds to separate layers and waiting at least 10 minutes, the hexane layer (top layer) was removed and the hexane rinse was repeated until all of the fat was removed. The samples were then stored at –20 °C overnight.

The samples in the glass test tubes were dried at 30 °C (Labconco CentriVap) and reconstituted in 500 µL of methanol to remove traces of the lysing matrix (debris from homogenization beads). After vortexing for 30 seconds, the samples were sonicated for 5 minutes at 20 °C, transferred to a centrifuge tube with a membrane-containing filter (Corning Costar Spin-X), and centrifuged at 4 °C and 7,300 RPM. The filtered sample was transferred to a new sterile glass tube and the original tube was rinsed with 500 µL methanol and vortexed for 30 seconds to extract additional supernatant. The samples were then dried, reconstituted in 150 µL

ACN, and transferred to an autosampler vial. All samples were stored at –20 °C until instrumental analysis.

3.3.5 Instrumental Analysis

For instrumental analysis, a 1.7 μm ACQUITY UPLC BEH C18 reversed-phase column by Waters (130Å, 1.7 μm, 2.1x150 mm) was used on a Thermofisher Vanquish UHPLC system coupled with an Orbitrap Fusion mass spectrometer. Reverse phase chromatographic separation was achieved using a programmed gradient with the following mobile phases: Eluent A (Milli-Q) with 0.1% (v/v) formic acid and eluent B (ACN) with 0.1% (v/v) formic acid were mixed with curve 5 to a flow rate 0.200 mL/min. The total run lasted 31 minutes with a 7 minute re-equilibration and the following gradient: 0 – 2 minutes hold at 5% B, ramp to 65% B for 18 minutes, ramp to 100% B for 1 minute, and hold at 100% B for 3 minutes. The heated electrospray ionization (H-ESI) setting was 3500 V for the positive spray voltage with the ion transfer tube temperature at 300 °C and vaporization temperature at 225 °C. The three gases on the H-ESI were 35 for sheath gas, 7 for aux gas, and 0 for sweep gas. The injection volume was adjusted to 5 μL to improve chromatographic peak shapes. The Orbitrap was run at 120,000 (FWHM at m/z 200) resolution and mass range 85 – 700 m/z with an RF lens at 40%. Following the full scan, two MS^2 were scanned with the ion trap via two filters, Dynamic Exclusion (n = 3 for 60 seconds) and intensity threshold (minimum = 1000). Both MS^2 scans were isolated with the Quadrupole (0.7 m/z), but one fragmentation scan was generated through CID with assisted energy collision, and the other fragmentation scan was generated through HCD with stepped energy collision. The MS^2 scan with CID had an automatic gain control (AGC) set at 3.0e4 and a maximum injection time of 50 milliseconds, and the MS2 scan with HCD had an AGC of 1.0e4 and a maximum injection time of 50 milliseconds. Labeled proline-$^{13}C_5$,^{15}N (Sigma-Aldrich) was

used as the internal locking mass standard, while labeled valine-$^{13}C_5$,^{15}N (Sigma-Aldrich) was used for evaluating the mass locking during the entire retention time. The internal standards for the *on-the-fly* calibration were added in a solution of 96.7% CH_3CN, 3% H_2O, and 0.3% HCOOH. The locking solution was introduced to the sample using a T-shaped connection after the column separation and before the H-ESI ion source using Dionex AXP-MS metering pump at a flow rate of 0.05 mL/min.

Skyline Software (MacCoss Lab, University of Washington) was used to determine each isotopically labeled and non-labeled hormone's explicit retention time, limit of detection (LOD), and limit of quantification (LOQ). Analytes were identified based on the retention time of the isotopically labeled standard (i.e., when the compound signal was detected) and the compound's molecular weight; the mass error (< 2 ppm) assigned to each signal peak was assessed to confirm analyte detection. A bilinear trendline was applied to the standard calibration curve of each hormone to assess the turning point (i.e., where horizontal and vertical trendlines converge) and sloped region of the regression (Galitzine et al., 2018). The LOD was identified as the concentration at the turning point. The LOQ was the next concentration above the LOD on the vertical slope that deviated by less than 20% from the fitted line. Concentrations were derived for analytes within quantifiable limits by first inputting the displayed peak area into each analyte's standard calibration curve formula. Samples were then corrected for potential loss during hormone extraction using the percent recovery of each analyte (see section 3.3.3). The final concentration (ng/g) was determined by dividing corrected values by the amount of blubber used (g).

3.3.6. Steroid Hormone Analysis of Blubber

Following validation analyses, all samples were processed in the same manner. Briefly, 150 mg of blubber tissue was prepared (see section 3.3.4) and concentrated to a final volume of 150 µL ACN for instrumental analysis (see section 3.3.5). A targeted analysis of all hormones was performed to simultaneously separate each analyte in the tissue (see section 3.3.5). The calibration curve of non-labeled standards (aldosterone, corticosterone, cortisol, cortisone, 17β-estradiol, progesterone, testosterone) was analyzed alongside the extracted analytes to quantify steroid hormone concentrations (see section 3.3.5).

3.4 Sex Determination

Genetic sexing of 2012 – 2014 samples was provided by the NOAA Southeast Fisheries Science Center Marine Mammal Molecular Genetics lab. Genetic sexing of all samples collected in 2022 was performed following a modified protocol (Rosel, 2003) using the biopsied skin layer. Regions of the X and Y chromosomes were amplified by targeting the ZFX and SRY genes, respectively (Table 1).

Table 1. Primer sequences for sex determination.

Primer	Sequence (5' – 3')	Reference
SRY Gene		
TtSRYR	ACCGGCTTTCCATTCGTGAACG	Rosel (2003)
PMSRYF	CATTGTGTGGTCTCGTGATC	Richard et al. (1994)
ZFX Gene		
ZFX0582F	ATAGGTCTGCAGACTCTTCTA	Berubé and Palsbøll (1996)
ZFX0923R	AGAATATGGCGACTTAGAACG	Berubé and Palsbøll (1996)

Aliquots of 20 µL of lysis buffer (2.25 mM MgCl2, 15 mM Tris pH 8.3, 75 mM Kcl, 0.0015% Gelatin, 0.3% Tween20, 0.3% NP-40) and 2 µL of proteinaseK (200 ug/mL) were pipetted into a PCR tube with approximately 0.05 mg of dolphin skin sample. DNA from known

female and male post-mortem dolphins were used as positive controls. Samples were incubated at 55 ℃ for approximately 1 hour and then heated for 10 minutes at 95 ℃ in a thermocycler (Bio-Rad C1000) to deactivate the proteinaseK. A 2 μL aliquot of the lysis mixture was added to 23 μL of the PCR reaction mix (10X PCR buffer (2.5 μL), 0.3 μM of primers ZFX0582F (0.75 μL), ZFX0923R (0.75 μL), PMSRYF (0.75 μL) and 0.06 μM of TtSRYR (0.15 μL), 1.5 mM MgCl2 (included in 10X buffer), 150 μM dNTPs (0.375 μL), 1.5 U Taq DNA polymerase (0.3 μL), and water (17.425 μL). The unidentified skin samples and positive and negative controls were run in the thermocycler at the following program: 92 ℃ for 30 seconds followed by 35 cycles of 94 ℃ for 30 seconds, 51 ℃ for 45 seconds, and 72 ℃ for 45 seconds. An aliquot of 3.5 μL of DNA was diluted in Milli-Q water to a 10 μL volume and run at 75 V on a 2.5% agarose gel. Fragment sizes produced by the ZFX and SRY primers were approximately 382 and 339, respectively. The presence of one band (female) or two bands (male) was used for sex determination.

3.5. Epidermal Assessments

3.5.1 Photograph Analysis

All photographs of dolphins collected in the 2012 – 2014 RB, 2013 MS, and 2022 RB and ULM biopsy surveys were viewed in Apple, Inc. Preview (v.11.0, 2021). Images were sorted by date and group sighting. The best photograph of each dolphin was selected for both the left and right sides of the animal, when possible, based on the amount of visible body (head, abdomen, and peduncle), angle (perpendicular), clarity (not blurry), and exposure (not backlit). Photographs with < 10% visible body, > 15° angle offset from perpendicular, blurry, or backlit were excluded from further processing. Dolphin dorsal fins are individually distinctive based on markings (Wursig & Wursig, 1979). Photographs of dorsal fins were matched by two interns in

the Functional Anatomy and Behavioral Ecology of Marine Mammals laboratory at Texas A&M University-Corpus Christi using finFindR (Thompson et al., 2021), a semi-automated fin-matching software. Resighted animals were identified both temporally and spatially across the three field sites.

All photographs were carefully analyzed for the presence of skin disorders (0 = no skin disorder detected, 1 = skin disorder detected) using a visual reference catalog of known lesion types (Toms et al., 2020). Two independent reviewers evaluated 20% of the photographs to ensure reliable identification of skin lesions. Once reviewer concordance was established, one reviewer assessed the remaining 80% of photographs and consulted with the second reviewer when there was uncertainty.

If a lesion was detected on either side of a dolphin, the photograph was identified as a potential positive case (i.e., lesion present) and retained for further evaluation. However, if no lesions were detected on one side of a dolphin, images of both sides of the dolphin were required to designate the dolphin as a negative case (i.e., lesion absent). Photographs retained for analysis were then evaluated based on photograph quality (resolution) (Figure 3A-B), lesion characteristics, and reviewer confidence. Images were required to be high quality for further consideration unless the reviewer had high confidence in the presence or absence of lesions. Positive cases required a minimum of five lesions that were approximately ≥ 20 mm in size (regardless of whether they were clustered together) (Figure 3C). Lesions near tooth rake marks (Figure 3D), shark bites (Figure 3E), and propeller/entanglement wounds were included in calculations if the other criteria were met. Cases of orange hue (Figure 3F) were excluded from analysis unless the dolphin had additional lesions or previous photographs of skin lesions underneath the orange hue. If reviewer confidence was intermediate or high and the other criteria

were met, the image was included in calculations. The proportion of dolphins with skin disorders (total # of dolphins with skin disorders / the total # of dolphins with both left and right dorsal images) was calculated per year for each field site.

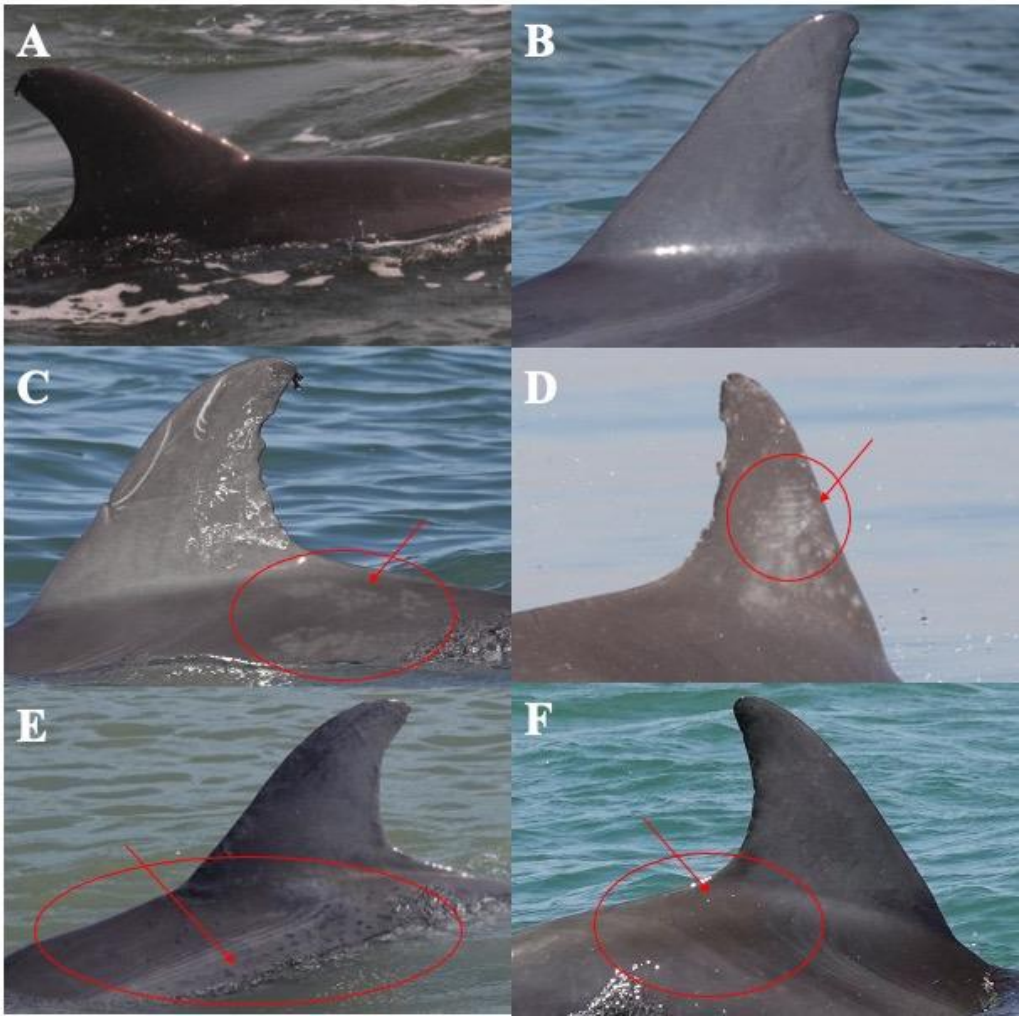


Figure 3. Examples of skin lesions. Evaluation metrics and criteria: A) poor quality, B) high quality, C) > 5 lesion spots, D) lesions on tooth rake marks, E) lesions on shark bite wound, and F) orange hue. Photographs collected under NMFS permit number 21938.

3.6. Statistical Analyses

Statistical analyses were conducted in the software R (v.4.1.2). All data were tested for assumptions of normality ("qqnorm" function) and homogeneity of variance ("leveneTest" function) prior to computational manipulation.

To address the hypothesis (H1) that steroid hormone concentrations will vary among field sites, a Pearson's R correlation test ("cor.test" function) was used. The prediction (P1) that aldosterone and cortisol are positively correlated with high and low salinities was tested using Pearson's R correlation test. A simple binomial logistic regression explored the relationships between hormone concentrations, sex, and season.

Before testing the hypothesis (H2) that there will be variation in the proportion of skin disorders across field sites, reviewer concordance was assessed using the "epi.kappa" function in R software to estimate Cohen's Kappa (*K*), a measure of agreement. $K = 0$ indicates a chance level of agreement (i.e., as if reviewers "guessed" every time), while $K = 1$ indicates complete concordance. *K* was tested for significance using a z-test (Ho: $K = 0.50$). Additional metrics were calculated including the prevalence index (range -1 – 1) (PI; the difference in the proportion of positive to negative cases), bias index (range |0 – 1|) (BI; the extent to which raters disagree in the proportion of positive or negative cases), prevalence-adjusted bias-adjusted kappa (PABAK), and K_{max}. PI=0 indicates an equal probability of positive and negative cases, while BI=0 indicates absolute symmetrical agreement (Byrt et al., 1993; Sim & Wright, 1995). The effects of bias were tested using a McNemar test, where $p < 0.05$ indicates a systematic difference in the proportion of positive responses between reviewers. Power analyses were conducted using the "N.cohen.kappa" function in R to determine the sample size needed for *K*. The percent agreement was calculated using the "agree" function in R.

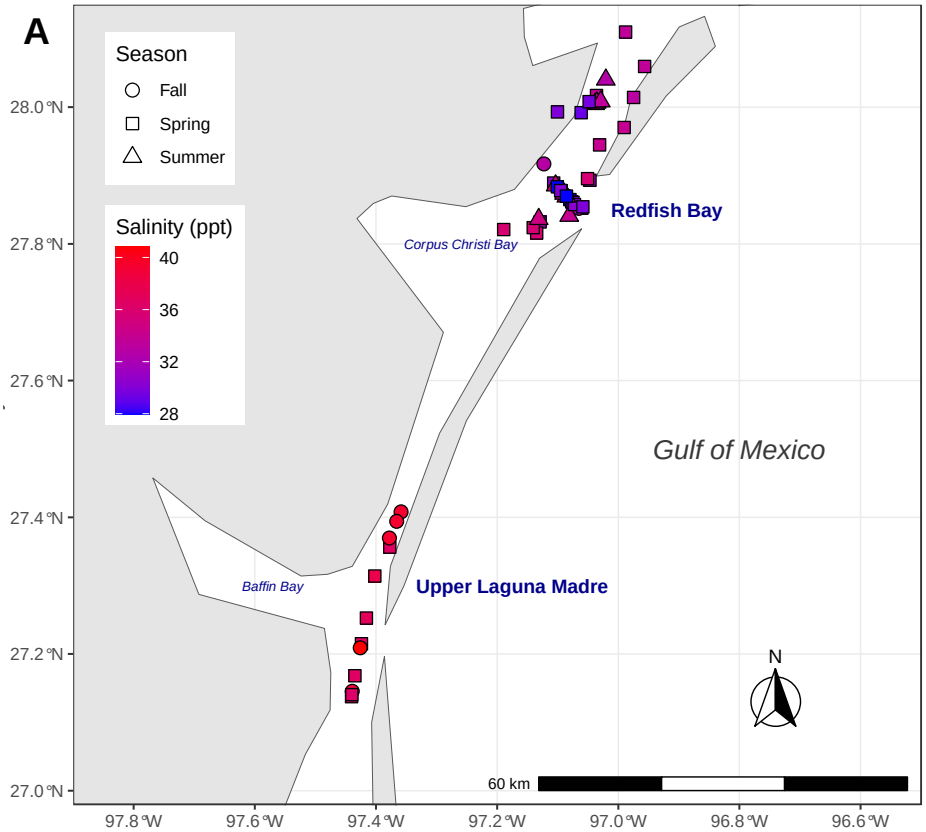
After reviewer concordance was ascertained, the hypothesis (H2) that there will be a difference in the proportion of skin disorders (total # of dolphins with skin disorders / the total # of dolphins with both left and right dorsal images) of dolphins across the field sites was assessed using a proportions test ("prop.test" function) in R. To test the prediction (P2) that the proportion

of dolphins with skin disorders will be negatively associated with salinity level, a simple binary logistic regression was run. The relationship between lesion prevalence and additional variables (pH, water temperature, dissolved oxygen, sex, season) was also tested using a logistic regression, with multicollinearity between all variables measured using the "vif" function. VIF $\leq$ 1 indicates no correlation, 1 – 5 indicates a moderate correlation, and > 5 indicates a strong correlation for which variables should be removed.

To test the hypothesis (H3) that there will be a difference in the physiological condition of RB dolphins sampled in 2012 – 2014 or 2022, a t-test and proportions test were used. The prediction (P3) that the current (2022) concentrations of aldosterone, corticosterone, cortisone, and/or cortisol and the proportion of dolphins with skin disorders in RB will be higher than previously measured in 2012 – 2014 was assessed using an unpaired two-sample t-test ("t.test" function) and two-sample proportions test ("prop.test" function), for hormones and skin disorders respectively.

4. RESULTS

In 2022, 56 km^2 of Redfish Bay (RB) and 46 km^2 of Upper Laguna Madre (ULM) were surveyed over 16 days (Figure 4A). Between 2012 - 2014, the NOAA Southeast Fisheries division spent 62 days surveying waters from Corpus Christi Bay to Matagorda Bay (including the same 56 km^2 of RB), and 24 days surveying 643 km^2 of Mississippi Sound (MS) (Figure 4B). A total of 82 blubber samples were obtained from different dolphins across the three field sites and utilized for steroid hormone analyses. A total of 49 samples used in this study were collected 10 or more years ago. Samples were collected in 2012 (RB, n=14), 2013 (MS, n=29; RB, n=5), 2014 (RB, n=3), and 2022 (RB, n=19; ULM, n=12). Based on genetic analyses, 53 males and 29 females were biopsied (Table 2). Since dolphins were not handled in this study, additional demographic data (e.g., size, age, sexual maturity) are unknown. A total of 432 dolphins were photographed from groups that were biopsy sampled.



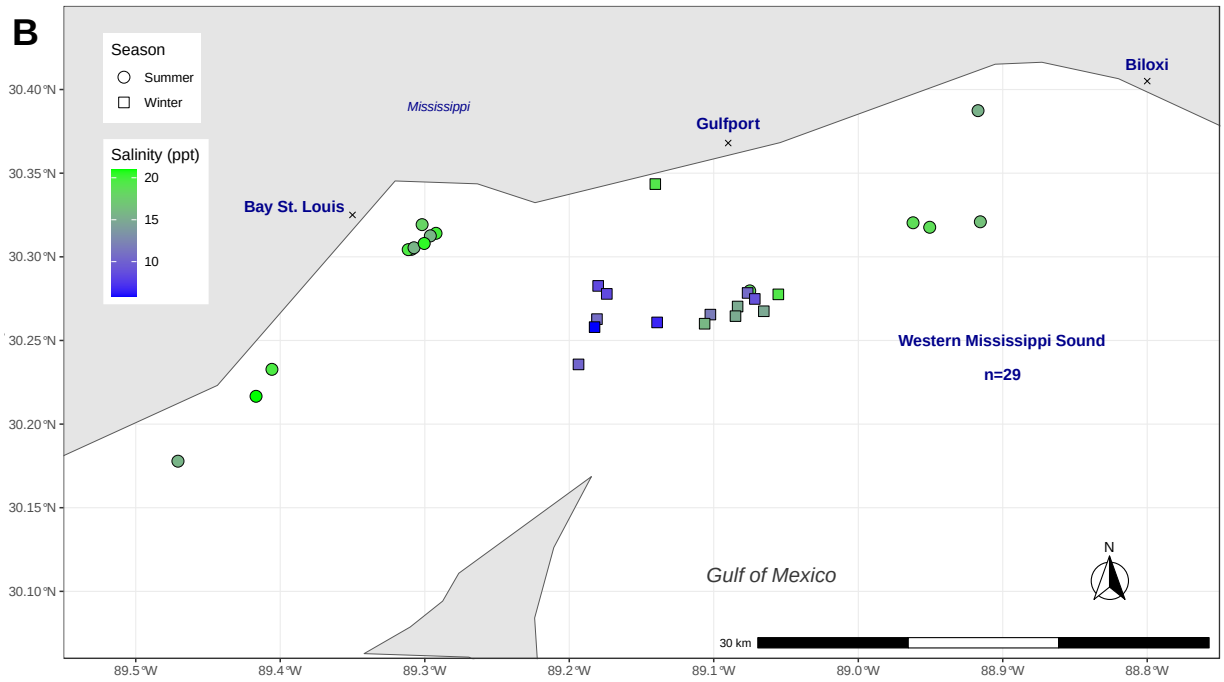


Figure 4. Distribution of blubber samples collected. Locations included A) Redfish Bay (2012-2014, 2022) and Upper Laguna Madre (2022), and B) Western Mississippi Sound (2013). Color coding denotes salinity levels. Symbols indicate the season of sampling.

Table 2. Demographic description of biopsy samples.

Year	Field Site	Number of males sampled	Number of females sampled
2012	Redfish Bay	9	5
2013	Redfish Bay	4	1
	Mississippi Sound	12	17
2014	Redfish Bay	2	1
2022	Redfish Bay	17	2
	Upper Laguna Madre	9	3

In MS, salinity and water temperature were highest in the summer and lowest in the winter (Figure 5). In RB, salinity and water temperature were generally highest in the summer and lowest in the fall (Figure 5). In ULM, salinity was highest in the fall and lowest in the spring, while the opposite trend was observed in water temperature (Figure 5). Salinity and water temperature conditions prior to the sampling periods were assessed to infer their potential impact on results (e.g., dolphins must be exposed to low salinity waters for at least a week before they

develop lesions; Takeshita et al., 2021). The observed salinity and water temperature conditions reported in this study were consistent for approximately 90 days in MS (2013), 30-120 days in RB (2012, 2013, 2014, 2022), and 30-60 days in ULM (2022) (NERR, 2023; TCEQ, 2023; USGS, 2023).

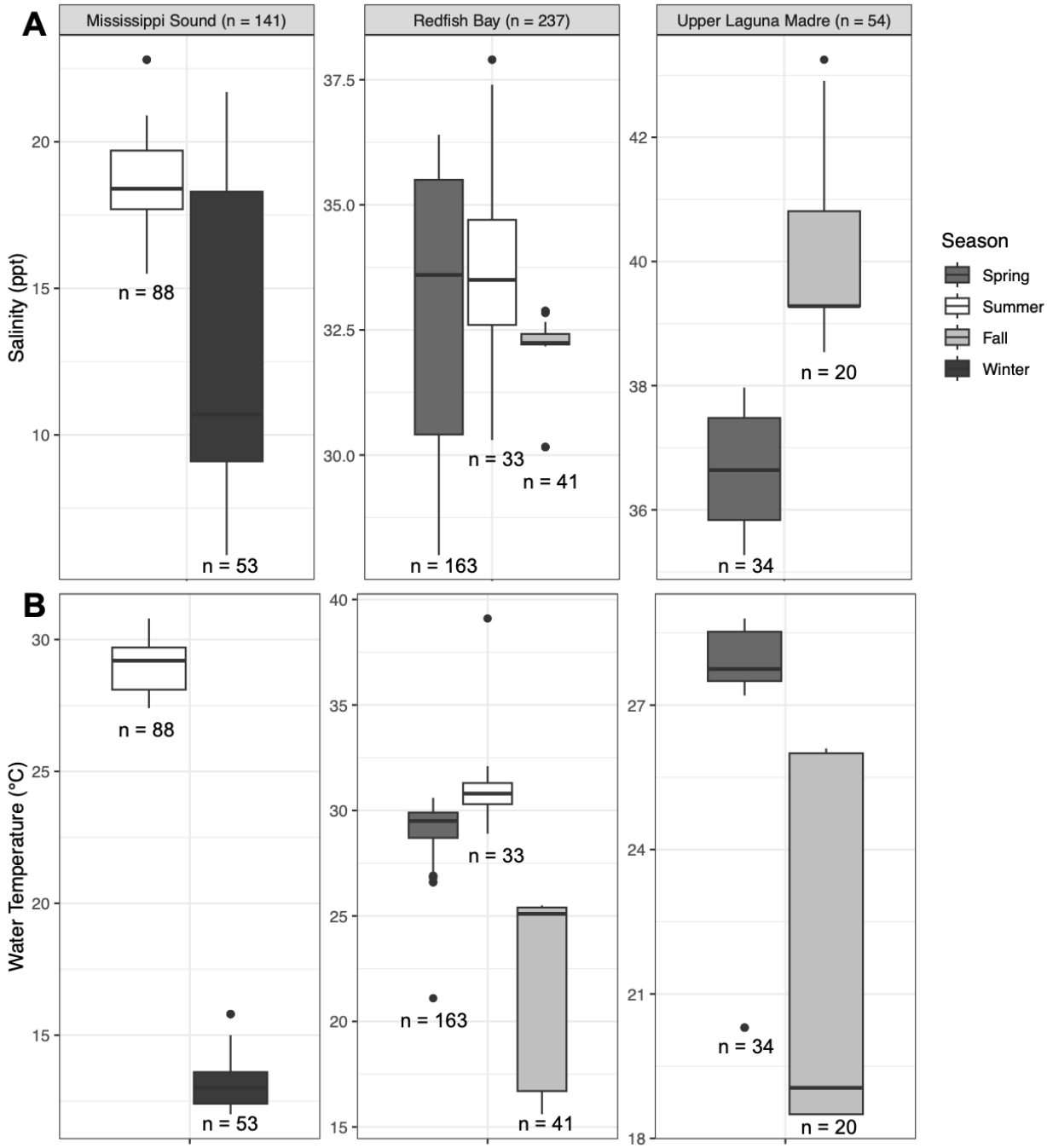


Figure 5. Seasonal variation in water quality parameters. Boxplots include A) salinity and B) water temperature across the three field sites. Spring corresponds with May and June, summer with August, fall with November, and winter with January. Seasons were determined using an annual season calendar. The black horizontal line within the bar indicates the median value. Lines extending from each bar indicate minimum and maximum values. Black dots outside of the bars indicate outliers.

4.1 Hormone Analyses

4.1.1 Recovery and Precision Assessment

Steroid hormones were detected in all replicate samples using different tissue sample masses (50 mg, 150 mg, 400 mg) and extraction solvents (Milli-Q water, acetonitrile). Positive hormone identification was achieved by running two chromatographic separations of internally labeled and non-labeled standards (Figure 2). Acetonitrile had a significantly higher extraction efficiency than Milli-Q water when mass replicates were averaged (t_{93} = -4.1, p = < 0.0001) and for individual hormones (Appendix B). Similarly, the percent recovery of replicates extracted with acetonitrile (range 2.22 – 17.60%) was much less variable than Milli-Q water (range 0.02 – 35.73%). Accordingly, all biopsied samples were processed using 150 mg of blubber with acetonitrile as the main extraction solvent.

Recovery (%) and precision (%) for all analytes except progesterone (36% recovery, 2.6% precision) were within accepted Environmental Protection Agency limits (51 – 74.5% recovery, 2.2 – 7.6% precision; Table 3). Since labeled standards are not manufactured for cortisone and corticosterone, recovery and precision were implied from quantified values for aldosterone and cortisol, respectively. Cortisone and aldosterone are isomers, while corticosterone is structurally very similar to cortisol. Potential matrix interference is reflected in the analyte recovery measurement, which includes the combined effects of both the matrix and extraction methods. Based on recovery and precision, matrix interference was minimal for the 9 blubber samples analyzed using ACN (Appendix B). Thus, this protocol is accurate and precise

in the measurement of known quantities of isotopically spiked samples for four steroid hormones (aldosterone, cortisol, testosterone, and progesterone).

4.1.2 Steroid Hormone Analysis of Blubber

Corticosterone, cortisol, testosterone, and progesterone were quantifiable in the blubber samples (Table 3; see full list in Appendix C). Hormone detection and quantification was confirmed based on retention times, LOD, and LOQ (Appendix A). Aldosterone, cortisone, and estradiol were not detected, thus the prediction (P1) about the relationship between aldosterone and salinity could not be tested. Of the 55 males sampled, corticosterone was detected in 7% (n=4) and quantified in 5% (n = 3), cortisol in 13% (n = 7), and testosterone in 42% (n = 23). Of the 30 females sampled, corticosterone was detected in 13% (n = 4), cortisol was quantified in 17% (n = 5), and progesterone was quantified in 20% (n = 6). Cortisol and progesterone were only detected and quantified in the spring and summer. Corticosterone was detected across all seasons except fall and was only quantifiable in the spring and summer. No seasonal differences were found for cortisol (t_9 = 0.47, p = 0.65), progesterone (t_6 = -0.56, p = 0.58), or corticosterone (t_1 = -1.06, p = 0.48). Testosterone was highest in the fall (t_{17} = 3.55, p = 0.002) and winter (t_{18} = 2.95, p = 0.008) months, and was only detected in males (Figure 6). Progesterone was only detected in females (Figure 7). A positive correlation was identified between cortisol and salinity (r(10)= 0.62, p= 0. 03) (Figure 8). Of the 12 animals with quantifiable cortisol, two dolphins had skin disorders, 11 were sampled in the spring and summer months, and seven were male. A simple binomial logistic regression showed no statistically significant relationship between sex and steroid hormones (R^2_{10} = -0.09, p = 0.99). There was not a significant difference in cortisol concentrations for dolphins sampled from RB in 2012-2014 compared to those sampled in 2022 (t(6) = 1.30, p = 0.24).

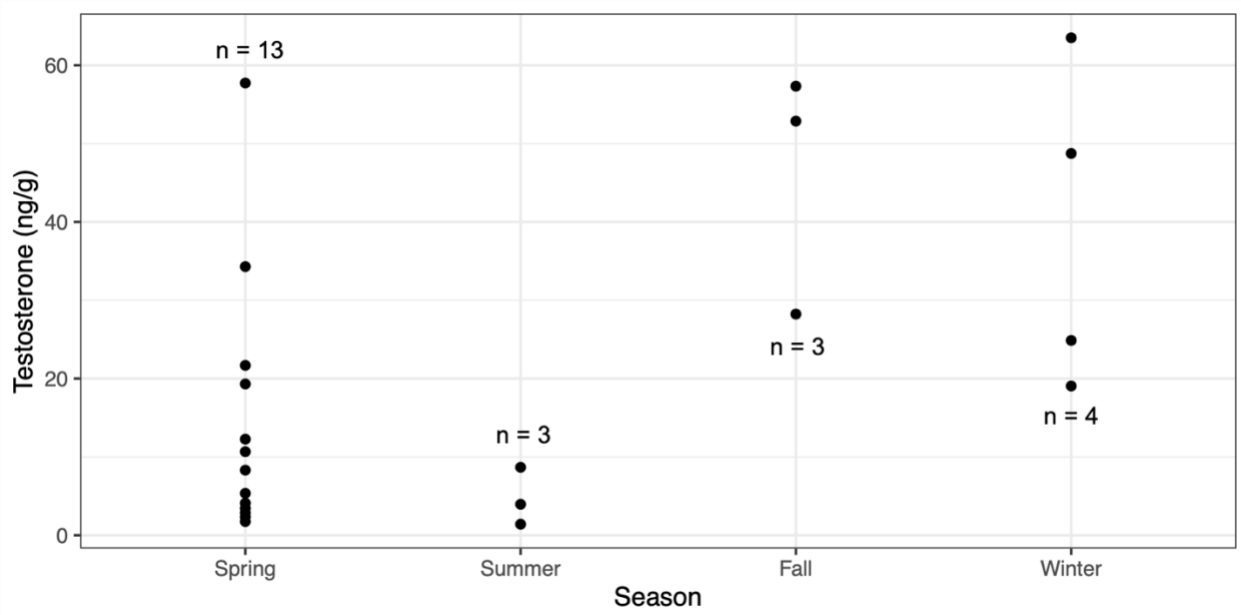


Figure 6. Seasonal distribution of testosterone concentrations. Samples measured in(ng/g) in male bottlenose dolphins. N value represents the number of dolphins with quantifiable testosterone per season.

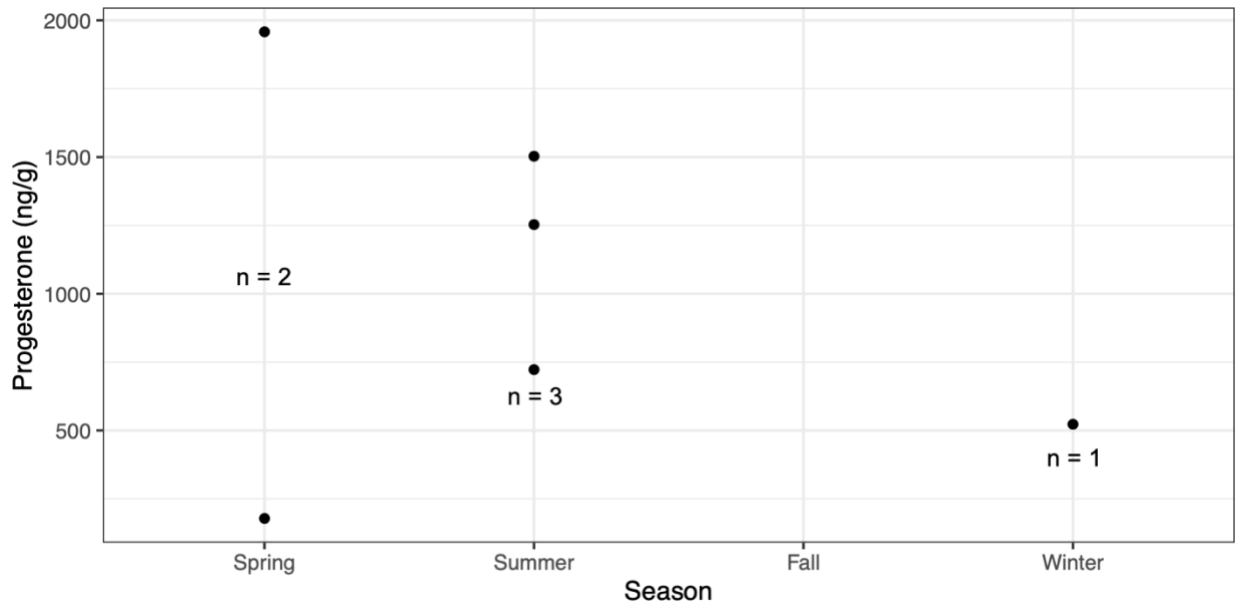


Figure 7. Seasonal distribution of progesterone concentrations. Samples measured in (ng/g) in female bottlenose dolphins. N value represents the number of dolphins with quantifiable progesterone per season. Progesterone was not detected in the fall.

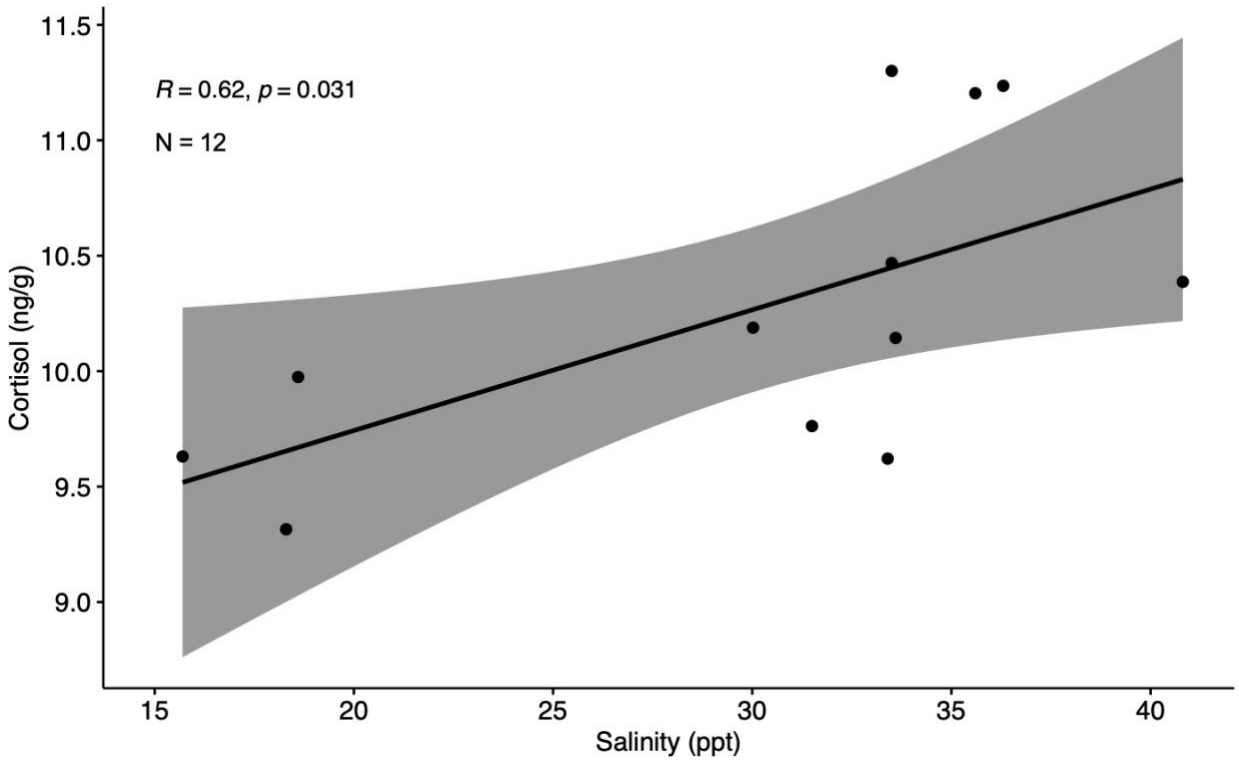


Figure 8. Positive correlation between cortisol and salinity level. The black line represents the fitted regression line. The gray shaded region represents the confidence interval. Data are from MS (n = 3), RB (n = 8), and ULM (n = 1). N value represents the number of dolphins with quantifiable cortisol.

Table 3. Mean blubber hormone concentrations. Concentrations ± standard error (ng/g) are corrected for extraction efficiency. Hormones that were below the limit of detection (LOD) or limit of quantification (LOQ) are identified. N indicates the number of animals for which the hormone was detected (if below LOQ) or quantified.

	Female			Male		
Hormone	Mean ± S.E.	Range	N	Mean ± S.E.	Range	N
Corticosterone	< LOQ	–	4	9.45 ± 0.53	8.88 – 10.67	3
				< LOQ	–	4
Cortisol	10.27 ± 0.40	9.32 – 11.20	5	10.27 ± 0.21	9.62 – 11.30	7
Progesterone	1022.9 ± 271.5	178 – 1958	6	< LOD	–	–
Testosterone	< LOD	–	–	21.4 ± 4.35	1.41 – 63.5	23

4.2 Skin Disorder Analyses

Two reviewers independently identified the presence or absence of skin lesions for 20% of the photographs (n = 80). High concordance was achieved between reviewers (93%) and minimal prevalence and bias effects were observed (Table 4). Accordingly, one reviewer evaluated the remaining 80% of photographs and consulted the other reviewer when confidence was low.

Table 4. Reviewer reliability test. Frequencies indicate the number of photographs with dolphin skin lesions present out of the 80 photographs assessed. Cohen's Kappa (K) indicates the level of agreement between reviewers. Prevalence (PI) and bias (BI) indices indicate the difference between the probability of positive and negative cases (PI) and the difference in the proportion of positive cases (BI), respectively. PABAK reflects the corrected Kappa accounting for PI and BI. Kmax is the upper limit of the confidence interval.

Frequency Reviewer #1	Frequency Reviewer #2	K	Agreement (%)	PI	BI	PABAK	Kmax
40	42	0.86	93	-0.01	-0.02	0.86	0.97

Skin disorders were observed on dolphins across all sites, seasons, and demographics. Lesion presence was significantly related to season (R^2_3= 20.4, p = 0.000138). In MS, a lower proportion of dolphins had skin disorders in the summer (48%, n = 42) than in the winter (57%, n = 30) (Figure 9; X^2_1= 1.043, p = 0.307). In RB, the presence of skin disorders was significantly higher in the fall (60%, 2022, n = 25) than in the spring (39%, 2012, n = 39; 24%, 2022, n = 12) or summer (31%, 2013, n = 8; 14%, 2014, n = 3) (Figure 9; X^2_2= 41.4, $p < 0.0001$). Additionally, the proportion of observed lesions in RB was not significantly different between 2012 – 14 and 2022 (Figure 10; X^2_1= 0.99, p = 0.3192). In ULM, the proportion of dolphins with skin disorders was higher in the fall (70%, n = 14) than in the spring (53%, n = 19), although not statistically significant (Figure 9; X^2_1= 1.5178, p = 0.2179). A simple binary logistic regression revealed that skin disorders were not significantly related to sex (z_{43} = 0.030, p = 0.976), salinity level (Figure

11; z_{431} = -1.3, p =0.193), nor cortisol concentration (z_6 = -0.267, p = 0.790). The relationship between corticosterone and lesions could not be tested due to a small sample size with quantifiable corticosterone (n = 3). However, a negative correlation was found between skin disorder presence and water temperature (Figure 12; r_{430} = -0.14, p = 0.003).

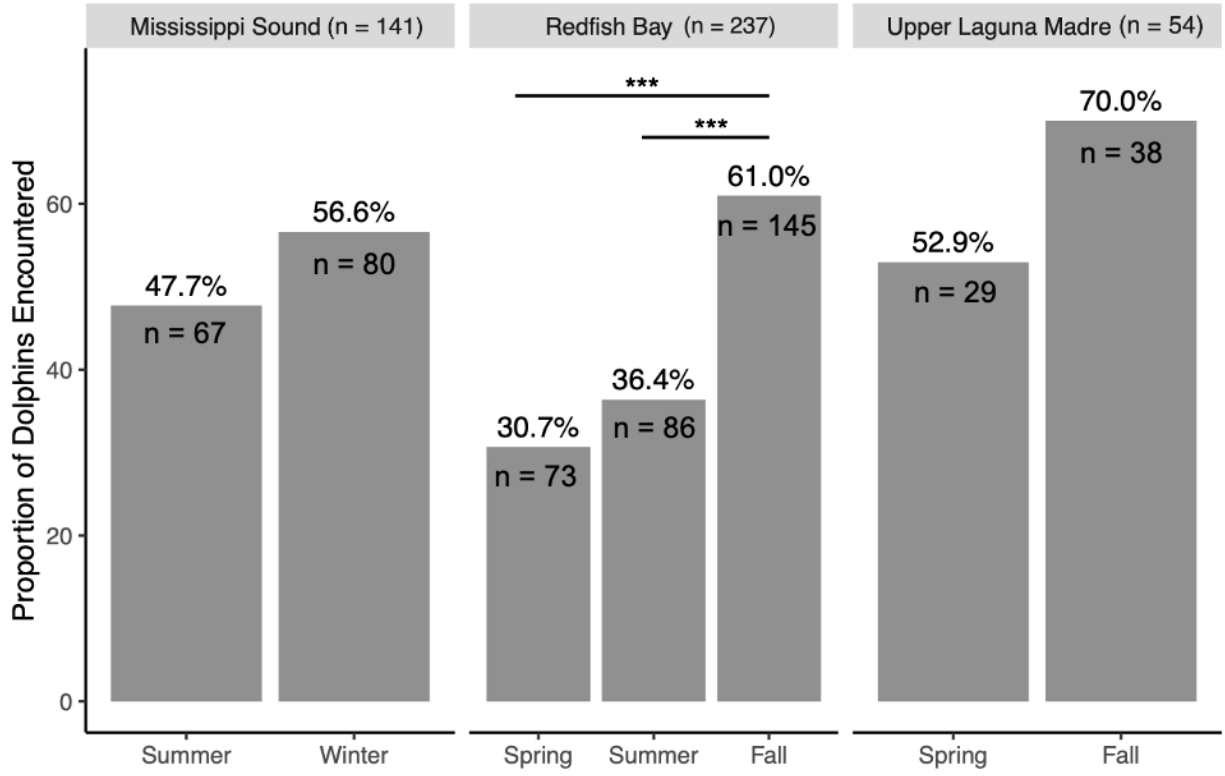


Figure 9. Seasonal patterns of skin disorders across field sites. N value next to site represents total number of dolphins encountered. Proportion values (%) and N listed below indicate the total number of positive cases (skin disorder present) out of all encountered animals. Spring corresponds with May and June, summer with August, fall with November, and winter with January. Seasons were determined using an annual season calendar.

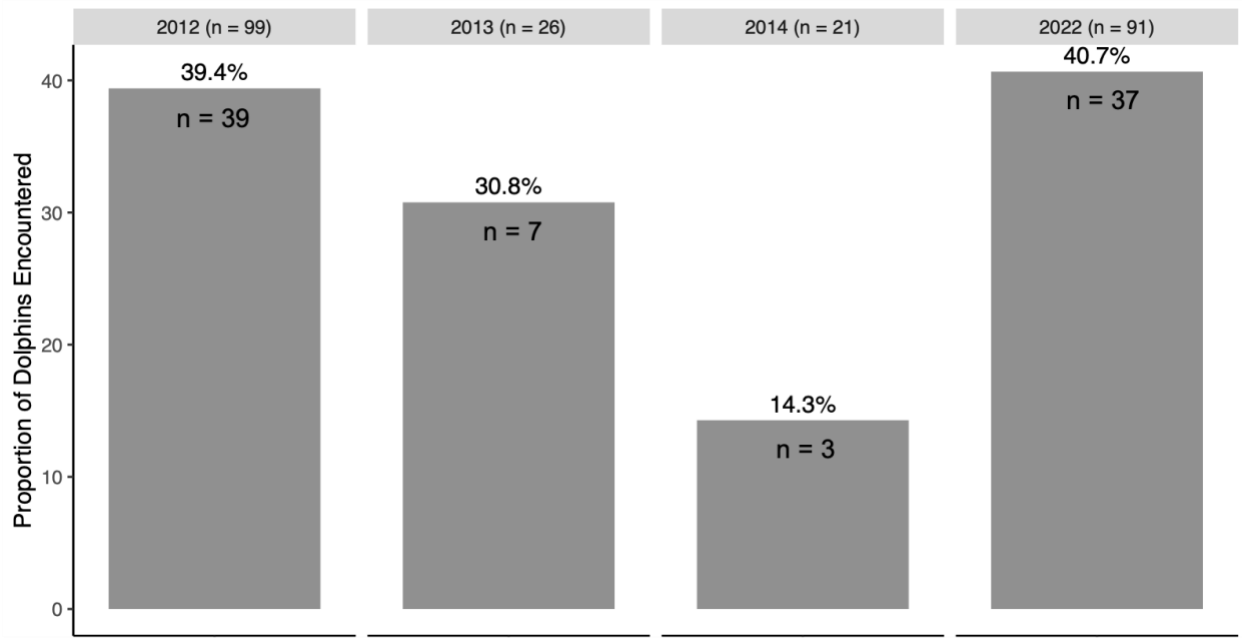


Figure 10. Skin lesions over time in Redfish Bay. Lesions observed in 2012, 2013, 2014, and 2022. N value next to year represents the total number of dolphins encountered. N value under percentage represents total number of dolphins with lesions.

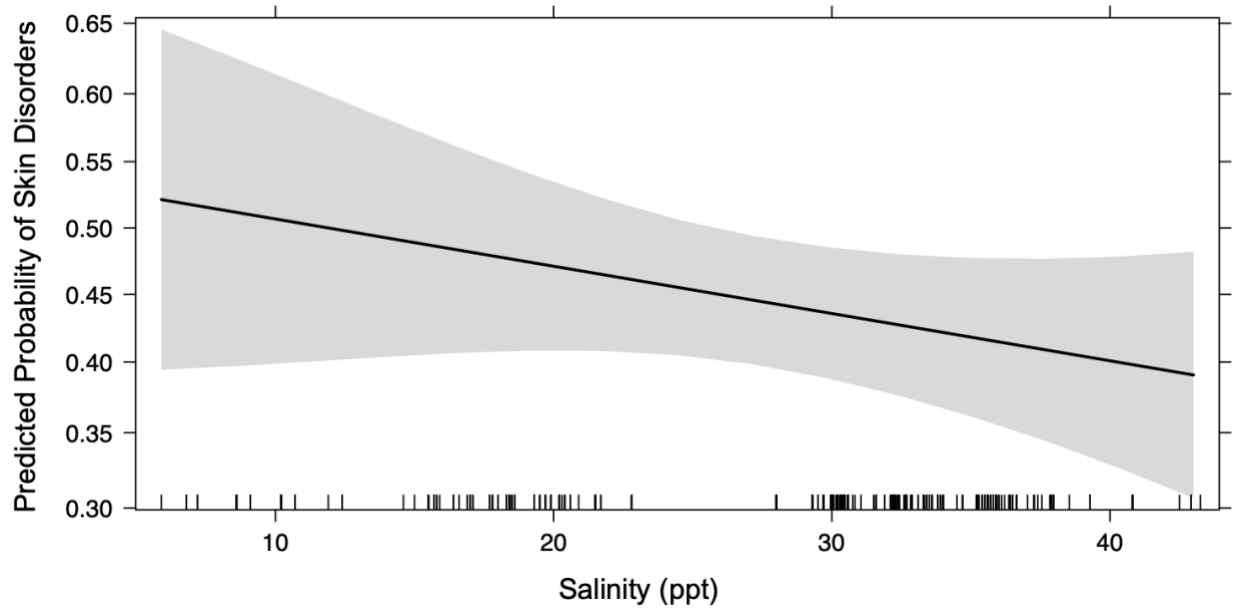


Figure 11. Salinity and skin lesions. The predicted probability of skin lesions is negatively related to salinity. The grey-shaded region depicts the confidence interval. Marks along the x-axis indicate salinity measurements for which a dolphin was assessed for the presence or absence of a skin disorder.

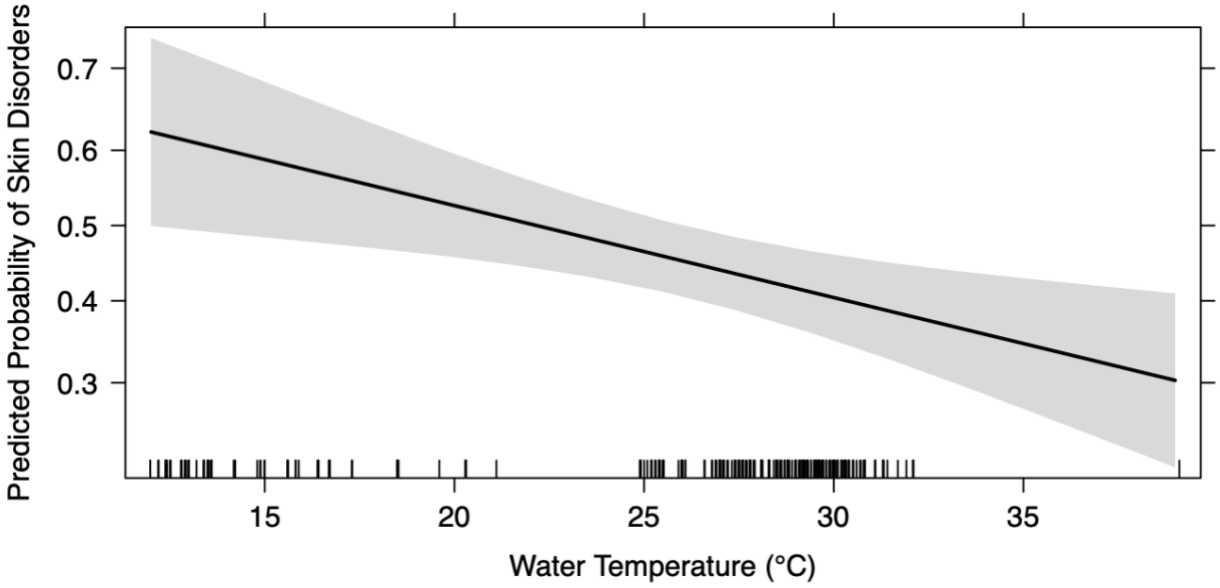


Figure 12. Water temperature and skin lesions. The predicted probability of skin lesions is negatively correlated with water temperature. The grey-shaded region depicts the confidence interval. Marks along the x-axis indicate water temperature measurements for which a dolphin was assessed for the presence or absence of a skin disorder.

5. DISCUSSION

Bottlenose dolphins in the Gulf of Mexico are increasingly exposed to variable salinity conditions as climate change intensifies extreme weather events. Understanding the tolerance of dolphins to environmental change is essential for sustainable management. While research has identified some of the health concerns for dolphins in hyposaline environments, this study is the first comprehensive assessment of blubber steroid hormones and epidermal health of free-ranging bottlenose dolphins across a salinity gradient that includes hypersaline conditions.

The proportion of males (65%) to females (35%) biopsy-sampled in this study suggests a strong male bias, consistent with other research findings (Quérouil et al., 2010). Potential drivers of sex bias during sampling may include geographic location and selectivity, differential sex-linked behaviors, nutritional requirements, reproductive condition, and season (Kellar et al., 2013). The observed male bias likely reflects efforts to avoid sampling groups with small calves (and their mothers).

Testosterone in male dolphins peaked in the fall and winter. In seasonally breeding cetaceans, increased androgenic stimulation prior to the breeding season activates morphological and physiological changes necessary for mating success (Boggs et al., 2019). Because spermatogenesis is a slow complex cycle (e.g., 60 days in Pacific white-sided dolphins (*Lagenorhynchus obliquidens*; Robeck et al., 2009), high testosterone levels prior to breeding help stimulate the spermatogenic cycle (Cates et al., 2019). In captive bottlenose dolphins, peak circulating testosterone preceded high frequencies of successful mating by one to two months (Harrison & Ridgway, 1971; Schroeder & Kellar, 1989); the period of greatest sperm production and density occurs during peak breeding season yet coincides with the lowest levels of serum testosterone (Schroeder & Kellar, 1989). Spring is the predominant mating and calving season

for dolphins in MS (Pitchford et al., 2016) and RB (Shane & Schmidly, 1978). Testosterone peaked in MS and RB stocks approximately three months prior to the reported mating season, supporting that the androgen secretion in males of this study may be in preparation for mating in the spring.

Using previously defined blubber progesterone concentrations for pregnancy (≥ 100 ng/g) in bottlenose dolphins (Galligan et al., 2020) and other dolphin species (short-beaked common dolphin, *Delphinus delphis*; northern right whale dolphin, *Lissodelphis borealis*; Dall's porpoise, *Phocoenoides dalli*; Pacific white-sided dolphin, *Lagenorhynchus obliquidens*; Kellar et al., 2006, 2013; Trego et al., 2013), all females with quantifiable progesterone in this study were identified as probable pregnant. Since progesterone levels can show considerable overlap between ovulating and pregnant females (Trego et al., 2013), it can be challenging to confirm pregnancy status in free-ranging animals without multiple samples from the same individual over time. Progesterone levels in odontocetes (toothed whales) generally exhibit bimodal peaks during early and late pregnancy (Duffield et al., 1995; Bergfelt et al., 2011; Zhang et al., 2021), although the progesterone levels measured here far exceeded concentrations in previous reports of free-ranging pregnant dolphins and were most similar to those of a stranded pregnant Dall's porpoise (Trego et al., 2013). Exceptionally high progesterone, however, may reflect recent parturition and residual progesterone in the blubber (Trego et al., 2013). This is consistent with the peak calving season being spring for MS and RB stocks (Shane & Schmidly, 1978; Pitchford et al., 2016).

Corticosterone was detected in two female bottlenose dolphins with detectable progesterone concentrations and is known to increase during normal pregnancy in rodents (Barzegar et al., 2015), fetal stress in humans (Wynne-Edwards et al., 2013), and pregnancy in

blue whales (*Balaenoptera musculus*; Valenzuela-Molina et al., 2018; Melica et al., 2022). The detection of corticosterone in pregnant females supports previous literature on the role of corticosteroids in the stress response of cetaceans, as gestation and parturition are inherently stressful events (Atkinson et al., 2015).

Although aldosterone was not detected in blubber in this study, research is needed to explore the potential relationship between aldosterone and high-saline environments. Aldosterone in bottlenose dolphins increases above baseline levels simultaneously with cortisol in response to acute stress events (Thomsan & Geraci, 1986; St. Aubin et al., 1996; Ortiz & Worthy, 2000; Houser et al., 2011; Champagne et al., 2018). Serum aldosterone but not cortisol was significantly elevated for dolphins exposed to low salinity conditions compared to average seawater (McClain et al., 2020). Low salinity, especially from intense and increasingly frequent tropical weather, is a physiological stressor linked to adverse health conditions in dolphins including skin disorders, electrolyte imbalances, secondary infections, and mortality (Booth & Thomas, 2021). However, the lack of association between circulating aldosterone and cortisol in low salinities (McClain et al., 2020) suggests that heightened aldosterone secretion may reflect natural homeostatic mechanisms for adapting to low salinity conditions instead of stress. Since cortisol was observed to be significantly related to high salinity exposure in this study, more research is needed to understand the role of aldosterone in high saline environments and in response to acute stress.

The low circulation of endogenous aldosterone in cetaceans could potentially be circumvented by the development of an extraction protocol more sensitive to the compound's structure and by testing the efficiency of different extraction methods to produce a cleaner sample prior to instrument injection. Specific extraction for different steroid targets may also

improve sensitivity and enhance detection capabilities (Boggs et al., 2016). Blubber is the only matrix in which aldosterone has yet to be quantified in bottlenose dolphins, which is surprising as aldosterone has been measured in the skin (Bechshoft et al., 2020) and feces (Champagne et al., 2018). The human epidermis is the largest steroid-producing organ for which aldosterone is known to be secreted independently from the adrenal gland (Slominski et al., 2013), however, this has not been thoroughly explored in cetaceans and could explain why aldosterone is more readily measured in skin than blubber. Applying a protocol to extract hormones from both the skin and blubber layer of the same sample could reveal differences in hormone deposition and concentration ratios between different tissues. Using the biopsied skin layers collected in this study, aldosterone and other undetected analytes could be measured to fill the gap in knowledge regarding response to high salinity. Because free unbound cortisol may diffuse into adipose tissue (Breuner et al., 2013) and aldosterone has a low affinity (< 20%) for binding to albumin and other proteins (Champagne et al., 2018), the capacity for aldosterone to bind to unknown proteins warrants further exploration.

Hormone concentrations that were quantifiable for archived samples from RB were consistent with those collected in 2022, indicating minimal blubber hormone degradation. Previous research has shown that storage time (i.e., 30 years) had no effect on blubber hormone concentrations when high sample quality was preserved (Trana et al., 2015). However, high variability in concentration can be expected when measuring blubber steroids. Factors such as sampling method, degree of perfusion into blubber, depth of blubber sampled, and changes in the levels of binding proteins prior to sampling can account for observed differences (Champagne et al., 2018). Generally, the compressive force of biopsy sampling reduces lipid values in extracted tissues (Ryan et al., 2013) and can lead to variation in concentrations (Kellar et al., 2009).

Additionally, blubber has the ability to metabolize steroids and likely shifts hormone profiles away from initial concentrations (Galligan et al., 2018, 2020).

The integration of archived and newly collected blubber samples provides a holistic picture of how environmental changes over time may alter dolphin stress levels. In particular, cortisol levels were consistent across years which may indicate dolphins have adapted to the increasing infrastructure development and port activity in RB. Baseline cortisol in captive bottlenose dolphin blubber has been reported to be 1.4 ng/g (Champagne et al., 2018), suggesting a nearly ten-fold increase in cortisol levels observed in this study. Since baseline cortisol levels in free-ranging bottlenose dolphin populations have not been established, the concentrations reported here may reflect normal levels since natural environments are inherently more stressful than aquaria. Cortisol levels measured in this study were similar to those reported for other biopsied free-ranging dolphins (Boggs et al., 2019). However, cortisol was positively associated with salinity in this study. One plausible explanation may relate to the permeability of dolphin skin. Dolphin skin is a major conduit for the ingress of water (Hui, 1981), particularly in low-salinity environments where barrier properties are reduced and dolphins are susceptible to freshwater intoxication (Andersen & Nielson, 1983; Ewing et al., 2017). Additionally, water loss has been reported for harbor porpoises (*Phocoena phocoena*) maintained in hyperosmotic conditions (Andersen & Nielson, 1983). The capacity for dolphins to experience considerable water flux when exposed to osmotic variability may lead to enhanced cortisol expression across a natural salinity gradient. In high salinity environments, dolphins may also experience mild dehydration due to the combined effects of net water loss and incidental seawater ingestion, which can increase both blood and urine osmolality (Kjeld, 2001; Ridgway and Venn-Watson, 2010). Thus, high salinity exposure may challenge physiological mechanisms used to reserve

fresh water, thereby enhancing glucocorticoid concentrations. Other factors such as limited prey distribution, mating, underlying physiological illness (i.e., skin lesions), or the interaction of multiple stressors may also account for the observed relationship between cortisol and salinity. With salinity levels continuing to rise in the southwestern U.S. (Asquith, 1997; Seager et al., 2007; Miller et al., 2021; Montagna et al., 2021), more research is needed to fully elucidate the physiological effects of high salinity exposure on coastal dolphins.

In addition to imposing osmoregulatory challenges, salinity influences the functional properties of cetacean skin. Prolonged exposure to low saline conditions (< 10 ppt) typically leads to hydropic degeneration (i.e., fluid accumulation and cell swelling) of the dolphin epidermis (Simpson & Gardener, 1972; Harrison & Thurley, 1972; Deming et al., 2020) resulting in lesions that may lead to secondary infection and mortality (Wilson et al., 1999; Hart et al., 2012; Mullin et al., 2015; Deming et al., 2020; Duignan et al., 2020; Fazioli & Mintzer, 2020; McClain et al., 2020; Toms et al., 2020). In natural high salinities, wounds on dolphins appear to heal faster compared to average saline conditions (Hurst & Orbach, 2022). However, despite increased salinity, the high proportion of lesions observed in ULM indicates lesion development may be linked to additional factors. The ability of cortisol to suppress aspects of the immune system when overproduced (Aguilar & Borrell, 1994; Miller et al., 2009; Morey et al., 2015; Liu et al., 2017) may indicate why dolphins inhabiting ULM had higher levels of cortisol and a higher proportion of lesions. However, cortisol generally enhances immunity to disease and may be independently produced in the skin (Slominski et al., 2013), so the lack of association between cortisol and skin lesions in this study suggests additional research with a larger sample size is needed to fully elucidate this relationship in dolphins.

The skin lesions assessed have characteristic patterns. General pallor is often associated with freshwater exposure (Ewing et al., 2017; Deming et al., 2020; Toms et al., 2020, 2021) and was seen most often in MS. Less is known about the etiology of hyperpigmentation and dark spots, which were most prevalent in RB and ULM. A few cases of poxvirus, which is characterized by ring-like, dark-fringed lesions (Geraci et al., 1979), were suspected in ULM dolphins in association with pallor. Hypopigmentation and hyperpigmentation on the same dolphin suggest a potential association between these lesion types. Dark lesions are precursors to pale lesions (Wilson et al., 2000). More pathological studies of biopsied tissue are needed to help clarify unknown etiology and elucidate the significance of associations between lesion types.

The proportion of dolphins encountered with lesions in MS and ULM was significantly higher than that observed in RB. However, more dolphins were encountered than were included in analyses since photographs of both the left and right side of an unaffected dolphin were needed to exclude the presence of lesions. The proportion of skin disorders reported for each stock may thus reflect an underestimation of true lesion prevalence based on highly selective photograph criteria. MS samples from the winter of 2013 were collected from dolphins five months after Hurricane Isaac during which southern Mississippi incurred > 10 inches of rain (Berg, 2013) and salinity levels were low. In contrast, salinity levels in RB and ULM increased during the fall and winter when skin disorders were most prevalent and water temperatures were lowest. Previous studies have reported a similar relationship between lesion occurrence and low water temperatures (Wilson et al., 1999; Hart et al., 2012), suggesting dolphin susceptibility to viable pathogens and disease may be heightened in cold waters. Cold water exposure may limit blood flow to the skin and impede immune protection, reducing epidermal cell regeneration (Feltz & Fay, 1966). Additionally, cold water may disrupt and shift the microbial community

inhabiting dolphin skin, rendering dolphins more susceptible to lesions and secondary disease (Carmichael et al., 2012).

Metabolic and genetic analyses of biopsied dolphin tissue are needed to better understand the physiological condition of free-ranging populations. For example, an untargeted evaluation of dolphin skin or blubber could reveal major metabolic pathway changes and metabolite segregation associated with specific environments (Suzuki et al., 2018). Untargeted analyses may also reveal the presence of accumulated toxic compounds that may be site-specific and of concern to local marine and human health. Assessing the skin microbiome of coastal dolphins using genetic sequencing can identify microbial species associated with terrestrial runoff and potentially localized disease (Russo et al., 2018). Since the microbiome is sensitive to changes in water chemistry, high salinity environments may support a unique microbial community that is conducive to lesion proliferation. Microbial skin assessments may also contribute to the determination of unknown etiologies of certain lesion types in small cetaceans. Improved characterization of lesions and their underlying sources would greatly enhance our understanding of local environmental stressors and further warrant the utilization of bottlenose dolphins as bioindicator species of marine environmental conditions.

Persistent organic pollutants (POPs) are ubiquitous in the marine environment and become concentrated through food webs. Dolphins are particularly susceptible to POP accumulation due to their long lifespan, large lipid reserves, high trophic position, and frequent strong site fidelity (Hickie et al., 2013), characteristics that also designate them as bioindicator species. POP burdens of individuals are often compounded by life history status, including size, nutritive condition, age, and prey selectivity (Aguilar et al., 1999). Diet thus plays a large role in contaminant exposure. Unprecedented levels of POPs continue to be reported in dolphins

residing in MS and coastal Texas (Kuehl & Haebler, 1995; Meador et al., 1999; Kucklick et al., 2011; Phillips & Rosel, 2014; Balmer et al., 2015, 2018; Smith et al., 2017). Legacy chemicals (i.e., polychlorinated biphenyls, polycyclic aromatic hydrocarbons) are endocrine disruptors linked to immune suppression in bottlenose dolphins (Schwacke et al., 2011), and have been found in individuals inhabiting MS and RB (Kucklick et al., 2011; Phillips & Rosel, 2014). Compromised immunity provides a direct opportunity for bacteria and fungi to invade host organisms (Wilson et al., 1997; Murdoch et al., 2010) and may be the precursor to severe cases of lesion outbreaks and disease in dolphins. Future studies of MS, RB, and ULM dolphin stocks are warranted to quantify POP concentrations and investigate the contribution of contaminant exposure to the abundant proliferation of lesions observed. Despite the inherent presence of 'stress' in a polluted environment, dolphin site fidelity likely reflects the perception of external conditions and overriding behavioral motivations for survival such as high prey availability, as found for dolphins in RB despite heavy vessel utilization (E. Mills unpublished data) and other stocks in areas following heavy oil spill events (Shane & Schmidly, 1978; Gruber, 1981; Smultea & Würsig, 1995).

Remotely biopsied blubber samples and images of free-ranging common bottlenose dolphins were utilized in this study to assess dolphin physiological condition across a natural salinity gradient. Steroid hormones were quantified and a relationship between cortisol and salinity was observed, potentially indicating increased stress at high salinities. Testosterone and progesterone exhibited seasonal patterns consistent with natural mating behaviors. Steroid hormones were consistent across the 10 years studied in RB, suggesting baseline levels can be established for this stock. Aldosterone was not detected in this study, and the role of aldosterone in response to high salinity exposure remains unclear. Skin lesions were negatively related to

salinity as expected and lesion prevalence was highest when dolphins were exposed to cold water, indicating enhanced lesion susceptibility under variable water conditions. This study provides evidence that high salinity may impose physiological challenges on bottlenose dolphins and warrants further exploration of the endocrine response and epidermal condition of dolphins inhabiting high saline environments.

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APPENDIX A: ANALYTICAL PARAMETERS FOR HORMONE STANDARDS.

Hormone	Molecular formula	Adduct	Precursor Ion (m/z)	Charge	Retention time (min)	LOD (ppb)	LOQ (ppb)
Aldosterone	C21H28O5	[M + H]	361.2010	+	11.85	3.12	5.0
Aldosterone ^{13}C	C18C'3H28O5	[M + H]	364.2110	+	11.85	-	-
Corticosterone	C21H30O4	[M + H]	347.2217	+	14.85	0.612	1.0
Cortisol	C21H30O5	[M + H]	363.2166	+	13.1	0.644	1.0
Cortisol D_4	C21H26H'4O5	[M + H]	367.2110	+	13.1	-	-
Cortisone	C21H28O5	[M + H]	361.2010	+	13.18	1.0	1.0
Progesterone	C21H30O2	[M + H]	315.2319	+	20.75	1.0	1.0
Progesterone ^{13}C	C18C'3H30O2	[M + H]	318.2419	+	20.75	-	-
Testosterone	C19H28O2	[M + H]	289.2162	+	16.8	0.1	0.1
Testosterone ^{13}C	C16C'3H28O2	[M + H]	292.2263	+	16.8	-	-

Isotopically labeled and non-labeled standards included for respective hormones. .

APPENDIX B: COMPARISON OF ANALYTE EXTRACTION EFFICIENCY.

Analyte	Milli-Q Water (n=15)		Acetonitrile (n=9)	
	Recovery (%)	Precision (%)	Recovery (%)	Precision (%)
Aldosterone				
50 mg	47.98	13.41	61.75*	7.72
150 mg	68.76*	21.71	51	4.82
400 mg	54.78	22.84	52.99	9.16
Cortisol				
50 mg	68.44	20.24	87.54*	8.68
150 mg	99.23*	31.72	74.54	7.64
400 mg	75.17	35.73	75.24	9.77
Testosterone				
50 mg	27.10	4.07	83.23*	11.63
150 mg	24.38	9.10	70.96*	2.22
400 mg	11.43	2.58	86.42*	17.67
Progesterone				
50 mg	0.18	0.04	42.65*	4.93
150 mg	0.33	0.02	36.18*	2.63
400 mg	1.04	0.03	39.90*	15.05

Asterisks (*) indicate significantly higher extraction in the respective solvent ($p < 0.05$).

APPENDIX C: DESCRIPTIVE PARAMETERS FOR SAMPLES WITH DETECTABLE AND QUANTIFIABLE HORMONES.

Dolphin ID	Sex	Site	Season/ Year	Lesion (Y/N)	Salinity ppt	Water Temp °C	F ng/g	B ng/g	T ng/g	P4 ng/g
12248	M	RB	SU ‘12	N	33.4	31.3		1.07		
12268	M	RB	SU ‘12	N	33.4	31.3	9.62			
7064	M	RB	SU ‘12	N	33.5	30.8		1.01		
12063	M	RB	SU ‘12	N	33.3	29.6		< LOQ		
12266	F	RB	SU ‘12	N	36.3	28.3	11.24			
6	F	RB	SU ‘12	N	35.6	29.2	11.20			
UNKN	M	RB	SU ‘12	N	33.4	31.3			1.41	
228	F	MS	SU ‘13	N	18.3	30	9.32			
211	F	MS	SU ‘13	N	16.6	28.5		< LOQ		1502.9
202	F	MS	SU ‘13	N	15.5	27.8		< LOQ		
116	F	MS	SU ‘13	N	20.9	29.2				1252.9
155	M	MS	SU ‘13	N	15.7	29.6			3.95	
6429	M	RB	SU ‘13	N	33.5	29.6	11.30			
20265	M	RB	SU ‘13	Y	33.5	29.6	10.47		21.69	
12024	M	RB	SU ‘13	N	33.5	29.6			4.10	
187	M	MS	SU ‘13	N	15.8	28.8			8.67	
171	F	MS	SU ‘13	N	15.7	27.8	9.63			
116	F	MS	SU ‘13	N	19.9	28.8		< LOQ		722.58

UNKN	F	MS	SU ‘13	N	18.6	30.3	9.97			
91	M	MS	WI ‘13	Y	8.6	13.5			63.51	
76	F	MS	WI ‘13	Y	10.2	12.4				522.56
53	M	MS	WI ‘13	N	9.1	12.8		< LOQ	24.87	
19	M	MS	WI ‘13	N	10.8	11.8			48.74	
UNKN	M	MS	WI ‘13	N	6.8	14.2			19.05	
7542	M	RB	SU ‘14	N	34	29.3			2.86	
7468	F	RB	SU ‘14	Y	34	29.1		< LOQ		
6635	M	RB	SU ‘14	N	33.6	28.8	10.14			
388	M	RB	SP ‘22	N	31.5	27.6			1.75	
399	F	RB	SP ‘22	N	30.22	27				1958.1
1834	M	RB	SP ‘22	N	30.48	27.2		< LOQ		
699	M	RB	SP ‘22	N	30	27.9			57.73	
914	M	RB	SP ‘22	N	29.97	26.9			12.26	
366	M	RB	SP ‘22	N	30.02	27	10.19			
1677	M	RB	SP ‘22	N	30.58	26.8			10.67	
1607	M	RB	SP ‘22	N	27.99	27.2			5.35	
1587	F	RB	SP ‘22	N	28	27				178.10
1576	M	RB	SP ‘22	Y	28.01	26.6		1.02		
1748	M	ULM	SP ‘22	Y	36.64	28.7			2.33	

1738	M	ULM	SP ‘22	N	37.85	28.3			32.29	
1802	M	ULM	SP ‘22	N	36.49	27.6			19.31	
1736	M	ULM	SP ‘22	N	35.71	28.3			8.31	
492	M	RB	FA ’22	N	32.17	25.3			57.33	
2507	M	ULM	FA ’22	N	40.81	26	10.39			
2553	M	ULM	FA ’22	Y	39.28	18.5			28.23	

UNKN corresponds to an unidentified dolphin ID. RB, MS, and ULM correspond to Redfish Bay, Mississippi Sound, and Upper Laguna Madre, respectively. SP, SU, FA, and WI correspond to spring, summer, fall, and winter, respectively. Hormone abbreviations are F (cortisol), B (corticosterone), T (testosterone), and P4 (progesterone). Hormones which were detected but not quantified are denoted as < LOQ.