UNIVERSITY OF CALIFORNIA
SANTA CRUZ

CHARACTERIZING ENDOCRINE VARIATION IN NORTH PACIFIC HUMPBACK WHALES: A BLUBBER-BASED INVESTIGATION ACROSS PHYSIOLOGICAL AND BEHAVIORAL CONTEXTS

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

ECOLOGY & EVOLUTIONARY BIOLOGY

By

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August 2026

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Abstract

Characterizing Endocrine Variation in North Pacific Humpback Whales: A Blubber-Based Investigation Across Physiological and Behavioral Contexts

by
Haley Robb Spears

Hormones provide a direct measure of the physiological state of wild animals. Yet, the natural variation that underlies endocrine biomarkers remains poorly characterized in North Pacific humpback whales (*Megaptera novaeangliae*), a population recovering from commercial whaling while facing increasingly variable ocean conditions. In this dissertation, I characterized variation in reproductive steroids and glucocorticoids measured from minimally invasive skin–blubber biopsies collected on the Hawai‘i breeding grounds (2014–2025) and the Southeast Alaska feeding grounds (2023–2025), spanning sex, reproductive class, season, social context, and region.

In male humpback whales (Chapter 1), androgens and progesterone were elevated on the breeding grounds, and androgens declined across the breeding season, consistent with males arriving in a heightened reproductive state and exhibiting reduced androgen production as the breeding season progressed. Reproductive hormone profiles did not differ among males occupying different social roles on the breeding grounds, indicating that endocrine profiles reflect broader reproductive physiology than short-term breeding behavior. In females (Chapter 2), progesterone identified

presumed pregnant females on the feeding grounds, while progesterone and estrone differentiated mothers from females without calves on the breeding grounds. Multihormone profiles further distinguished these reproductive classes, demonstrating that coordinated endocrine variation provides greater insight into reproductive physiology than individual hormones alone. For glucocorticoids (Chapter 3), cortisol was the predominant hormone measured in blubber and was elevated on the breeding grounds. Seasonal glucocorticoid trajectories differed among reproductive classes, with mothers maintaining relatively constant concentrations while females without calves declined across the breeding season. In males, cortisol was positively associated with testosterone and varied with social-group composition.

Across all three chapters, reproductive state and seasonal timing—rather than sex or social role—were the primary drivers of endocrine variation. Taken together, this work establishes a multihormone framework for interpreting endocrine variation in North Pacific humpback whales and providing the biological context needed to interpret endocrine responses to environmental change in this recovering population.

Acknowledgments

This dissertation was made possible through the commitment, collaboration, and support of many people whose contributions have spanned several years. From fieldwork and data collection to laboratory analyses, writing, and revisions, I have been fortunate to have an incredible team supporting me throughout this journey. I am deeply grateful to everyone who contributed to making this work possible.

This work was supported by Whale Trust, with additional funding provided by the Whale Tales Beneficiary Fund, the Hawaiian Islands Humpback Whale National Marine Sanctuary, the American Cetacean Society, Monterey Bay Chapter, the Lewis and Clark Fund for Exploration and Field Research of the American Philosophical Society, and the Department of Ecology and Evolutionary Biology at the University of California, Santa Cruz.

First, I would like to thank my committee, Dan Costa, Ari Friedlaender, Terrie Williams, Nick Kellar, and Logan Pallin, for their expertise, guidance, and support throughout this process. Dan, I am grateful to you for accepting me into the lab, despite it not "doing cetacean work," and for allowing me the flexibility to pursue this degree while traveling back and forth to Maui. I have learned a tremendous amount about ecophysiology from you, which has shaped how I approached this dissertation.

Ari, thank you for welcoming me into your lab and providing the same support and opportunities that you provide your own graduate students. Terrie, thank you for joining my dissertation committee with enthusiasm and kindness. Nick, thank you for laying the foundation for this work through the initial hormone analyses and for generously sharing your expertise throughout this project. Logan, thank you for becoming the person I turned to whenever I needed help, for supporting me throughout this degree, and for becoming truly one of my dearest friends.

As this work was done in collaboration with Whale Trust, I want to express my deepest gratitude to the team and my Maui community. Meagan, thank you for making it possible for me to pursue whale research, for guiding me every step of the way, and for being my hānai mom. This project, and ultimately this dissertation, would not exist without you, and I appreciate that you have so willingly taken me under your wing. Your mentorship, support, and knowledge have been critical and mean more to me than I can express. Jim and Flip, thank you for always being advocates for me, for jumping in whenever I have asked for help, and for helping pave the way for my success. Ralph, thank you for your contributions to the science and fieldwork, and for keeping things light and fun on the water. A huge mahalo to the rest of the Whale Trust team, field support, volunteers, and supporters, including Linda Nicklen, Renee and Winston Warr, Annemarie Hoffman, Lorraine Arenberg, Curtis and Julie Robb, Sven and Kristin Lindblad, Yumi Kuwana, the Wanderlust Fund, and the Makana Aloha Foundation, whose dedication to whale research and

education over the past 25 years has made this work possible and established Whale Trust as an enduring presence in Maui.

I would like to thank Debbie Steel at the Hatfield Marine Science Center, Oregon State University, and Elyse Wurster at the Southwest Fisheries Science Center, La Jolla, for their contributions to the laboratory analyses. Pete, thank you for your statistical guidance, and Linn, thank you for your contributions to manuscript preparation and editing.

I would like to thank my lab mates and colleagues in both the Costa Lab and the Friedlaender Lab, as well as the EEB 2020 cohort, for creating such a welcoming and supportive community. I particularly want to express my gratitude to my friends Ryan, Noah, Joe, Victoria, Thompson, and Logan, who have been a family to me and have made my time in Santa Cruz enjoyable and memorable.

Finally, I would like to thank my family. Chase, thank you for your patience, love, support, and for always showing up, sometimes from thousands of miles away, whenever I needed you. I am grateful to our dog, Louie, who has been a constant companion, traveling back and forth with me. To Parker and Tyler for providing levity, not always when asked, but appreciated. Granny, thank you for your encouragement, and Nana, thank you for your generosity and support.

Lastly, I would like to thank my parents, Julie and Curtis, who have gone far above and beyond in their support of me throughout this journey. My dad has captained the research boat for the entirety of this dissertation, maintained the boat and truck, and never hesitated to do whatever was needed. He has generously given his time, skills, and effort without expecting anything in return. My mom has spent many hours as a field assistant and outreach volunteer, cared for Louie whenever I needed her to, provided endless emotional support, and was always available to help in whatever way she could. The success of this dissertation reflects not only years of my own work, but also the countless ways they have supported me behind the scenes. I would not be where I am today without their love, encouragement, generosity, financial support, and belief in me.

Mahalo,

Haley

Dedication

To my parents

whose time, love, and support made this endeavor possible

Introduction

Broad conservation and ecological context

Humpback whales within the North Pacific have experienced tremendous recovery following the cessation of commercial whaling in the 1980s (International Whaling Commission, 1982; Cheeseman et al., 2024; Calambokidis et al., 2008). Touted as a conservation success story, the Hawai‘i breeding population, which represents the largest aggregation of humpbacks in the North Pacific (Calambokidis et al., 2008), was removed from the endangered species list in 2016 (NOAA, 2016). However, during this period of recovery, the North Pacific was undergoing a marine heatwave (2014–2016) colloquially known as “the blob” (Cornwall, 2019; Di Lorenzo and Mantua, 2016), which impacted prey species at higher latitudes with cascading effects on the marine ecosystem (Suryan et al., 2021). Humpback whales were among those affected, with an observed 76.5% decline in mother–calf encounter rates within Hawai‘i in the years during and immediately after the marine heatwave (Cartwright et al., 2019). Similarly, in the Alaskan foraging grounds, non-calf abundance declined by 56%, with severe declines in calf production (0.27 to 0.041) and calf survival (0.396 to 0.032) observed during the same period (Gabriele et al., 2022). These declines highlight the vulnerability of this population to severe weather events and environmental changes that are predicted to increase in severity and frequency with continued climate change (Oliver et al., 2018).

As humpback whale populations continue to recover and face increasingly variable environmental conditions, there is a growing need for tools that can assess individual

physiological condition beyond simple counts of abundance. Hormones provide insight into the underlying physiology, offering an opportunity to understand how individuals are physiologically coping with these changes (Hunt et al., 2013). Steroid reproductive hormones govern key processes including ovarian cyclicity, pregnancy, and lactation, directly linking individual physiology to population-level calving rates and reproductive success (Atkinson et al., 2023a; Kellar et al., 2006; Pallin et al., 2018; Pallin et al., 2022b). Glucocorticoids, in contrast, reflect an individual's energetic state and capacity to respond to internal and external stressors (Atkinson et al., 2015; Reeder and Kramer, 2005). These two hormone systems are not independent, sharing common biosynthetic origins. Collection of these steroid hormones can be done using minimally invasive techniques, such as remote blubber collection (Hunt et al., 2013), providing an opportunity to extend our understanding of this species beyond what was previously possible from examinations of harvested individuals during the commercial whaling era (Chittleborough, 1958, 1965; Dawbin, 1966).

Blubber hormone levels have been shown to vary across demographic and life-history contexts, as well as exposure to stressors (Cates et al., 2019, 2020; Dalle Luche et al., 2020; Kellar et al., 2009, 2013; Pallin et al., 2022a, 2024; Melica et al., 2022; Trana et al., 2016). As a result, characterizing the natural variability that occurs within a population is critical to our understanding of how endocrine profiles are interpreted and their application to monitoring efforts. Despite the growing interest in using remotely collected hormonal data, comprehensive blubber hormone examination across sex and reproductive class remains limited for North Pacific humpback whales.

This dissertation addresses these gaps by characterizing endocrine variation in North Pacific humpback whales across both the Hawai‘i breeding grounds and the Southeast Alaska feeding grounds. Together, these profiles provide a necessary foundation for detecting physiological changes associated with environmental and anthropogenic stressors in this recovering population.

Humpback whale life history, reproductive biology, and study system

As capital breeders, humpback whales temporally separate energy acquisition from reproduction across their annual migratory cycle (Baker et al., 1986; Dawbin, 1966). During the boreal summer, North Pacific humpback whales forage in productive, high-latitude waters, such as those off the coast of Alaska, accumulating blubber reserves that will support reproductive activity during the subsequent breeding season (Baker et al., 1986; Dawbin, 1966). This period of intensive foraging results in substantial gains in body mass (Irvine et al., 2017; van Aswegen et al., 2025a). As boreal winter approaches, the majority of North Pacific humpback whales migrate to low-latitude breeding grounds, such as Hawai‘i, to reproduce (Baker et al., 1986; Calambokidis et al., 2008; Cheeseman et al., 2023). This seasonal shift between intensive foraging and reproductive fasting creates fundamentally different physiological contexts across the annual cycle, contributing to the natural variation in reproductive hormones and glucocorticoids investigated in this dissertation.

Hawai‘i represents the largest breeding aggregation of North Pacific humpback whales (Calambokidis et al., 2008; Cheeseman et al., 2023) and serves as the primary study site for this dissertation. Biopsy samples were collected in the Au‘au, Pailolo,

and Kalohi channels during the boreal winter breeding season (December–April) from 2014–2025. Individual whales were identified using ventral fluke photographs submitted to Happywhale, which provided minimum age estimates, sighting histories, and calving histories where available (Cheeseman et al., 2023). To provide a regional comparison between breeding and feeding ground hormone expression, additional samples were collected in Southeast Alaska (Chatham Strait) during the boreal summer feeding season (July) from 2023–2025. Together, sampling across both sites provides a seasonal comparison of hormone variation across the annual migratory cycle of North Pacific humpback whales.

In Hawai‘i, male humpback whales occupy a range of social roles, including singing alone, escorting females, and competing in multi-male groups for access to females (Darling et al., 1983; Darling and Bérubé, 2001; Tyack and Whitehead, 1983). These roles are not fixed — males may alternate between singing, escorting, and competing over short timescales, suggesting a flexible, context-dependent breeding strategy (Darling and Bérubé, 2001; Dunlop and Frere, 2023). The function of song remains debated, though it has been proposed to serve as a mechanism for organizing males within the breeding grounds rather than directly attracting females (Darling et al., 2006). Behavioral context, female reproductive status, and individual condition may all influence which strategy a male employs at a given time, as males have been shown to preferentially compete for larger females (Pack et al., 2009). The mating system is presumed to be polygynous (Cerchio et al., 2005), though copulation has never been formally documented in this species (Clapham, 1996). Given the ephemeral

and flexible nature of male social roles, in which individuals may occupy multiple strategies over short timescales, discrete hormonal differences among roles may not be expected — a prediction explored and supported by the findings of Chapter 1.

In contrast to males, female humpback whales travel to Hawai‘i to breed and give birth, and are represented by three reproductive classes: immature females, females without calves, and mothers with calves (Darling et al., 1983). Females in the North Pacific typically produce their first calf after the age of eight (Gabriele et al., 2007), with gestation lasting approximately 11 months (Chittleborough, 1958) and calving intervals typically ranging from two to three years (Straley et al., 1994). On the breeding grounds, females are outnumbered by males by approximately 1.8:1 (Herman et al., 2011), such that females are rarely observed without a male escort, except for some mother–calf pairs. Despite the direct relevance of female reproductive state to population dynamics and calving success, hormone profiles across these reproductive classes within the breeding grounds remain poorly characterized in North Pacific humpback whales, a gap that Chapter 2 of this dissertation addresses.

The seasonal and reproductive-class contexts described above influence not only reproductive hormone profiles, but also glucocorticoid dynamics. On the Hawai‘i breeding grounds, where whales are fasting while engaging in energetically costly reproductive behaviors, glucocorticoid concentrations are expected to reflect the combined influence of nutritional state, reproductive class, and social context. Despite growing interest in glucocorticoids as indicators of physiological condition in humpback whales, variation across sex, reproductive class, and season remains poorly

characterized in the North Pacific population (Cates et al., 2020), a gap that Chapter 3 of this dissertation addresses.

Endocrinology as a tool

Steroid hormones provide a particularly informative method for investigating these patterns and characterizing the underlying physiology of humpback whales in their natural environment. Reproductive (progesterone, androgens, and estrogens) and glucocorticoid (cortisol and corticosterone) steroid hormones participate in maturation, reproduction, metabolism, and the stress response, playing a central role in physiological regulation (Atkinson et al., 2015, 2018; Norris and Carr, 2020; Robeck and O'Brien, 2018). As reproductive hormones and glucocorticoids share common upstream precursors, such as progesterone, and are regulated by the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal (HPA) axes respectively, their production is linked through a common biosynthetic pathway (Atkinson et al., 2015, 2018; Norris and Carr, 2020; Reeder and Kramer, 2005; Robeck and O'Brien, 2018; Sapolsky et al., 2000). This dissertation therefore characterizes both reproductive hormones and glucocorticoids across three complementary chapters, providing a more comprehensive assessment of physiological condition than has previously been available for this population.

In female mammals, reproductive hormone concentrations vary across the reproductive cycle. During follicular development, ovarian follicles synthesize estrogens (estradiol and estrone) from androgen precursors, resulting in elevated concentrations before ovulation. Following ovulation, the mature follicle transforms

into the corpus luteum, which secretes progesterone to support implantation and pregnancy maintenance (Norris and Carr, 2020; Pineda, 2003). While elevated estrogens and androgens are generally associated with follicular development and ovulation, these hormones may also increase during pregnancy, resulting in overlap among endocrine profiles across reproductive states (Norris and Carr, 2020; Robeck et al., 2017; Steinman et al., 2021). In males, testosterone and its precursor androstenedione regulate spermatogenesis and reproductive behavior, and have been shown to peak before or during the breeding season in several cetacean species (Cates et al., 2019; Kellar et al., 2009). Progesterone and estrogens are also present in males, where progesterone serves as a precursor in androgen biosynthesis, and estrogens — converted from androgens — play important roles in modulating reproductive physiology, including influencing hypothalamic function and stimulating sexual behavior (Abney, 1999; Norris and Carr, 2020; Pineda, 2003). Because hormone profiles can overlap across reproductive states, evaluating multiple hormones simultaneously provides a more comprehensive assessment of reproductive condition than relying on any single biomarker. The biosynthetic relationships among these five reproductive steroids—progesterone, androstenedione, testosterone, estrone, and estradiol—are summarized in Figure I.1.

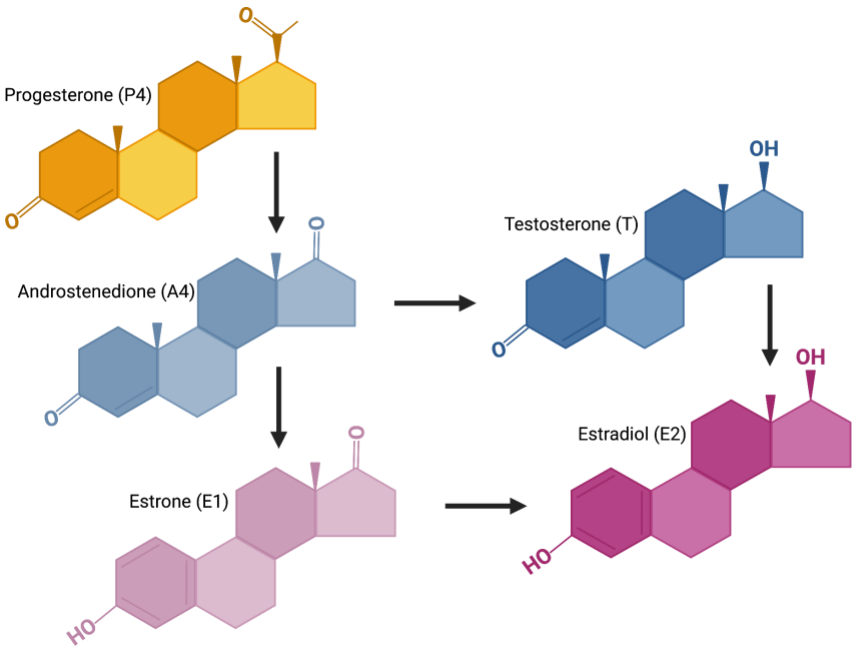

Figure I.1. Simplified biosynthetic pathway of the five reproductive steroid hormones examined in this dissertation. Progesterone (P4) is converted to androstenedione (A4), which gives rise to testosterone (T) and estrone (E1); both testosterone and estrone are converted to estradiol (E2). This shared pathway underlies the reproductive hormone relationships examined in Chapters 1 and 2.

The physiological demands of fasting and reproduction are also supported by glucocorticoids, which play an important role in regulating metabolism and the stress response. Glucocorticoids (cortisol and corticosterone) are steroid hormones released through activation of the hypothalamic–pituitary–adrenal (HPA) axis in response to increased physiological demand (Norris and Carr, 2020; Reeder and Kramer, 2005). In addition to mediating stress responses, glucocorticoids regulate gluconeogenesis, lipolysis, and protein catabolism, thereby supporting energy mobilization during periods of fasting, reproduction, and increased energetic demand (Atkinson et al., 2015; Crocker et al., 2012; Norris and Carr, 2020; Sapolsky et al., 2000). In migratory capital breeders, glucocorticoids may play an especially important role in mobilizing stored energy reserves to support breeding activity (Crocker et al., 2012; Fowler et al., 2016).

Steroid hormones can be measured from several biological matrices in cetaceans, including blood, feces, respiratory vapor, baleen, and blubber (Hunt et al., 2013). However, practical limitations constrain which matrices are suitable for quantifying hormone concentrations in humpback whales. While respiratory blow can detect the presence of hormones, quantification has proven challenging (Burgess et al., 2018; Hunt et al., 2013). Fecal sampling is not practical on the Hawai‘i breeding grounds, where whales are fasting, and blood collection is not feasible in large wild cetaceans. Steroid hormones are derived from cholesterol and are lipophilic, and accumulate within blubber tissue, making remotely collected skin-blubber biopsies a practical and reliable method for quantifying hormone concentrations across the full annual life history of humpback whales (Hunt et al., 2013). Although blubber is metabolically active and capable of locally interconverting steroid hormones (Galligan et al., 2018), numerous studies have validated blubber hormones as biologically meaningful biomarkers in cetaceans, including progesterone as a marker of pregnancy, testosterone as an indicator of male maturation, and cortisol as a measure of physiological stress (Champagne et al., 2017; Kellar et al., 2006, 2009, 2015; Pallin et al., 2018). In humpback whales specifically, progesterone has been validated as a pregnancy biomarker (Atkinson et al., 2023b; Pallin et al., 2018), testosterone and corticosterone have been characterized in North Pacific males (Cates et al., 2019, 2020), and cortisol has been examined in relation to physiological condition and disturbance (Pallin et al., 2022a; Teerlink et al., 2018). Together, these studies established the methodological foundation upon which this dissertation expands by integrating multiple reproductive

hormones and glucocorticoids to investigate endocrine variation across biological contexts.

Knowledge gap

Despite the growing interest in applying endocrinology to population monitoring of cetaceans, critical gaps remain in our understanding of hormone variation in North Pacific humpback whales.

In male humpbacks, previous blubber hormone studies in the North Pacific have focused primarily on testosterone as a single biomarker of reproductive activity (Cates et al., 2019), and while testosterone has also been examined in Southern Hemisphere males in relation to reproductive behavior (Mingramm et al., 2020b), multi-hormone approaches have been applied only during migration rather than within a breeding ground (Dalle Luche et al., 2021). For females, endocrine characterization of reproductive states remains limited beyond the use of progesterone as a biomarker of pregnancy in the feeding grounds (Atkinson et al., 2023a; Dalle Luche et al., 2020; Pallin et al., 2024). More recent studies have begun to expand beyond progesterone to include androgens and estrogens across reproductive states (Dalle Luche et al., 2020; Mingramm et al., 2020b; Pallin et al., 2024), demonstrating that several reproductive hormones vary with reproductive condition and demographic class. However, comparable multi-hormone analyses have not been conducted for female humpback whales in the North Pacific, particularly within breeding-ground populations. For glucocorticoids, previous work in North Pacific humpback whales has been limited to corticosterone in males (Cates et al., 2020) and cortisol in relation to vessel disturbance

(Teerlink et al., 2018), with broader glucocorticoid characterization across sex, reproductive class, and season largely restricted to Southern Hemisphere populations (Dalle Luche et al., 2021; Mingramm et al., 2020a; Pallin et al., 2022a). No study has simultaneously examined both cortisol and corticosterone across sex, reproductive class, and season within a North Pacific population.

Without this integrated understanding of natural hormone variation across both reproductive and stress endocrine axes, detecting or interpreting physiological departures associated with environmental change or anthropogenic disturbance remains limited. Establishing this physiological foundation will allow for improved monitoring of this population.

Dissertation objectives

This dissertation aims to establish a comprehensive endocrine characterization of North Pacific humpback whales across sex, reproductive class, and season, addressing the gaps identified above through three complementary chapters. Together, these chapters build toward a better understanding of endocrine regulation within this population, across both the Hawai‘i breeding grounds and the Southeast Alaska feeding grounds (Figure I.2).

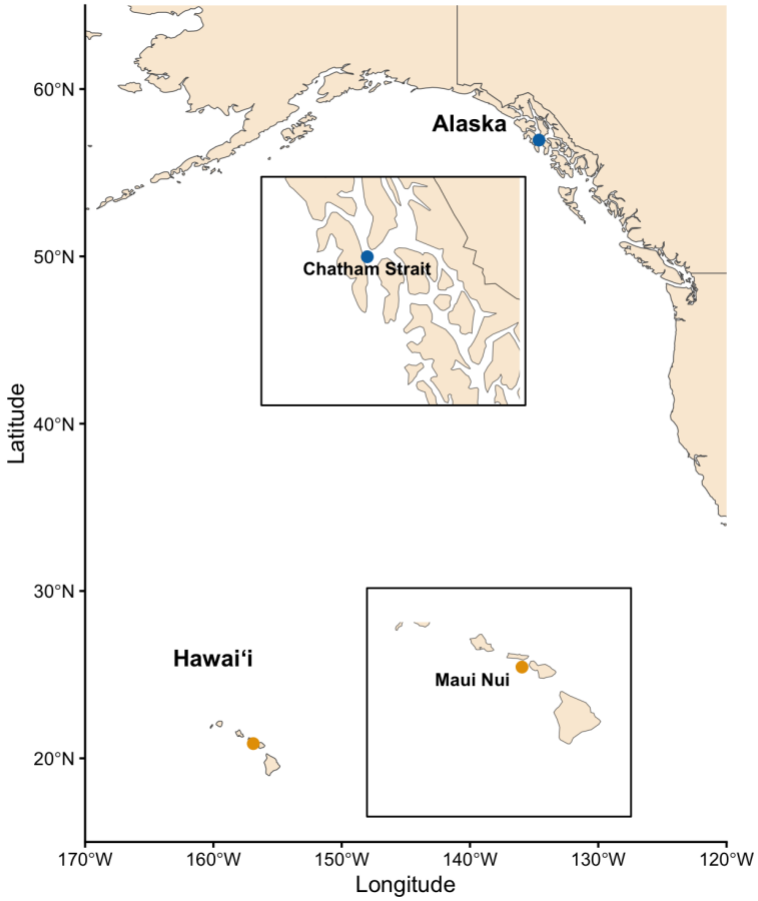

Figure I.2. Map of study sites. Data on humpback whales were collected from the Au‘au, Pailolo, and Kalohi channels of Hawai‘i during the breeding season (December–April) from 2014–2025. Additional data were collected from male humpback whales in Chatham Strait, Southeast Alaska, during July 2023–2025, corresponding to the feeding season.

Dissertation Summary

The three chapters are presented as an integrated examination of endocrine variation in North Pacific humpback whales rather than as independent studies. Chapters 1 and 2 characterize reproductive-hormone variation in males and females, respectively, and Chapter 3 applies the same regional and seasonal sampling framework to the glucocorticoid axis, so that together they describe how a shared

steroid biosynthetic pathway is expressed across sex, reproductive class, season, and region.

Chapter 1 characterizes the reproductive hormone profiles of male humpback whales within the North Pacific, examining how progesterone, androgens (androstenedione and testosterone), and estrogens (estrone and estradiol) vary across the breeding season and between Hawai‘i and Alaska. Additionally, this chapter examines whether reproductive hormone profiles differ among males occupying different social roles — including singing, escorting, and competing — to evaluate whether endocrine profiles reflect the flexible, context-dependent breeding strategies observed in this species. This represents the first multi-hormone characterization of male reproductive physiology conducted within a North Pacific breeding ground.

Chapter 2 similarly characterizes reproductive hormone variation in female humpback whales, examining how progesterone, androgens (androstenedione and testosterone), and estrogens (estrone and estradiol) vary between females without calves and mothers with calves on the Hawai‘i breeding grounds, with a comparison to the Southeast Alaska feeding grounds. Within Alaska, hormone profiles are compared across females without calves, mothers, and pregnant females. By moving beyond progesterone alone to include androgens and estrogens, this chapter provides a more comprehensive picture of female reproductive endocrinology than has previously been available for North Pacific humpback whales, and contributes to our understanding of how reproductive state shapes hormone profiles in humpback whales.

Chapter 3 characterizes glucocorticoid variation (cortisol and corticosterone) across sex, reproductive class, social group composition, and season in North Pacific humpback whales. This chapter examines regional differences in glucocorticoid concentrations between the Hawai‘i breeding grounds and the Southeast Alaska feeding grounds, and evaluates how glucocorticoid profiles vary among reproductive classes and social group compositions within Hawai‘i. This represents the first simultaneous characterization of both cortisol and corticosterone across sex, reproductive class, and season within a North Pacific humpback whale population.

Ethics/Permits

All data presented in this dissertation were collected under National Marine Fisheries Service (NMFS) permit numbers 13846, 19225, and 25987, in accordance with established guidelines.

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Chapter 1: Reproductive hormone trends in male humpback whales during the breeding season

1.1 Abstract

Reproductive hormones regulate breeding behavior and reproductive physiology, yet endocrine patterns in male humpback whales (*Megaptera novaeangliae*) remain poorly understood. This study examined regional, seasonal, and behavioral variation in five reproductive steroid hormones—androstenedione, testosterone, estrone, estradiol, and progesterone—measured in blubber biopsies collected from male humpback whales on the Hawai‘i breeding grounds (n = 178) and Alaska feeding grounds (n = 40).

Androstenedione, testosterone, and progesterone concentrations were higher in Hawai‘i than in Alaska, whereas estradiol concentrations were higher in Alaska, and estrone did not differ between regions. Within Hawai‘i, both androgens declined across the breeding season, while progesterone exhibited a nonlinear U-shaped pattern, decreasing through mid-season before increasing later in the season. Neither estrone nor estradiol varied with sampling date. Correlation analyses revealed a strong positive relationship between androstenedione and testosterone, consistent with known steroidogenic pathways. Despite clear seasonal endocrine trends, neither individual hormone concentrations nor multivariate hormone profiles differed among males occupying different social roles, including lone males, escorts, and males in competitive groups.

These findings indicate that reproductive hormone profiles in male humpback whales vary predictably across seasons and regions but are not strongly associated with social role at the time of sampling. Elevated breeding-season androgen concentrations and their subsequent decline suggest that males arrive on the breeding grounds physiologically prepared for breeding. Establishing endocrine profiles across seasonal and breeding contexts provides a foundation for monitoring reproductive health and assessing population responses to environmental change.

1.2 Introduction

Steroid hormones are increasingly used as biomarkers of health and reproduction in wildlife because they regulate development, reproduction, and metabolism (Hunt et al., 2013). Hormones have been successfully used to assess reproductive status across a wide range of species (Corkeron et al., 2017; Larsen Tempel and Atkinson, 2020; Galligan et al., 2020; Rolland et al., 2005). Interpreting these biomarkers, however, requires an understanding of natural variation in hormone concentrations.

In marine mammals, remotely collected blubber biopsies have greatly expanded opportunities to investigate endocrine physiology in wild populations. Because steroid hormones are lipophilic, blubber provides a minimally invasive tissue for quantifying reproductive hormones and has been widely used to assess reproductive status, maturation, and seasonal reproductive activity (Carone et al., 2019; Kellar et al., 2006; Pallin et al., 2018; Rolland et al., 2005). Increasingly, endocrine measurements are also being integrated with behavioral observations to better understand reproductive

strategies in cetaceans, including humpback whales (Burgess et al., 2012; Carone et al., 2019; Mingramm et al., 2020b).

Humpback whales migrate seasonally from their high-latitude foraging grounds to their low-latitude breeding grounds each year (Chittleborough, 1965; Dawbin, 1966). The water surrounding the Hawaiian Islands is the largest assemblage utilized by the North Pacific humpback population during the boreal winter breeding season (Calambokidis et al., 2008; Cheeseman et al., 2023). Males have been known to stay within the breeding grounds for extended periods (8–11 weeks), likely to maximize mating opportunities (Craig et al., 2001; Darling et al., 1983). Because males may remain on the breeding grounds for several weeks, they likely encounter multiple mating opportunities and may shift reproductive strategies as the availability of receptive females and the level of male competition change throughout the season. On the breeding grounds, males are observed occupying different social roles, including lone males (singing, traveling, or resting), escorting females (with or without a calf), or engaging in competitive groups, where multiple males compete for the principal escort position of a single female (Darling et al., 1983). These social roles among males are ephemeral, as individuals may alternate between singing, escorting, and competing over short time scales (Darling and Bérubé, 2001; Dunlop and Frere, 2023). It remains unclear whether changes in hormonal profiles initiate these behavioral changes.

Although the breeding behavior of male humpbacks in Hawai‘i has been documented (Darling and Bérubé, 2001; Pack et al., 2009; Spitz et al., 2002; Tyack and Whitehead, 1983), few studies have investigated the underlying male reproductive

physiology in live individuals (Cates et al., 2019; Mingramm et al., 2020b). Reproductive hormones regulate changes in male physiology and behavior during the breeding season (Norris and Carr, 2020). In particular, testosterone promotes spermatogenesis and has been shown to peak before the breeding season in several cetacean species, including humpbacks (Cates et al., 2019; Kellar et al., 2009; Vu et al., 2015). Furthermore, high levels of testosterone have been linked to increased aggression and competition amongst other species (Bouissou, 1983; Surbeck et al., 2012). Elevated testosterone concentrations have previously been associated with socially dominant male humpbacks, although this pattern was documented primarily from samples collected along migratory routes rather than on the breeding grounds (Mingramm et al., 2020b). Accordingly, higher androgen concentrations were predicted in males occupying more competitive social roles (e.g., escorts and males in competitive groups) than in lone males and singers, consistent with a role for androgens in regulating reproductive behavior and social competition during the breeding season.

Because multiple steroid hormones regulate different aspects of reproduction (Norris and Carr, 2020), a multihormone approach provides a more comprehensive understanding of male reproductive physiology than testosterone alone. Comparing breeding and feeding habitats establishes endocrine differences associated with reproductive state, while seasonal sampling within Hawai‘i provides insight into physiological changes across the breeding season.

This study measured how several reproductive hormones (testosterone, androstenedione, progesterone, estradiol, and estrone) varied over the breeding season

in male humpback whales within the Hawai‘i breeding grounds, and how these levels compared to hormone concentrations measured during the feeding season in Alaska. Potential differences in hormonal profiles among males occupying different social roles (i.e., lone, singing, escorting females with and without calves, and competing) were examined to evaluate whether reproductive hormone concentrations were associated with breeding behavior. These patterns were assessed using remote skin-blubber biopsy samples collected from males across a range of social roles throughout the breeding season in Hawai‘i, spanning multiple years.

The objectives were to (1) investigate regional trends in two androgens (androstenedione and testosterone), two estrogens (estrone and estradiol), and progesterone between the Hawai‘i breeding grounds and Alaska feeding grounds; (2) assess correlations among reproductive hormones within the breeding season; (3) evaluate intra-seasonal variation in reproductive hormones across the Hawai‘i breeding season; and (4) test whether male reproductive hormone profiles differed among male social roles during the breeding season.

1.3 Methods

1.3.1 Field observations and social-role classification

Biopsy samples from male humpback whales (*Megaptera novaeangliae*) were collected in Hawai‘i (Au‘au, Pailolo, and Kalohi channels; 20.88°N, 156.89°W) during the boreal winter breeding season (December–April) from 2014–2025 (Figure I.2). Each whale was assigned to an age class (immature or mature) based on relative body

size, with immatures defined as individuals larger than a calf but appreciably smaller than full-sized adults. When possible, social role was recorded following the classifications of Darling et al. (1983), and males were categorized into one of six roles (Table 1.1). Of the 178 males sampled in Hawai‘i, 5 were excluded from intraseasonal analysis due to immaturity status and unknown group composition.

Photographs of the ventral flukes of sampled whales were taken for individual identification. Images were uploaded to Happywhale to obtain minimum age estimates from first-sighting dates and to compile sighting histories.

To provide a regional comparison, supplementary biopsy samples were collected in Southeast Alaska (Chatham Strait; 56.95°N, 134.62°W) during July 2023–2025 (Figure I.2). Four additional samples were collected in British Columbia, Canada, on 16 and 28 July 2012. Due to the small sample size and single-year representation, the British Columbia samples were excluded from statistical analyses but are included in the Supplemental Materials.

1.3.2 Biopsy sample collection

Skin/blubber biopsies were collected using a crossbow fitted with a modified bolt and a 40-mm barbed hollow biopsy tip (CetaDart). Remote biopsy sampling is a minimally invasive and widely used technique for studying free-ranging whales, producing only a brief behavioral response and no documented long-term effects on the animal (Weinrich et al., 1992). Samples were taken from the mid-lateral region beneath the dorsal fin. After sampling, the bolt and sample were retrieved from the water within 3 minutes, placed immediately into a sterile vial or bag, and then stored

on ice until returned to shore. For each biopsy, the sample location, time, GPS coordinates, and whale reaction (scored 0–3 following Weinrich et al., 1992) were recorded. Upon return to shore, samples were stored at −20 °C and transferred to −80 °C at the end of each field season.

1.3.3 Genetic sex determination

Skin subsamples (~25 mg) were used for genetic sex determination. Samples collected before 2022 were processed at the NOAA Southwest Fisheries Science Center (SWFSC; La Jolla, CA). DNA from samples collected in 2022 or later was extracted at the University of California, Santa Cruz, using a Qiagen DNEasy kit and subsequently sent to the Cetacean Conservation and Genetics Laboratory at Oregon State University for sex assignment.

1.3.4 Hormone extraction and quantification

Steroid hormone extraction followed a protocol adapted by N. Kellar (SWFSC; pers. comm.) from Kellar et al. (2006). Blubber samples were cut perpendicular to the skin into 0.05–0.1 g subsamples, placed in stainless-steel microvials with a ¼″ ceramic bead, and homogenized in ethanol. Two quality-control samples were created from a stranded humpback whale blubber sample: a negative control with no added hormones and a positive control spiked with known concentrations of progesterone, estradiol, estrone, testosterone, and androstenedione.

Following homogenization, samples underwent sequential ethanol and 4:1 ethanol:acetone washes, followed by centrifugation. The resulting supernatant was

evaporated in a dry bath, and the remaining lipid pellet was weighed. Acetonitrile and hexane were added to separate steroids from remaining lipids; the hexane (lipid) layer was removed, and the steroid-containing acetonitrile phase was dried and reconstituted in PBS buffer with BSA.

Commercial enzyme immunoassay (EIA) kits were used to quantify androstenedione (A4), estrone (E1), 17β-estradiol (E2), progesterone (P4), and testosterone (T) (Table S1.1). Extraction efficiency was calculated as the ratio of the measured positive-control concentration to the negative-control concentration, with efficiencies >60% considered acceptable. Samples exceeding kit detection limits were diluted and reanalyzed; samples below detection limits were assigned a value equal to one-half of the lowest standard to avoid censoring.

To avoid pseudoreplication, only the first biopsy collected from each individual was retained for analysis. Juvenile males were excluded from the main statistical analyses but are summarized relative to mature males in the Supplemental Materials (Table S1.2).

1.3.5 Assay validation

Parallelism and accuracy tests were conducted on the androstenedione and estrone EIAs to assess assay suitability for humpback whale blubber extracts. Validations for progesterone, testosterone, and estradiol were previously completed and are reported elsewhere (Pallin et al., 2018; Pallin et al., 2024).

For parallelism, pooled blubber extract from 10 individuals was serially diluted (neat to 1:64, twofold dilutions) and run in duplicate alongside kit standards. Percent

binding curves for standards and pooled extract were compared to assess parallelism, indicating that the assay detected the target antigens similarly. Accuracy was assessed by regressing observed against expected concentrations for the dilution series and calculating the slope and R^2.

1.3.6 Statistical analysis

All statistical analyses were performed in R (R Core Team, 2025). Statistical support for effects was interpreted using the evidence framework outlined by Muff et al. (2022), where *p*-values were categorized as little or no evidence ($p > 0.1$), weak evidence (0.05–0.1), moderate evidence (0.01–0.05), strong evidence (0.001–0.01), and very strong evidence (≤0.001).

Regional comparisons. Differences in mean hormone concentrations between Hawai‘i (breeding) and Alaska (feeding) were evaluated using one-way ANOVA or Kruskal–Wallis tests when assumptions of normality or homogeneity of variance were not met. Boxplots were generated to visualize regional variation. For four males sampled in both Hawai‘i and Alaska, within-individual regional comparisons are reported in the Results and presented in Figure S1.1 and Table S1.3.

Correlations among hormones. To investigate correlations between hormones, Spearman’s rank correlations were calculated for each pair of log-transformed hormone concentrations, as these data did not meet the assumptions of normality required for parametric correlation. Squared correlation coefficients (R^2) were used to represent the proportion of shared variance between hormone pairs.

Intra-seasonal variation. To evaluate seasonal trends in reproductive hormone values within the Hawai‘i breeding season, linear mixed-effects models were used to model log-transformed hormone concentrations (ng/g). Fixed effects included ordinal date, minimum age (from sighting history), social role (defined as the role occupied within the group at the time of sampling), and an ordinal date^2 term to test for nonlinear patterns. Year was included as a random intercept to account for interannual variation. Model assumptions were evaluated using Q–Q plots and residual diagnostics. Fixed effects were retained based on Type II ANOVA results. Social role and ordinal date were retained in all final models to facilitate consistent evaluation of temporal and social effects across hormones.

Scatterplots were generated to illustrate intraseasonal variation. To visualize general seasonal patterns independent of interannual variation, hormone concentrations were normalized within year (mean-centered and scaled by annual SD). These intra-seasonal trends reflect pooled samples across individuals, with one biopsy collected per individual per season, rather than repeated measurements of the same individuals over the season; only a small number of incidental repeat samples occurred.

Social-role comparisons. Estimated marginal means for each social role were extracted from final linear mixed-effect models, and pairwise comparisons were conducted using Tukey’s Honest Significant Difference tests. Estimated means with 95% confidence intervals were plotted for visualization.

To evaluate multivariate hormone signatures among social-role categories, residuals from linear regressions excluding role were extracted to remove seasonal and

interannual effects. A principal component analysis (PCA) was performed on these residuals, and role differences in multivariate hormone profiles were tested using PERMANOVA on Euclidean distances. Because the PCA required complete data across all five hormones, multivariate analyses were restricted to 130 of the 173 males with complete hormone profiles.

1.4 Results

1.4.1 Assay validation

Androstenedione (A4) and estrone (E1) exhibited strong parallelism and accuracy, demonstrating that both EIAs reliably quantified target hormones in humpback whale blubber extract. Serial dilutions of pooled extract generated curves parallel to kit standards (Figure S1.3a,c). Accuracy assessments revealed strong linear relationships between expected and observed concentrations (A4: $R^2 = 0.9644$, slope = 1.1192; E1: $R^2 = 0.9942$, slope = 0.9877; Figure S1.3b,d).

1.4.2 Regional differences in hormone concentrations

Reproductive hormone concentrations differed between males sampled on the Alaska feeding grounds ($n = 40$) and the Hawai‘i breeding grounds ($n = 178$) (Table 1.2; Figure 1.1). Androgens were higher in Hawai‘i (A4: $F_{1,40.50} = 214.87$, $p < 0.001$; T: $F_{1,218} = 345.01$, $p < 0.001$), as was progesterone (P4: $\chi^2_1 = 43.8$, $p < 0.001$). Estradiol (E2) concentrations were higher in Alaska ($F_{1,179} = 21.69$, $p < 0.001$), whereas estrone (E1) did not differ between regions ($\chi^2_1 = 0.056$, $p = 0.813$).

Within-individual comparisons were available for four males sampled in both Hawai‘i and Alaska. Testosterone was higher in Hawai‘i than Alaska for all four individuals, and androstenedione was higher in Hawai‘i for all three individuals with available data. Progesterone was higher in Hawai‘i for three of the four individuals, whereas estrogen concentrations varied among individuals. Individual hormone profiles are presented in Figure S1.1 and Table S1.3.

1.4.3 Correlations among reproductive hormones within the breeding season

Correlation patterns among the five hormones generally reflected known steroidogenic pathways. Androstenedione and testosterone exhibited the strongest positive association among males ($\rho = 0.67$, $R^2 = 0.45$, $p < 0.001$; Figures 1.3–1.4). Weaker positive correlations were observed among androstenedione, testosterone, progesterone, and estrone ($\rho = 0.24$–0.41, $p \leq 0.002$; Figure 1.2), while estrone and estradiol were also positively correlated ($\rho = 0.30$, $R^2 = 0.09$, $p < 0.001$). All remaining hormone pairings showed little or no evidence of correlation (Figure 1.2).

1.4.4 Intra-seasonal variation in reproductive hormones

Within Hawai‘i, both androgens declined across the breeding season (Table 1.3; Figure 1.4). Androstenedione concentrations decreased with day (logA4 ~ day: -0.0021 ± 0.0010, $t_{160} = -2.18$, $p = 0.031$), equivalent to an estimated ~0.5% reduction per day. Testosterone showed a stronger negative trend (logT ~ day: -0.004 ± 0.0010, $t_{168} = -5.14$, $p < 0.001$), corresponding to nearly a 1% daily decrease (Table 1.3). No evidence of intra-seasonal change was detected for either estrogen measured (logE1 ~

day: 0.0007 ± 0.0009, $t_{153} = 0.81$, $p = 0.422$; logE2 ~ day: −0.001 ± 0.001, $t_{128} = -0.83$, $p = 0.408$). Progesterone exhibited a non-linear pattern consistent with a U-shaped relationship across the season. Concentrations were higher early in the season, declined through mid-season, and increased again late in the season. Both the linear and quadratic day terms contributed to the observed U-shaped pattern (logP4 ~ day: −0.010 ± 0.002, $t_{165} = -5.80$, $p < 0.001$; logP4 ~ day²: 0.0001 ± 0.00002, $t_{164} = 5.29$, $p < 0.001$). Interannual variation accounted for approximately 30%, 11%, and 25% of the variance in androstenedione, testosterone, and progesterone, respectively.

1.4.5 Social-role comparisons of reproductive hormones

The influence of social role on hormonal variation independent of the intraseasonal decline was estimated using the marginal means from the final mixed-effects model. The estimated marginal means showed no differences in reproductive hormone concentrations among the six male role categories (Figure 1.5). Multivariate analyses likewise indicated no distinct hormone profiles associated with social role.

The PCA of residual hormone values showed no visible separation among groups (Figure 1.6), and a PERMANOVA confirmed that social role explained only 3.8% of variation in multivariate hormone profiles ($F_{5,124} = 0.99$, $p = 0.457$). PC1 accounted for 42.2% of the total variance and reflected shared variance across all hormones. PC2 contrasted androstenedione and testosterone with estradiol, while PC3 was driven primarily by progesterone (Table 1.4). Together, these results indicate that male social roles do not correspond to distinct reproductive hormone patterns.

1.5 Discussion

This study characterized variation in multiple reproductive steroid hormones in male humpback whales across breeding and feeding habitats and throughout the Hawai‘i breeding season. Reproductive hormone profiles varied with season, with elevated androgen and progesterone concentrations on the breeding grounds and declining androgen concentrations across the breeding season. In contrast, hormone profiles showed little association with male social role, despite substantial behavioral variation among individuals. Together, these findings suggest that endocrine variation in male humpback whales reflects broader seasonal changes in reproductive investment more strongly than short-term social or behavioral context.

1.5.1 Regional differences

Androgens. Male humpback whales exhibited higher androstenedione and testosterone concentrations in Hawai‘i during the breeding season than in Alaska during the feeding season, consistent with increased reproductive activity on the breeding grounds. Similar seasonal increases in testosterone have been reported in humpback whales (Cates et al., 2019; Dalle Luche et al., 2021; Vu et al., 2015), supporting the interpretation that elevated androgen concentrations reflect heightened reproductive investment during the breeding season. Comparisons of four males sampled in both Alaska and Hawai‘i further supported this pattern, with testosterone and androstenedione concentrations consistently higher in Hawai‘i than in Alaska. Although regional differences in water temperature could potentially influence hormone incorporation into blubber, the consistency of seasonal androgen cycles

reported across multiple humpback whale studies, together with similar seasonal patterns observed in baleen from other whale species (Hunt et al., 2018), suggests that the elevated androgen concentrations observed on the breeding grounds reflect genuine physiological changes rather than a sampling artifact.

Progesterone. Elevated progesterone concentrations during the breeding season in male humpback whales may support androgen synthesis, as progesterone is converted to 17-hydroxyprogesterone and subsequently used in androgen production. Similar patterns of elevated progesterone and androgen concentrations during the breeding season have been reported in male cetaceans, along with positive correlations between progestogens and androgens, suggesting that progesterone may be produced by the testes during this period and serve as a precursor in androgen biosynthesis (Boggs et al., 2019; Dalle Luche et al., 2021).

Estrogens. Estradiol concentrations were higher in Alaska than Hawai‘i, although the biological significance of this pattern remains unclear. In males, estradiol contributes to the regulation of spermatogenesis and androgen production through feedback within the hypothalamic–pituitary–gonadal axis (Abney, 1999; Pineda, 2003), suggesting that seasonal changes in reproductive physiology may contribute to the observed differences. However, the mechanisms underlying elevated estradiol concentrations during the feeding season remain uncertain and warrant further investigation.

In contrast, estrone concentrations did not differ between regions. This finding contrasts with Dalle Luche et al. (2021), who reported higher estrone concentrations in

male humpback whales sampled along the migratory route toward the breeding grounds in the Southern Hemisphere compared to individuals sampled during the return migration to the feeding grounds, while estradiol concentrations did not differ between migrations. Differences in hormone quantification methodology, as well as sampling along migratory corridors rather than terminal feeding and breeding grounds, may partially explain these discrepancies.

1.5.2 Correlations among hormones

The observed correlation structure generally reflected known steroidogenic pathways (Figure I.1), providing additional support that blubber hormone concentrations capture biologically meaningful endocrine variation in male humpback whales. The strongest relationship was observed between androstenedione and testosterone, which are directly linked through conversion by the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD). Both hormones play important roles in male reproductive physiology and behavior (Burgess et al., 2012; Carone et al., 2019; Chittleborough, 1955; Dixson and Anderson, 2004; Negro et al., 2010). Similar positive relationships between androstenedione and testosterone have been reported in male humpback whales during migration (Dalle Luche et al., 2021) and in bottlenose dolphins during the breeding season (Boggs et al., 2019), suggesting this relationship is conserved across cetaceans.

Weaker positive correlations among progesterone, androstenedione, testosterone, and estrone were likewise consistent with shared steroidogenic pathways. Progesterone serves as a precursor for androgen synthesis through 17α-hydroxyprogesterone, while

androstenedione can be converted to estrone through aromatase activity (Boggs et al., 2019; Dalle Luche et al., 2021). Estrone and estradiol also exhibited a weak positive relationship, reflecting their close biochemical association within estrogen metabolism. Together, these correlations support the interpretation that variation in blubber hormone concentrations reflects underlying reproductive endocrine processes.

1.5.3 Intra-seasonal trends

Male humpback whales exhibited clear intra-seasonal variation in reproductive hormones during the Hawai‘i breeding season, with declining androgens, a U-shaped progesterone pattern, and stable estrogens.

Androgens. Among the androgens, both androstenedione and testosterone concentrations declined throughout the breeding season, with testosterone exhibiting a more pronounced decrease. Although known immature males were excluded from analysis (Table S1.2), some sexually immature individuals may have been present in the dataset. To evaluate whether the intra-seasonal decline in androgens was driven by an increase of immature males within the dataset towards the latter half of the season, males were grouped by minimum age (≥8 vs. <8 years), based on estimated sexual maturity of humpback whales (Clapham, 1992; Glockner-Ferrari and Ferrari, 1990), and intra-seasonal testosterone trends were compared. Because testosterone values in both groups fell within the range observed for known mature males (≥8 years), the testosterone decline is unlikely due to a change in age composition within the dataset (Figure S1.2). Furthermore, immature males are often among the first to arrive in Hawai‘i (Craig et al., 2003; Darling, 1983), making it less likely that the decline in

androgens towards the end of the season resulted from sampling younger individuals. The trend, therefore, likely represents a decrease in androgen production within individuals towards the end of the season, and is supported by other studies on male humpback whales (Cates et al., 2019; Dalle Luche et al., 2021; Mingramm et al., 2020b).

Although declining testosterone would be expected to affect sperm production, a temporal lag likely exists between testosterone influx and spermatogenesis. In seasonal breeding cetaceans, peak testosterone concentrations have been observed before periods of breeding activity (Cates et al., 2019; Vu et al., 2015; Schroeder and Keller, 1989). Moreover, studies of captive killer whales, which are not seasonal breeders, have documented multiple annual peaks in serum testosterone without corresponding changes in sperm density (Robeck and Monfort, 2006). These findings suggest that males arrive on the breeding grounds already reproductively primed. Given the extended residency of males on the breeding grounds despite the energetic costs of fasting, mating opportunities are likely available throughout much of the breeding season rather than being restricted to a discrete breeding window, and males remain reproductively active even as androgen concentrations decline.

Progesterone. Progesterone declined early in the breeding season and increased slightly later in the season. This pattern may reflect the use of progesterone as a precursor to androgen production during peak reproductive activity, followed by reduced demand as the breeding season progresses (Pineda, 2003; Norris and Carr, 2020). Similar seasonal patterns were observed for cortisol and corticosterone in male

humpback whales (Chapter 3), suggesting that progesterone may also contribute to glucocorticoid synthesis. As a precursor to both reproductive and stress-related steroids, seasonal changes in progesterone may reflect shifts in multiple steroidogenic pathways. A comparable overall decline in progesterone has been reported in male humpback whales during migration in the Southern Hemisphere (Dalle Luche et al., 2021), although within-season trends on the breeding grounds have not previously been described.

Estrogens. No intraseasonal trends were observed for estrogens, which is consistent with their more limited role in male reproductive physiology during the breeding season. Likewise, Dalle Luche et al. (2021) found no differences in estradiol concentrations between migration to and from the breeding grounds, although they reported higher estrone concentrations during migration toward the breeding grounds.

1.5.4 Social-role comparisons

There was no evidence of differences in hormone levels among male social roles, nor was there a distinct multivariate hormone profile associated with role. A previous study reported higher testosterone concentrations in principal escorts than in secondary escorts or singers, supporting the challenge hypothesis that dominant males exhibit elevated androgen concentrations (Mingramm et al., 2020b). However, most samples in that study were collected during migration, and only a limited number were obtained on the breeding grounds. Similarly, Dalle Luche et al. (2021) reported inconsistent differences among social groups during migration, with patterns varying between

migratory phases. Interpretation was further limited because the maturity status of sampled males was unknown.

In the present study, males were classified by social role rather than individual rank because no differences in hormone concentrations were detected among principal escorts, secondary escorts, and challengers within competitive groups. If males consistently maintained distinct mating strategies (e.g., singing, escorting, or competing), differences in endocrine profiles might be expected, particularly given the association between androgens and competitive behavior in other species (Negro et al., 2010; Surbeck et al., 2012). However, the absence of hormonal differences among social roles, combined with observations of males readily switching between roles and strategies, sometimes within a single day (Darling and Bérubé, 2001; Dunlop and Frere, 2023), is consistent with a flexible breeding system. Alternatively, any hormone–behavior associations may have been obscured by the temporal integration of hormones within blubber, such that hormone concentrations at the time of sampling may reflect physiological conditions from days or weeks earlier rather than the individual's immediate social role.

1.5.5 Limitations

While this study characterizes population-level patterns in male reproductive hormones, it does not capture the individual-level variability likely associated with differences in arrival timing, residency duration, and short-term endocrine fluctuations. Because large numbers of whales move through the Hawai‘i breeding grounds each

season, determining when individuals arrive and how long they remain within the study area is challenging, making it difficult to account for these sources of variation.

Although minimum age derived from sighting histories was included as an explanatory variable, it was not retained in the final models for any hormone. Previous studies have reported age-related differences in hormone concentrations in humpback whales (Cates et al., 2019, 2020) and other cetaceans (O'Brien et al., 2017), suggesting that age may still contribute to endocrine variation. The lack of evidence observed here may reflect uncertainty associated with minimum-age estimates derived from photo-identification histories. Additional sources of variability, including size and body condition, may also contribute to observed differences in hormone concentrations, but were not measured in this study.

Finally, the hormonal perfusion rate from the blood to the outer blubber layers, where biopsy samples are collected, is not fully understood and is difficult to validate in large, wild cetaceans. Current understanding is derived primarily from studies of captive odontocetes, suggesting this process may occur over timescales ranging from tens of minutes to several hours (Champagne et al., 2018). Consequently, hormone–behavior relationships may exist but remain difficult to detect given uncertainty in blubber hormone integration times and the highly dynamic social behavior exhibited by males on the Hawai‘i breeding grounds.

1.5.6 Future directions

Building on these findings, future studies should prioritize repeated sampling of known individuals to better characterize within-individual variation in hormone

profiles and evaluate how endocrine patterns relate to reproductive strategies and social behavior over time. The present study was largely limited to a single biopsy per individual per season, and repeated sightings in Hawai‘i are uncommon due to the large number of whales using the breeding grounds (Cheeseman et al., 2023). Although satellite tags could facilitate resampling of individuals over time, their use must be balanced against potential impacts on the animal (Gulland et al., 2024; Guzmán and Capella, 2017). Because the timescale over which hormones are incorporated into blubber remains poorly understood, blubber biopsies are also unlikely to capture rapid endocrine changes associated with shifts in social role occurring within a single day. Consequently, alternative approaches with higher temporal resolution, such as blow sampling, may provide additional insight into short-term hormone–behavior relationships, despite current challenges associated with variable sample dilution (Burgess et al., 2018).

Integrating hormone measurements with emerging technologies such as unmanned aircraft systems (UAS) would further strengthen the interpretation of endocrine profiles. UAS-derived body measurements can be used to estimate age class and body condition (van Aswegen et al., 2025), while aerial imagery can facilitate individual identification and longitudinal sighting histories (Evans et al., 2026). Such information would improve our ability to distinguish maturation classes, evaluate the influence of body condition on hormone concentrations, and better understand the physiological drivers of reproductive behavior.

1.6 Conclusions

Reproductive hormone profiles in male humpback whales varied predictably across breeding and feeding habitats and changed throughout the Hawai‘i breeding season but showed little association with male social role. Elevated androgen and progesterone concentrations on the breeding grounds, together with declining androgen concentrations across the breeding season, are consistent with males arriving in Hawai‘i physiologically prepared for reproduction and gradually reducing reproductive investment as the season progresses. In contrast, the absence of endocrine differences among males occupying different social roles suggests that reproductive hormone profiles reflect broader seasonal reproductive investment more strongly than short-term behavioral context, despite the dynamic social interactions observed on the breeding grounds.

By characterizing five reproductive hormones simultaneously, this study expands beyond previous work focused primarily on testosterone and provides a more comprehensive understanding of male reproductive physiology and steroidogenic pathways. The successful application of minimally invasive blubber biopsies further demonstrates the value of multihormone endocrine profiling for investigating reproductive physiology in large whales. Together, these findings characterize natural endocrine variation in male humpback whales and provide a framework for future studies examining reproductive health, population monitoring, and how environmental change may influence reproductive physiology and long-term population resilience.

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1.8 Tables

Table 1.1. Social-role classification of breeding humpback whales sampled in Hawai‘i (2014–2025).

Social group class	Social role description	Sample size
Lone	Lone non-singing male	7
Singer	Mature male singing humpback whale song	16
Escort to mother	Male escorting a mother–calf pair	48
M/F pair	Male escorting a female without a calf	20
Pair unknown	Male in unknown pair composition, either male–male or male–female	14
Competitive group	Male in a competitive group, typically with a single female	68

Table 1.2. Mean ± SD and range of regional hormone concentrations (ng/g) for male humpback whales sampled in Hawai‘i and Alaska. Androgens: A4, T; estrogens: E1, E2; progesterone: P4. Ranges are shown in italics.

Region	A4	T	E1	E2	P4
Alaska (n = 40)	0.82 ± 0.61 *(0.019–2.11)*	0.16 ± 0.12 *(0.050–0.677)*	0.11 ± 0.07 *(0.014–0.32)†*	0.45 ± 0.31 *(0.017–1.27)*	0.17 ± 0.10 *(0.037–0.376)‡*
Hawai‘i (n = 178)	19.13 ± 24.29 *(1.87–166)*	1.71 ± 1.33 *(0.134–10.4)*	0.11 ± 0.08 *(0.010–0.433)*	0.23 ± 0.26 *(0.008–1.50)*	0.48 ± 0.30 *(0.114–1.66)*

‡ Progesterone in Alaska was quantified for n = 25.

Table 1.3. Linear mixed-effects model results for the effect of sampling date on log-transformed hormone concentrations. Estimates are back-transformed to the corresponding daily percent change. Year was included as a random intercept.

Term (Day)	$\log_{10}$ estimate	SE	df	*t*	*p*	Fold-change	% difference
Androstenedione	−0.002	0.001	160.123	−2.175	0.031	0.995	−0.488
Testosterone	−0.004	0.001	168.263	−5.139	<0.001	0.991	−0.929
Estrone	0.001	0.001	153.136	0.805	0.422	1.002	0.168
Estradiol	−0.001	0.001	128.451	−0.830	0.408	0.997	−0.256
Progesterone (Day)	−0.010	0.002	164.947	−5.803	<0.001	0.977	−2.328
Progesterone (Day^2)	0.0001	0.00002	163.723	5.288	<0.001	1.000	0.024

Table 1.4. Principal-component loadings for the five reproductive hormones (A4, T, E1, E2, P4) from the PCA of residual hormone values. The first three components together explain 80% of the total variance.

Hormone	PC1	PC2	PC3
Androstenedione (A4)	−0.479	0.514	0.288
Testosterone (T)	−0.523	0.415	−0.094
Estrone (E1)	−0.484	−0.315	0.201
Estradiol (E2)	−0.302	−0.608	0.479
Progesterone (P4)	−0.414	−0.308	−0.799

1.9 Figures

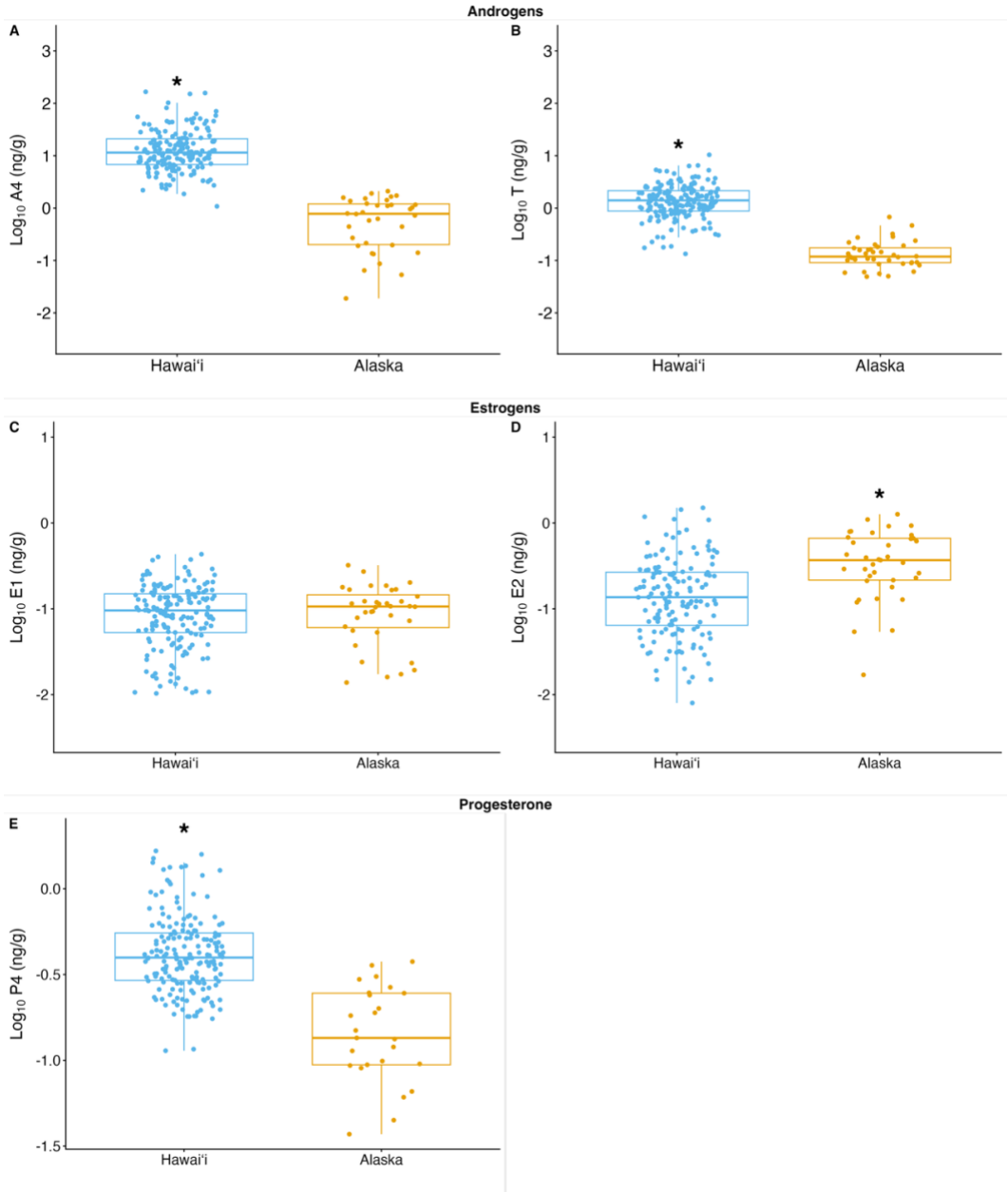


Figure 1.1. Regional differences in androstenedione (A), testosterone (B), estrone (C), estradiol (D), and progesterone (E) concentrations in male humpbacks sampled in Hawai'i (breeding region; 2014–2025) and Alaska (feeding region; 2023–2025). *P*-values < 0.05 are denoted by an asterisk (*).

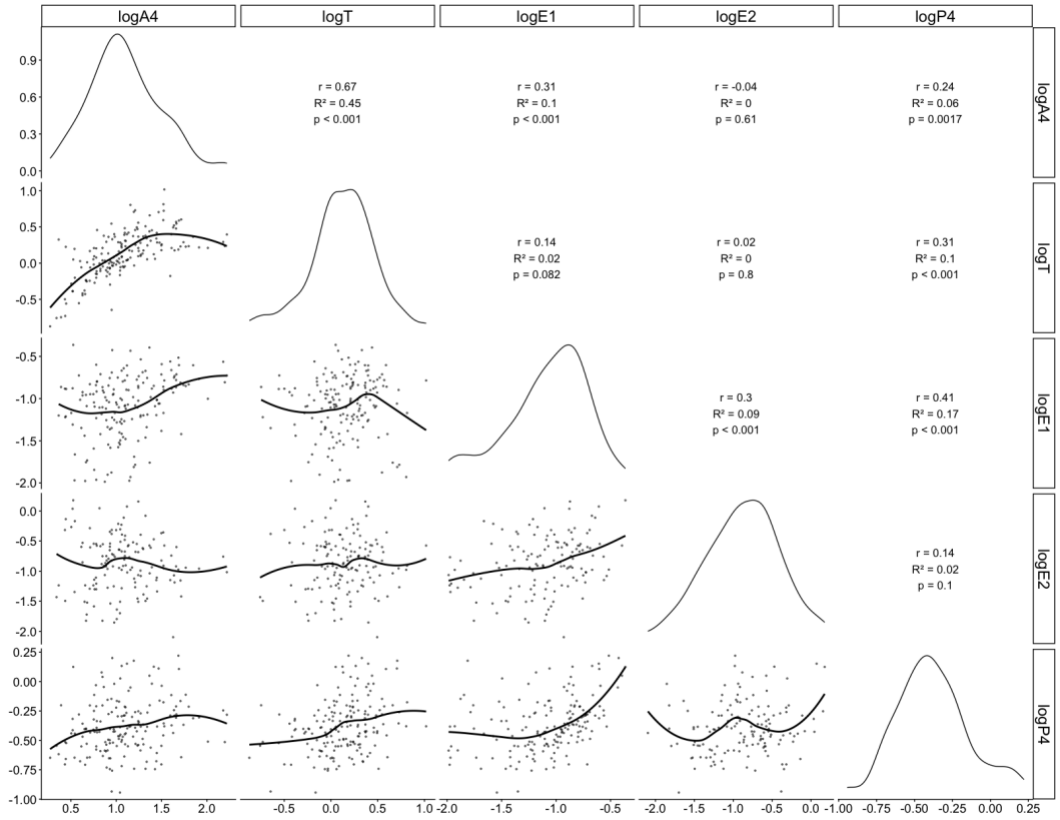


Figure 1.2. Pairwise correlations among log-transformed steroid reproductive hormone concentrations in adult male humpbacks sampled in Hawai‘i during the breeding season between 2014 and 2025. Upper-right panels display Spearman correlation coefficients (r), associated R^2, and p-values. Lower-left panels show LOESS-smoothed scatterplots. Diagonal panels depict density distributions for each hormone.

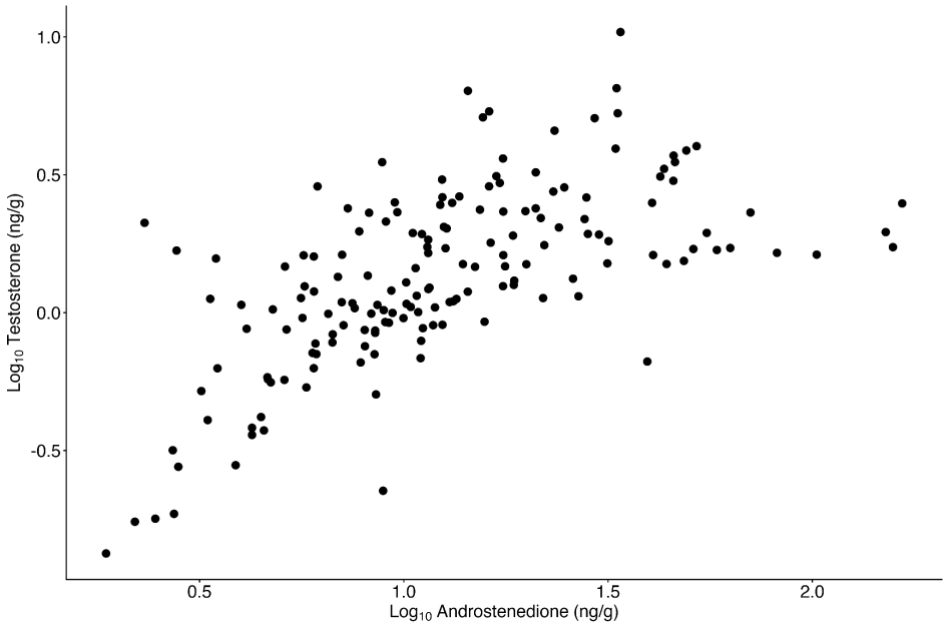


Figure 1.3. Relationship between log-transformed androstenedione and testosterone concentrations in male humpback whale blubber samples from the Hawai‘i breeding season (Spearman's $\rho = 0.67$, $R^2 = 0.45$, $p < 0.001$).

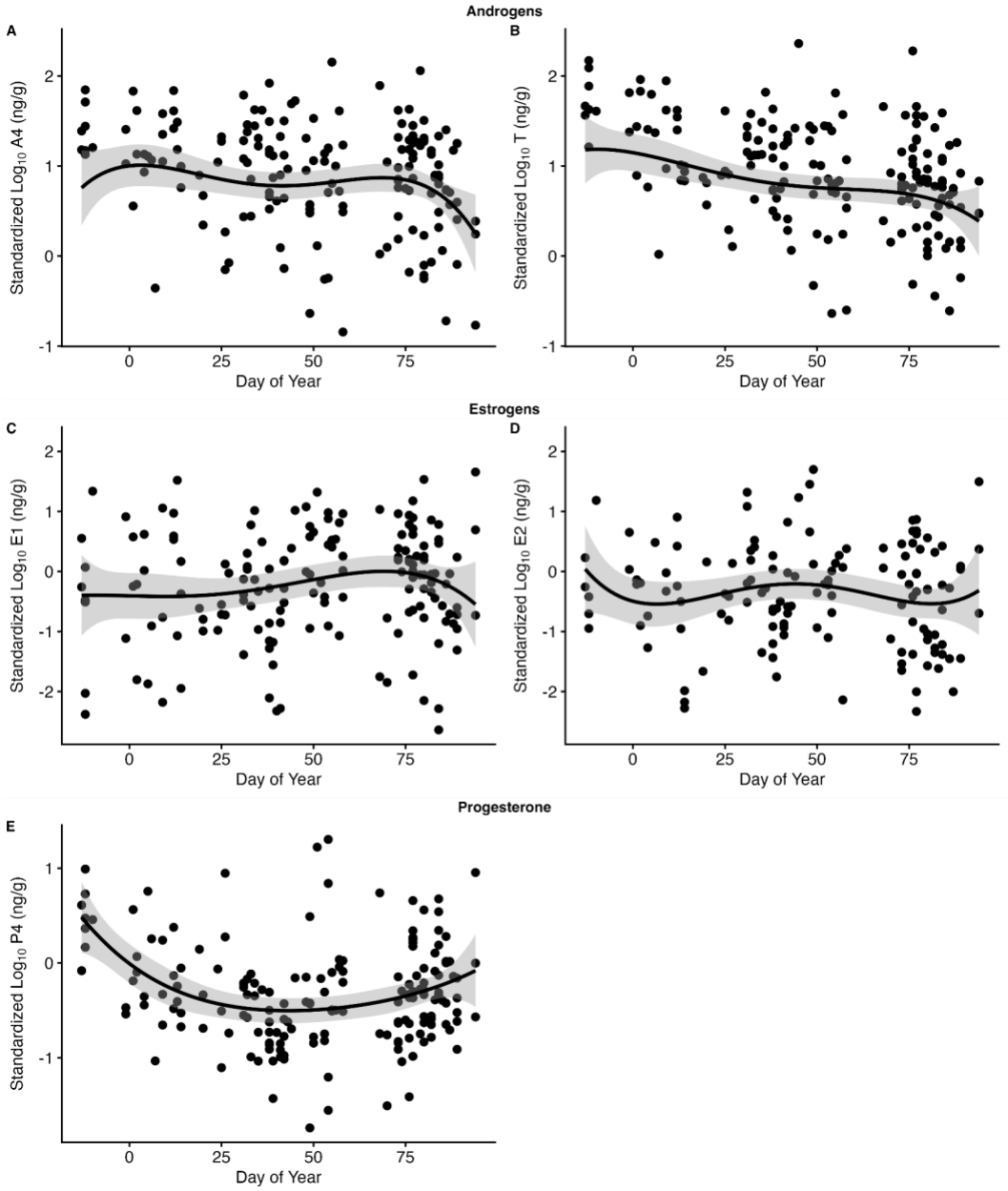


Figure 1.4. Intra-seasonal variation in steroid reproductive hormones from male humpback whale blubber collected in Hawai‘i between 2014 and 2025. Years were standardized to reduce inter-annual variability to better visualize trends. The x-axis represents sampling day within the season (Day 0 = December 17). Shaded areas represent 95% confidence intervals. Androgen (A, B) concentrations show a temporal decline across the breeding season; progesterone (E) exhibits a U-shaped intraseasonal pattern.

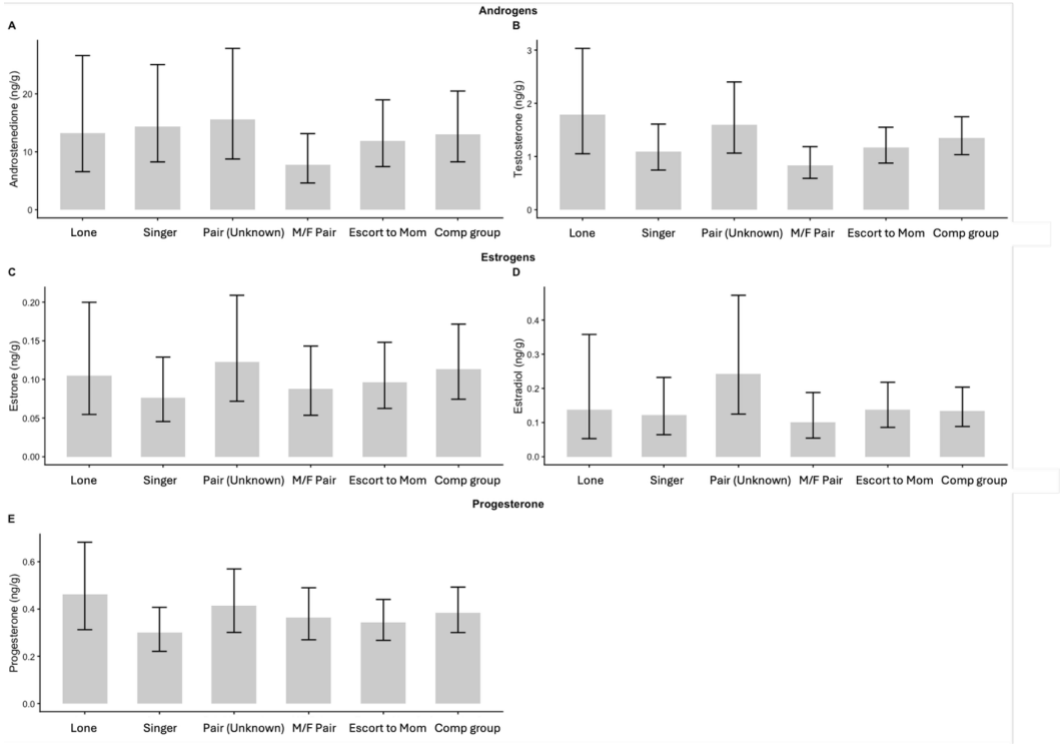


Figure 1.5. Estimated mean reproductive steroid concentrations for male humpback whales during the Hawai‘i breeding season across social-role categories. Estimated marginal means were derived from the final linear mixed-effects models for each hormone, which accounted for intra-seasonal variation and interannual variability.

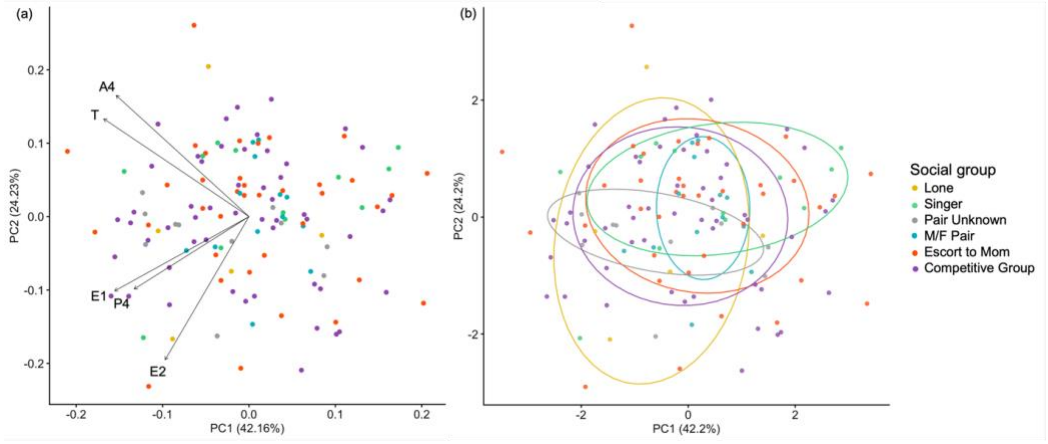


Figure 1.6. Principal component analysis (PCA) of residual reproductive hormone values from male humpback whales after accounting for seasonal and interannual effects. PCA biplot showing individual males colored by social role with hormone loadings.

1.10 Supplemental Materials

Table S1.1. EIA kit descriptions for the five target reproductive hormones.

Target hormone	EIA kit	Manufacturer	Reactivity	Detection limit
Androstenedione (A4)	11-ANRHU-E01	ALPCO	100%	0.1–10 ng/mL
Estrone (E1)	K031-H1	Arbor Assays	100%	31.25–2,000 pg/mL
17β-Estradiol (E2)	900-008	Enzo Life Sciences	100%	29.3–30,000 pg/mL
Progesterone (P4)	900-011	Enzo Life Sciences	100%	15.62–500 pg/mL
Testosterone (T)	900-065	Enzo Life Sciences	100%	7.81–2,000 pg/mL

Table S1.2. Mean ± SD reproductive hormone concentrations (ng/g) for juvenile and adult male humpback whales sampled in Hawai‘i (2014–2025). Juveniles were excluded from main analyses and are shown here for context.

Age class	A4	T	E1	E2	P4
Juvenile (n = 2)	8.39 ± 10.33	0.94 ± 0.90	0.020	0.25 ± 0.29	0.32 ± 0.15
Adult (n = 178)	19.13 ± 24.39	1.71 ± 1.33	0.11 ± 0.08	0.23 ± 0.26	0.48 ± 0.30

Table S1.3. Reproductive hormone concentrations (ng/g wet mass) for four male humpback whales sampled in both Hawai‘i (breeding) and Alaska (feeding).

Whale	Region (date)	A4	T	E1	E2	P4
Whale 3	Alaska (28 Jul 2023)	0.772	0.275	0.037	0.119	0.296
	Hawai‘i (17 Dec 2024)	13.675	2.638	0.126	0.270	0.835
Whale 11	Hawai‘i (12 Feb 2022)	8.549	0.505	0.007	0.287	0.396
	Alaska (27 Jul 2024)	NA	0.104	0.056	0.054	0.135
Whale 12	Hawai‘i (31 Jan 2022)	18.592	1.261	0.063	0.402	0.537
	Alaska (28 Jul 2023)	0.581	0.164	0.004	0.241	0.358
Whale 20	Hawai‘i (25 Feb 2024)	7.118	0.900	0.125	0.280	0.308
	Alaska (25 Jul 2024)	0.133	0.201	0.094	0.266	0.233

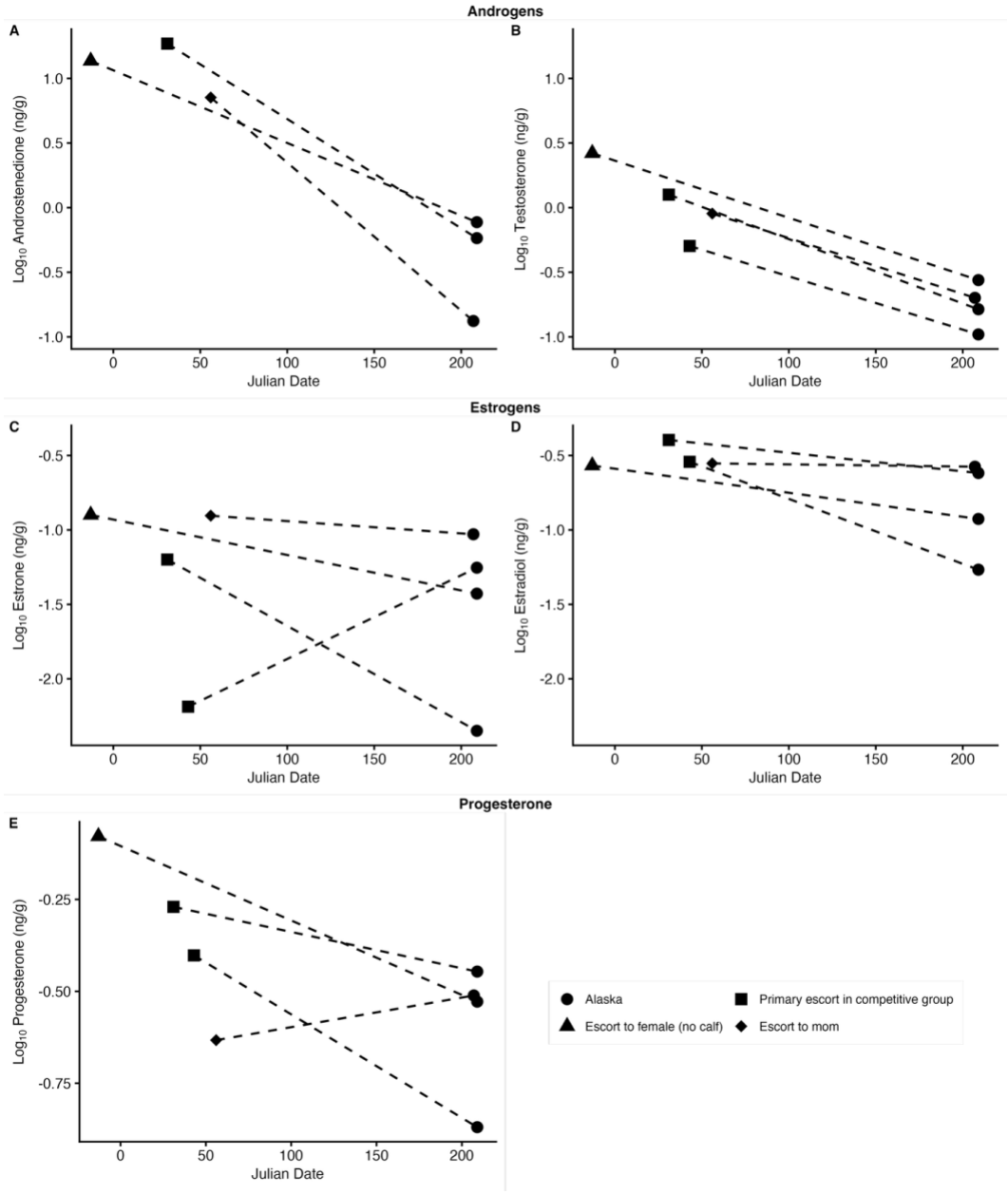


Figure S1.1. Within-individual seasonal variation in reproductive hormone concentrations for four male humpback whales. Log-transformed concentrations of (a) androstenedione, (b) testosterone, (c) estrone, (d) estradiol, and (e) progesterone are shown for each individual sampled once in Hawai‘i and once in Alaska. Point shapes indicate social role: circles = Alaska samples; squares = principal escort in a competitive group; triangles = male escorting a female without a calf; diamonds = male escorting a female with a calf. Dashed lines connect the two sampling points for each individual across regions. Julian dates for December samples were shifted to negative values to retain seasonal continuity.

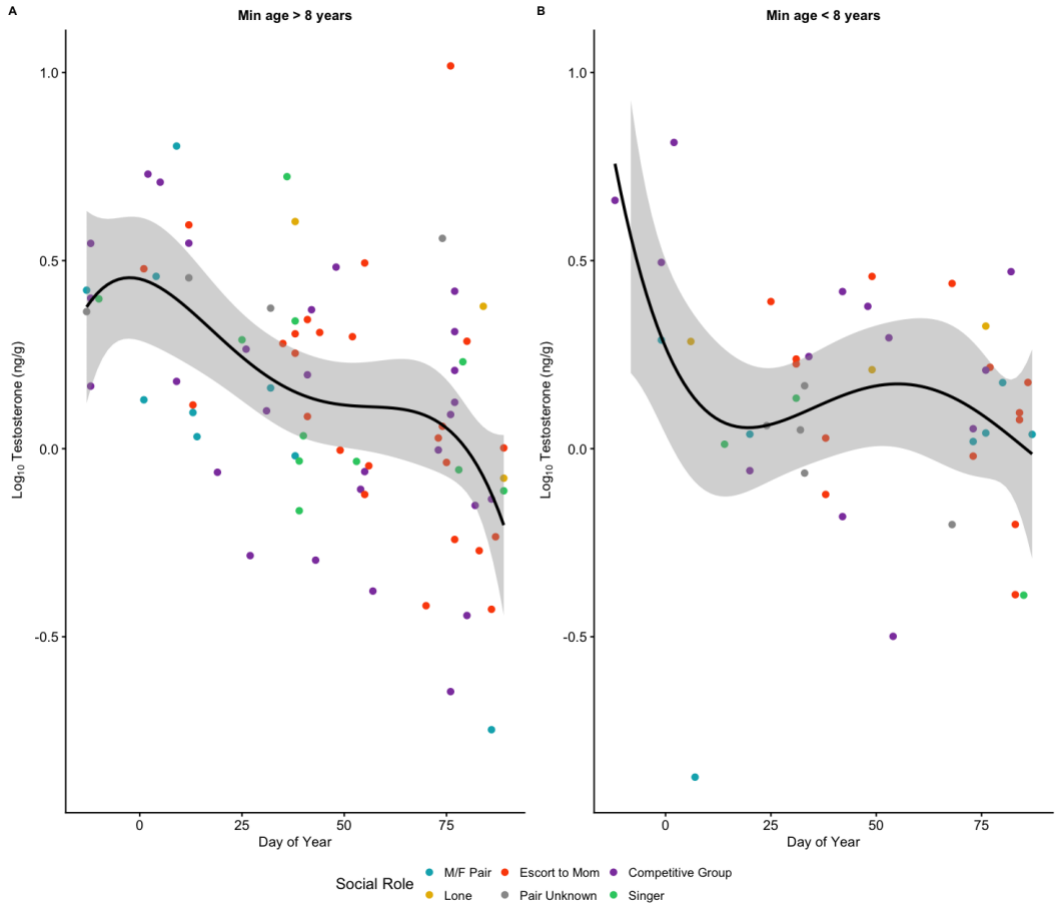
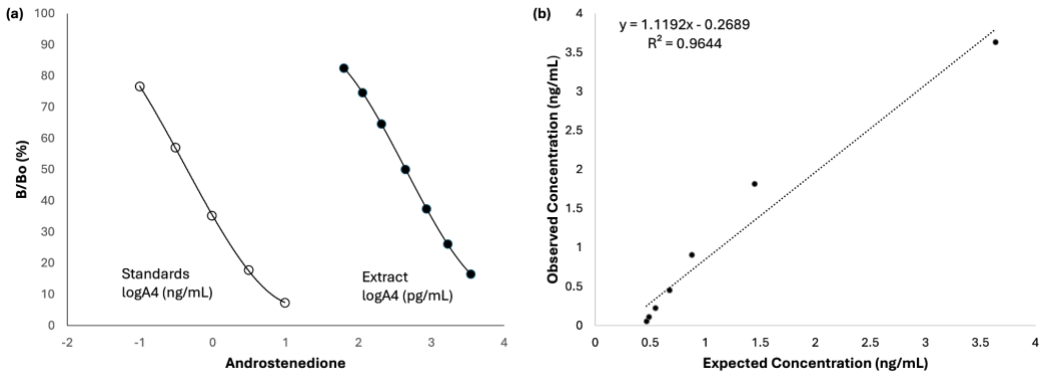


Figure S1.2. Minimum age-class differences in testosterone trends across the breeding season. Males were grouped into two minimum-age categories (<8 years, ≥8 years) based on sighting histories. Both age classes exhibited similar distributions and seasonal trajectories of testosterone concentrations, indicating that the observed intra-seasonal decline is not driven by increased presence of younger males later in the season.



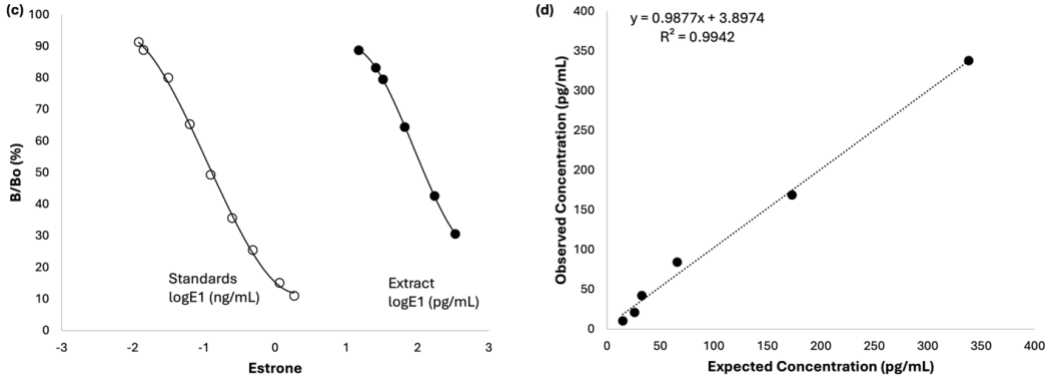


Figure S1.3. Validation of androstenedione (A4) and estrone (E1) EIAs for humpback whale blubber. (a, c) Serial-dilution curves for pooled blubber extract and kit standards demonstrating parallelism. (b, d) Accuracy plots showing linear regressions of observed vs. expected concentrations (A4: R^2 = 0.9644, slope = 1.1192; E1: R^2 = 0.9942, slope = 0.9877).

Chapter 2: Reproductive hormone trends in female humpback whales during the breeding season

2.1 Abstract

Understanding reproductive physiology is essential for assessing reproductive performance and population health in recovering baleen whale populations, yet endocrine variation among female humpback whales (*Megaptera novaeangliae*) remains poorly characterized in the North Pacific. This study investigated regional, seasonal, and reproductive-state variation in five reproductive steroid hormones (androstenedione, testosterone, estrone, estradiol, and progesterone) measured in blubber biopsies collected from female humpback whales on the Hawai‘i breeding grounds (females without calves, $n = 55$; mothers, $n = 95$) and Alaskan feeding grounds (females without calves, $n = 15$; mothers, $n = 4$; pregnant females, $n = 6$). Linear mixed-effects models, principal component analysis, and hormone correlation analyses were used to describe endocrine variation across reproductive classes, seasons, and social contexts.

Progesterone and estradiol concentrations were higher in Alaska than Hawai‘i; however, the regional progesterone difference was largely attributable to pregnant females, whereas elevated estradiol concentrations occurred across reproductive classes. Within Hawai‘i, progesterone concentrations were higher in females without calves, whereas estrone concentrations were higher in mothers. Across both reproductive classes, androstenedione increased, and testosterone declined over the breeding season, while estradiol exhibited distinct seasonal trajectories, peaking earlier

in females without calves than in mothers. Neither social group composition nor escort presence was associated with hormone concentrations. Principal component analysis revealed distinct multihormone endocrine profiles between reproductive classes, with greater endocrine heterogeneity among females without calves, reflecting the diversity of reproductive states represented within this group. These findings demonstrate the potential of multihormone endocrine approaches to improve reproductive-state assessment within breeding populations, while the observed regional and seasonal variation emphasizes the importance of validating blubber endocrine biomarkers across habitats before they are broadly applied to reproductive monitoring and conservation.

2.2 Introduction

Assessing reproductive performance and population health in recovering humpback whale populations requires a thorough understanding of reproductive physiology. Although many populations have recovered substantially following the cessation of commercial whaling (Thomas et al., 2016), the physiological processes underlying reproductive success remain poorly understood because much of our current knowledge is still derived from whales examined during the commercial whaling era (Chittleborough, 1958, 1965; Dawbin, 1966; Nishiwaki, 1959). As these populations face changing environmental conditions (Cartwright et al., 2019; Gabriele et al., 2022), there is increasing interest in developing non-invasive endocrine methods to assess reproductive state.

North Pacific humpback whales migrate annually between high-latitude feeding grounds, including Alaska, and low-latitude breeding grounds, including Hawai‘i

(Baker et al., 1986; Calambokidis et al., 2008). During the breeding season, females may occupy several reproductive states, including immature, cycling, pregnant, and lactating (Chittleborough, 1958, 1965; Darling, 1983; Glockner-Ferrari and Ferrari, 1985; Nishiwaki, 1959). Females reach sexual maturity at approximately five years of age but typically do not produce their first calf in the North Pacific until around eight years of age (Chittleborough, 1958; Gabriele et al., 2007). Gestation lasts approximately 11 months, and females generally produce calves every two to three years due to the energetic demands of gestation and lactation (Chittleborough, 1958; Straley et al., 1994). As females without calves may include individuals representing multiple reproductive states, distinguishing reproductive state from behavioral observations alone is challenging.

Historical evidence from commercial whaling suggests that female humpback whales are seasonally polyestrous and may undergo multiple estrous cycles if conception is unsuccessful (Chittleborough, 1965). Ovulation is thought to peak in February, coinciding with peak humpback whale abundance in Hawai‘i (Baker and Herman, 1981; Herman and Antinoja, 1977; Mobley et al., 1999; Nishiwaki, 1959), although the timing, duration, and endocrine characteristics of estrus remain poorly understood. Females observed within multiple-male competitive groups have long been hypothesized to be in estrus (Clapham, 1992; Darling et al., 1983), yet this assumption has not been validated through observations of copulation or direct assessments of ovarian activity. Behavioral observations alone cannot reliably distinguish among reproductive states, including pregnancy. Consequently, reproductive status on the

breeding grounds has largely been limited to distinguishing mothers from females without calves based on the presence or absence of a calf (Cartwright et al., 2019; Craig et al., 2003; Jones, 2010). Hormone profiles therefore offer an opportunity to improve reproductive-state assessment during the breeding season. Interpreting these endocrine profiles requires understanding how reproductive hormones vary across the reproductive cycle.

In mammals, ovarian steroid production varies predictably across the reproductive cycle: estrogens generally increase during follicular development and ovulation, whereas progesterone predominates during the luteal phase and pregnancy (Atkinson et al., 2018; Norris and Carr, 2020; Pineda, 2003). However, endocrine profiles overlap, as estrogens and androgens may also increase during pregnancy (Legacki et al., 2020; Norris and Carr, 2020; Robeck et al., 2017; Rolland et al., 2005; Steinman et al., 2021; Zhang et al., 2021). Consequently, evaluating multiple hormones simultaneously may provide a more complete assessment of reproductive state than relying on a single biomarker.

Because steroid hormones are lipophilic, they can be readily measured in cetacean blubber using remotely collected biopsy samples (Hunt et al., 2013). Blubber hormone analyses have emerged as valuable biomarkers of reproductive physiology because they integrate endocrine activity over longer timescales than circulating blood (Hunt et al., 2013; Kellar et al., 2013). Blubber progesterone has been successfully validated for pregnancy detection in humpbacks, although pregnancy classification has largely been limited to feeding grounds where pregnant females are sampled during mid-gestation

and are more readily distinguished from non-pregnant individuals (Atkinson et al., 2023; Pallin et al., 2018). More recent studies have expanded beyond progesterone to examine additional reproductive hormones associated with estrous cycling, reproductive behavior, and seasonal habitat use (Atkinson et al., 2023; Dalle Luche et al., 2020; Mingramm et al., 2020b; Pallin et al., 2024). However, relatively little is known about how multiple reproductive hormones vary among females occupying different reproductive states on the breeding grounds, how endocrine profiles change throughout the breeding season, or how they differ between breeding and feeding regions.

This study aimed to investigate variation in reproductive hormones among female humpback whales using steroid concentrations measured from blubber biopsies. Specifically, the objectives were to: (1) characterize variation in androgen (androstenedione and testosterone), estrogen (estradiol and estrone), and progesterone concentrations among female humpback whales sampled on the Hawai‘i breeding grounds and Alaska feeding grounds, including females without calves, mothers, and pregnant females (Alaska only); (2) examine seasonal variation in reproductive hormone concentrations throughout the Hawai‘i breeding season; and (3) evaluate multivariate endocrine profiles and correlations among reproductive hormones across reproductive classes and regions to identify multihormone endocrine patterns associated with underlying reproductive state.

2.3 Methods

2.3.1 Field observations and classifications

Biopsy samples from female humpback whales (*Megaptera novaeangliae*) were collected in Hawai‘i (Au‘au, Pailolo, and Kalohi channels; 20.88°N, 156.89°W) during the winter breeding season (December–April) between 2014 and 2025 (Figure I.2). Individuals were assigned to one of three reproductive classes based on relative body size and calf association: immature females, mature females without calves, and mothers with calves. Immature females were defined as whales larger than calves but noticeably smaller than full-grown adults. Calves less than one year old were not sampled. Group composition was recorded for each encounter (Table 2.1). Mature females were sampled across a range of social contexts, including solitary individuals (typically mothers only), male–female pairs, and competitive groups.

Photographs of ventral flukes were obtained from sampled individuals for photo-identification purposes (Katona et al., 1979). Images were submitted to Happywhale to determine minimum age estimates from first sighting records, compile sighting histories, and assess calving histories where available.

To provide a regional comparison between breeding and feeding grounds, an additional 25 biopsy samples were collected from female humpback whales in Southeast Alaska (Chatham Strait; 56.95°N, 134.62°W) during the summer feeding season (July) from 2023 to 2025 (Figure I.2). Whales sampled in Alaska were assigned to one of three reproductive classes: females without calves, pregnant, or mothers. Pregnancy status in Alaska was determined using a validated binomial regression

model based on blubber progesterone concentration, following Pallin et al. (2018). No immature whales were sampled based on minimum age obtained from sighting history.

2.3.2 Biopsy sample collection

Skin and blubber samples were collected using a minimally invasive remote biopsy system consisting of a crossbow fitted with a modified bolt and a 40-mm stainless steel barbed biopsy tip (CetaDart). Biopsies were obtained from the mid-lateral region below the dorsal fin. Following sampling, the biopsy dart and tissue sample were recovered from the water, typically within 3 minutes, and immediately transferred to a sterile collection vial or bag stored on ice until processing on shore.

For each sample collection, the date, time, GPS location, biopsy site, and the whale's behavioral response were documented. Reactions to sampling were scored on a 0–3 scale following Weinrich et al. (1992). Upon return to shore, samples were frozen at −20 °C and subsequently transferred to −80 °C for long-term storage at the conclusion of each field season.

2.3.3 Genetic sex determination

Field sex was initially assigned based on behavioral observations or close association with a calf. Sex was subsequently confirmed genetically through DNA extraction from approximately 25 mg of skin tissue using a Qiagen DNeasy extraction kit. Genetic analyses were conducted at the NOAA Southwest Fisheries Science Center and the Cetacean Conservation and Genetics Laboratory at Oregon State University. All samples included in analyses were genetically confirmed as female.

2.3.4 Hormone extraction and quantification

Steroid hormone extraction followed a protocol adapted by N. Kellar (SWFSC; pers. comm.) from Kellar et al. (2006). Blubber samples were cut perpendicular to the skin into 0.05–0.1 g subsamples, placed in stainless-steel microvials with a ¼″ ceramic bead, and homogenized in ethanol. Following homogenization, samples underwent sequential ethanol and 4:1 ethanol:acetone washes, followed by centrifugation. The resulting supernatant was evaporated in a dry bath, and the remaining lipid pellet was weighed. Acetonitrile and hexane were added to separate steroids from remaining lipids; the hexane (lipid) layer was removed, and the steroid-containing acetonitrile phase was dried and reconstituted in PBS buffer with BSA.

Two control samples were created from a stranded humpback whale blubber sample: a negative control with no added hormones and a positive control spiked with known concentrations of progesterone, estradiol, estrone, testosterone, and androstenedione. Extraction efficiency was calculated as the ratio of the measured positive-control concentration to the negative-control concentration, with efficiencies >60% considered acceptable.

Commercial enzyme immunoassay (EIA) kits were used to quantify androstenedione (A4), estrone (E1), 17β-estradiol (E2), progesterone (P4), and testosterone (T) (Table S2.1). Progesterone, testosterone, and estradiol assays have previously been validated for measuring steroid hormones in humpback whale blubber using parallelism and accuracy tests (Pallin et al., 2018, 2024). The androstenedione and estrone assays were validated for humpback whale blubber using the same

procedures in Chapter 1 of this dissertation. Samples exceeding kit detection limits were diluted and reanalyzed; samples below detection limits were assigned a value equal to one-half of the lowest standard to avoid censoring.

2.3.5 Statistical analysis

All statistical analyses were performed in R (R Core Team, 2025) on $\log_{10}$-transformed hormone concentrations to improve normality. Statistical support for effects followed the evidence framework of Muff et al. (2022), where *p*-values were categorized as little or no evidence ($p > 0.1$), weak evidence (0.05–0.1), moderate evidence (0.01–0.05), strong evidence (0.001–0.01), and very strong evidence (≤0.001). To avoid pseudoreplication, only the first biopsy sample collected from each identified individual was included in analyses. Immature females were excluded from primary analyses but are summarized in the Supplemental Materials (Table S2.2).

Regional differences between Hawai‘i and Alaska were evaluated using Welch’s t-tests on females pooled across reproductive classes to account for unequal sample sizes and variances between regions. Hormone relationships were assessed using Spearman correlations and visualized with correlation heatmaps. Principal component analysis (PCA) of mean-centered hormone concentrations, based on correlation matrices, was used to examine multivariate endocrine structure within each region. Varimax rotation was applied to the Hawai‘i PCA to improve interpretation of cross-loading hormones across principal components. Components with eigenvalues >1 were retained, and PC scores were evaluated relative to reproductive class and sampling date using linear models.

Seasonal trends were evaluated using linear mixed-effects models with reproductive class, sampling day, group composition, and relevant interactions as fixed effects, and year as a random intercept. Separate mixed-effects models were used to compare hormone concentrations among reproductive classes in Alaska. Model fit was assessed using residual diagnostics and Q–Q plots, and predicted values from final models were used to visualize seasonal trends. Exploratory analyses examining relationships between minimum age and hormone concentrations are presented in the Supplemental Materials (Figure S2.2).

2.4 Results

2.4.1 Regional comparisons

Reproductive hormone concentrations differed between females sampled on the Hawai‘i breeding grounds and Alaska feeding grounds, although patterns varied among hormone classes. Among the androgens, there was weak evidence that androstenedione concentrations were lower in Alaska than in Hawai‘i, whereas testosterone concentrations did not differ between regions (Welch's t-test A4: $t_{23.3} = -1.770$, $p = 0.089$; T: $t_{51.3} = -1.218$, $p = 0.229$) (Figure 2.1a,b).

Among estrogens, estrone concentrations also did not differ between regions (Welch's t-test: $t_{33.9} = -1.654$, $p = 0.107$). However, there was strong evidence that estradiol concentrations were higher in Alaska than in Hawai‘i (Welch's t-test: $t_{37.9} = 3.358$, $p = 0.002$) (Figure 2.1c,d).

There was very strong evidence that progesterone concentrations were higher in Alaska than in Hawai‘i (Welch’s t-test: $t_{26.5} = 3.629$, $p = 0.001$) (Figure 2.1e).

2.4.2 Hormone correlations

Relationships among reproductive hormones were examined to assess how hormone concentrations varied together within and between regions and to provide insight into the steroidogenic pathways underlying hormone production. The simplified steroid pathway shared by the five hormones examined here is presented in the General Introduction (Figure I.1).

Hawai‘i. During the breeding season in Hawai‘i, correlations among reproductive hormones were more pronounced and differed somewhat by reproductive class (Figure 2.2). Across all females, the strongest positive correlations were observed between progesterone and androstenedione ($r_s = 0.26$, $p = 0.001$), progesterone and testosterone ($r_s = 0.22$, $p = 0.007$), androstenedione and estrone ($r_s = 0.35$, $p < 0.001$), and testosterone and estradiol ($r_s = 0.24$, $p = 0.005$). Additionally, there was moderate evidence for an association between androstenedione and estradiol ($r_s = 0.20$, $p = 0.019$), and weak support for a relationship between progesterone and estradiol ($r_s = 0.14$, $p = 0.1$).

Hormone correlations examined within each reproductive class revealed distinct patterns (Figure 2.3). For mature females without calves, there was moderate evidence for positive correlations between progesterone and both estrone and estradiol ($r_s = 0.28$, $p = 0.047$; $r_s = 0.32$, $p = 0.027$, respectively), as well as between androstenedione and both estrone and estradiol ($r_s = 0.33$, $p = 0.018$; $r_s = 0.29$, $p = 0.041$, respectively).

There was also a weak association between progesterone and testosterone ($r_s = 0.23$, $p = 0.086$).

For mothers, there was very strong evidence for positive correlations between progesterone and androstenedione ($r_s = 0.39$, $p < 0.001$), progesterone and estrone ($r_s = 0.38$, $p < 0.001$), and androstenedione and estrone ($r_s = 0.49$, $p < 0.001$). Additionally, testosterone and estradiol ($r_s = 0.26$, $p = 0.013$), as well as estrone and estradiol ($r_s = 0.31$, $p = 0.004$), showed positive associations. As with females without calves, there was weak evidence for a relationship between progesterone and testosterone ($r_s = 0.18$, $p = 0.083$; Figure 2.3).

Alaska. Among females sampled in Alaska, androstenedione and estradiol showed the strongest supported positive correlation ($r_s = 0.60$, $p = 0.004$; Figure 2.2). This pattern appeared to be driven primarily by the strong relationship between these hormones in females without calves ($r_s = 0.80$, $p = 0.002$), which was the only supported correlation within this reproductive class. For pregnant females, there was strong evidence of a correlation between testosterone and estradiol ($r_s = 0.89$, $p = 0.033$), and weak evidence of a relationship between progesterone and estrone ($r_s = 0.77$, $p = 0.10$). Correlation tests for mothers in Alaska could not be conducted due to the low sample size for this class ($n = 3$).

2.4.3 Multivariate structure (PCA)

Hawai'i. Principal component analysis (PCA) of reproductive hormones using varimax-rotated components revealed separation in multivariate hormone profiles and

differences in dispersion between females without calves and mothers sampled on the Hawai‘i breeding grounds (Figure 2.4). PC1 and PC2 explained 31.2% and 27.2% of the total variance, respectively (Table 2.2). PC1 was characterized primarily by high loadings of androstenedione, estrone, and estradiol, whereas PC2 was defined primarily by progesterone and testosterone (Table 2.2).

Linear models of rotated PCA scores indicated that multivariate hormone structure varied with reproductive class and sampling date. Mothers exhibited higher PC1 scores than females without calves ($F_{1,132} = 12.01$, $p < 0.001$), and PC1 scores increased across the breeding season ($F_{1,132} = 8.02$, $p = 0.005$), with little evidence of a reproductive class × sampling date interaction (Figure 2.4b). Females without calves exhibited higher PC2 scores than mothers ($F_{1,131} = 42.19$, $p < 0.001$), and there was moderate evidence of a reproductive class × sampling date interaction ($F_{1,131} = 4.83$, $p = 0.030$), indicating that PC2 scores declined more strongly across the breeding season in females without calves than in mothers (Figure 2.4c).

Alaska. Principal component analysis (PCA) of reproductive hormones in females sampled on the Alaska feeding grounds showed partial separation among reproductive classes and distinct hormone loading patterns (Figure 2.5). PC1, PC2, and PC3 explained 61.3%, 21.2%, and 9.54% of the total variance, respectively (Table 2.3). Progesterone loaded most strongly onto PC1, androstenedione and estradiol onto PC2, and estrone onto PC3 (Table 2.3). Testosterone loaded most strongly onto PC5, which explained little additional variance and is therefore not shown.

Linear models of PCA scores indicated that multivariate hormone structure varied strongly with reproductive class for PC1 ($F_{2,18}$ = 70.62, p < 0.001), but not for PC2 ($F_{2,19}$ = 0.22, p = 0.804) or PC3 ($F_{2,19}$ = 1.81, p = 0.191). PC1 was dominated by progesterone loadings, and mothers exhibited lower PC1 scores than females without calves (t = −4.73, p < 0.001), whereas pregnant females exhibited substantially higher PC1 scores (t = 8.56, p < 0.001). In contrast, PC2 and PC3, which were associated primarily with androstenedione/estradiol and estrone variation, respectively, showed little evidence of reproductive class differences.

2.4.4 Seasonal hormone trends

Intraseasonal hormone trends in females sampled on the Hawai‘i breeding grounds differed among hormone classes, with androgens showing broadly similar patterns between mothers and females without calves, while estrogens and progesterone exhibited greater reproductive-class separation (Figure 2.6).

Among androgens, androstenedione increased across the breeding season by approximately 0.5% per day (Day: 0.002 ± 0.001 SE, t_{140} = 3.88, p < 0.001), and did not differ between mothers and females without calves. Testosterone exhibited a nonlinear seasonal decline with no overall reproductive-class differences (Day: −0.011 ± 0.003 SE, t_{141} = −3.678, p < 0.001; Day^2: $7.4 \times 10^{-5} \pm 3.0 \times 10^{-5}$ SE, t_{142} = 2.479, p = 0.014).

Estrogens showed greater separation between reproductive classes than androgens. Estrone concentrations were approximately 138% higher in mothers than in females without calves (Mother: 0.378 ± 0.054 SE, t_{141} = 6.998, p < 0.001), but did not vary

across the season. Estradiol showed a lack of overall separation between reproductive classes, although trajectories differed strongly across the breeding season (reproductive class × sampling day: $p < 0.001$). Both reproductive classes exhibited an increase followed by a decline in estradiol concentrations across the season, although females without calves peaked earlier in the breeding season than mothers (Day: 0.011 ± 0.005 SE, $t_{133} = 2.375$, $p = 0.019$; Day^2: $-1.75 \times 10^{-4} \pm 5.2 \times 10^{-5}$ SE, $t_{133} = -3.348$, $p < 0.001$).

Progesterone concentrations differed strongly between reproductive classes but showed no seasonal trend. Females without calves exhibited progesterone concentrations approximately 145% higher than mothers (Female no calf: 0.391 ± 0.074 SE, $t_{147} = 5.281$, $p < 0.001$).

In contrast to Hawai‘i, reproductive hormone variation in Alaska was limited. Progesterone was the only hormone that differed among reproductive classes, with pregnant females exhibiting progesterone concentrations approximately 24-fold higher than females without calves (Pregnant: 1.385 ± 0.135 SE, $t_{21} = 10.29$, $p < 0.001$), whereas mothers had the lowest progesterone concentrations of all reproductive classes (Mother: −1.056 ± 0.167 SE, $t_{20} = -6.327$, $p < 0.001$; Figure S2.1).

2.5 Discussion

2.5.1 Key findings

Female humpback whale reproductive hormones were primarily influenced by reproductive state but also varied across regions and throughout the breeding season.

Regional differences between Hawai‘i and Alaska were limited to progesterone and estradiol, with progesterone clearly distinguishing females classified as pregnant on the Alaska feeding grounds and estradiol showing an unexpected elevation in Alaska that was not explained by pregnancy status. Within the Hawai‘i breeding grounds, hormone profiles varied among reproductive classes and across the breeding season, reflecting the diversity of reproductive states occupied by females throughout the breeding season. Multivariate analyses further demonstrated that mothers and females without calves differed in overall endocrine profiles, even when individual hormone concentrations overlapped. Collectively, these findings provide a biological context for interpreting blubber steroid profiles across reproductive states, seasonal contexts, and regions.

2.5.2 Regional comparisons

Overall, reproductive hormone concentrations differed less between Hawai‘i and Alaska than expected (Figure 2.1). Because humpback whales are highly seasonal breeders (Dawbin, 1966), endocrine profiles would be expected to differ between females sampled during the breeding and feeding seasons, as seasonal fluctuations in reproductive hormones have been documented across marine mammals (Atkinson et al., 2023; Carone et al., 2019; Melica et al., 2021; Zhang et al., 2021). However, except for progesterone and estradiol, most hormones showed little evidence of variation between Hawai‘i and Alaska, suggesting that reproductive state exerts a stronger influence on endocrine profiles than season alone.

The higher progesterone concentrations observed in Alaska were largely driven by females classified as pregnant. Progesterone is produced primarily by the corpus luteum following ovulation and later by the placenta during pregnancy, where it promotes implantation, suppresses uterine contractions, and supports fetal development throughout gestation (Atkinson and Yoshioka, 2007; Norris and Carr, 2020). Because of its central role in the establishment and maintenance of pregnancy, progesterone has become one of the most widely applied endocrine biomarkers for pregnancy determination in wild cetaceans, including humpback whales (Mansour et al., 2002; Kellar et al., 2006; Trego et al., 2013; Pallin et al., 2018). Pregnancy status for females sampled in Alaska was determined using the blubber progesterone-based model of Pallin et al. (2018). Although only one female classified as pregnant has so far been confirmed with a calf, this most likely reflects the relatively small number of females sampled in Alaska and the limited opportunity for resighting to date, rather than uncertainty in the pregnancy classifications. Continued long-term sampling and photo-identification should increase opportunities for retrospective confirmation of pregnancy status. Furthermore, progesterone concentrations exceeded the pregnancy threshold independently established for this population by Atkinson et al. (2023) (28.58 ng/g) in all but one female classified as pregnant (26.07 ng/g), providing additional support for the pregnancy classifications assigned in this study.

Multivariate analyses further supported the influence of pregnancy status on endocrine profiles in Alaska. Principal component analysis revealed that progesterone-dominated PC1 explained over 60% of the total variation in reproductive hormones and

strongly separated pregnant females from both mothers and females without calves. In contrast, PC2 and PC3, which were associated primarily with androstenedione, estradiol, and estrone variation, showed little evidence of reproductive class differences. However, testosterone and estradiol were positively correlated within pregnant females, suggesting coordinated activity between these hormones that was not captured by the PCA. Given the small sample size (n = 6), however, this pattern warrants confirmation with additional individuals.

Unlike progesterone, elevated estradiol concentrations in Alaska could not be readily explained by pregnancy. Although estrogens typically increase during pregnancy (Norris and Carr, 2020), pregnant females exhibited estradiol concentrations similar to other reproductive classes, perhaps because the sampled whales were likely in mid-gestation rather than near parturition, when estradiol increases have been reported in captive dolphins (Steinman et al., 2021; Zhang et al., 2021). Furthermore, progesterone and estradiol were not positively correlated, as might be expected if elevated estradiol concentrations were primarily driven by pregnancy-related hormone production. Instead, non-pregnant females without calves exhibited the greatest range of estradiol values (Table S2.2).

Another possible explanation for the elevated estradiol concentrations observed in Alaska is local steroid production within blubber. Blubber is metabolically active, and adipose tissue is a recognized site of steroidogenesis, with enzymes such as aromatase and 17β-HSD expressed in human and rodent adipose tissue (Corbould et al., 1998; Stocco, 2012). Although their activity in cetacean blubber remains unconfirmed,

glucocorticoid interconversion has been demonstrated in cetacean blubber (Galligan et al., 2018), suggesting that local steroid metabolism may also occur for reproductive hormones. As androstenedione concentrations did not differ substantially between regions, the conversion of existing androgens into estradiol could explain the elevated concentrations observed in Alaska. This hypothesis is supported by the strong correlation between androstenedione and estradiol among females without calves sampled in Alaska, the strongest hormone correlation observed in either region. The feeding season also corresponds to a period of substantial blubber deposition (Lockyer, 1981; van Aswegen et al., 2025b), when local steroid conversion within blubber may be most pronounced. These results suggest that reproductive state explains much of the endocrine variation observed between Hawai‘i and Alaska, but does not fully account for the elevated estradiol concentrations observed in Alaska.

2.5.3 Hawai‘i: single-hormone trends

During the breeding season, reproductive hormone concentrations differed among reproductive classes and varied across the breeding season. Although progesterone concentrations were highest among females without calves overall, this pattern was attributable to higher concentrations in a minority of these females (Figure 2.6E). Most females without calves fell within the progesterone range observed for mothers, whereas a smaller number exhibited concentrations above that range. This extensive overlap is consistent with Southern Hemisphere humpback whales, in which Pallin et al. (2024) reported no difference in blubber progesterone between mature females without calves and mothers during the breeding season.

Elevated progesterone concentrations in some females without calves likely reflect different reproductive states, including the luteal phase, early pregnancy, or pseudopregnancy. In each of these states, the corpus luteum secretes progesterone following ovulation (Norris and Carr, 2020; Robeck et al., 2001, 2018). Furthermore, because blubber progesterone concentrations are thought to integrate circulating progesterone concentrations over periods of weeks to months rather than days (Kellar et al., 2013), recently conceived pregnancies may not yet be fully distinguishable from other post-ovulatory states. Similar challenges in distinguishing early pregnancy from post-ovulatory states using progesterone concentrations have been reported in other cetaceans (Carone et al., 2019; Mingramm et al., 2020b). These findings suggest that progesterone alone cannot distinguish among the reproductive states represented by females without calves on the breeding grounds.

Although androgen concentrations did not differ among reproductive classes, androstenedione and testosterone exhibited contrasting seasonal trajectories. Androstenedione increased steadily across the breeding season, whereas testosterone declined nonlinearly. In contrast, Pallin et al. (2024) reported lower testosterone concentrations in mothers than in non-mother adults in Southern Hemisphere humpback whales, a reproductive-class difference that was not detected here. Instead, testosterone varied primarily across the breeding season rather than among reproductive classes, suggesting that androgen production may change across the breeding season.

The seasonal increase in androstenedione parallels findings from Southern Hemisphere humpback whales, where Dalle Luche et al. (2020) reported the highest concentrations during migration toward the breeding grounds and linked elevated androgens to late gestation, consistent with increases observed before parturition in several cetacean species (Legacki et al., 2020; Robeck et al., 2017; Steinman et al., 2021). Since births are rarely observed on the breeding grounds (Patton and Lawless, 2021; Ransome et al., 2022), many females likely give birth before arrival or shortly after reaching Hawai‘i. Consequently, the seasonal increase in androstenedione may reflect changing steroidogenic activity across the breeding season rather than late gestation alone.

Because mothers are the only reproductive class with a known recent pregnancy, they provide the clearest opportunity to examine endocrine signals that may persist following parturition. Estrone concentrations were higher in mothers than in females without calves. Estrone has been identified as the predominant estrogen during pregnancy in bottlenose dolphins (Steinman et al., 2016), and in Southern Hemisphere humpback whales, estrone was detected almost exclusively in females with elevated androstenedione concentrations presumed to be near parturition (Dalle Luche et al., 2020). Elevated estrone concentrations observed in mothers may represent residual endocrine signals from late gestation that persist following birth, with changes in blubber hormone concentrations lagging behind changes in circulating hormone concentrations (Galligan et al., 2020; Kellar et al., 2013).

Unlike estrone, estradiol showed no overall difference between reproductive classes, though seasonal trajectories differed between them. Estradiol is the primary estrogen associated with follicular development and ovulation in mammals, increasing during the follicular phase before ovulation (Norris and Carr, 2020). Females without calves exhibited an earlier estradiol peak than mothers, occurring approximately in February. This timing closely aligns with historical estimates of peak ovulation in North Pacific humpback whales in Hawai‘i (reviewed in Darling, 2001), suggesting that the observed estradiol peak reflects follicular development preceding ovulation. Similarly, Pallin et al. (2024) reported higher estradiol relative to progesterone early in the breeding season, followed by increasing progesterone later in the season as females transitioned into the luteal phase.

In contrast, mothers exhibited a later estradiol peak, consistent with delayed resumption of ovarian activity following parturition. Pallin et al. (2024) similarly observed that mothers had the lowest overall estradiol concentrations among adult female demographic groups in New Caledonia but exhibited a late-season increase in estradiol that was interpreted as renewed ovarian activity toward the end of the breeding season. Although the timing of postpartum ovarian cycling in humpback whales remains unknown, the later estradiol peak is consistent with mothers resuming ovarian activity later in the breeding season than females without calves.

2.5.4 Hawai‘i: multihormone trends

No single hormone completely resolved reproductive state, motivating multivariate analyses to evaluate whether combined hormone profiles better differentiated

reproductive classes. Principal component analysis revealed separation between reproductive classes: mothers scored higher on the estrogen/androstenedione-dominated PC1, whereas females without calves scored higher on the progesterone/testosterone-dominated PC2. These patterns indicate that reproductive state is better reflected by multihormone endocrine profiles than by any single hormone alone.

The androstenedione-, estrone-, and estradiol-dominated PC1 profile characterizing mothers likely reflects residual endocrine signals from late gestation that persist following parturition. Because humpback whale mothers fast throughout the breeding season and mobilize substantial lipid reserves (Christiansen et al., 2016; Lockyer, 1981; van Aswegen et al., 2025b), changes in blubber composition may also influence hormone concentrations, making the relative contributions of hormone production and lipid metabolism difficult to separate.

In contrast, females without calves scored higher on PC2 and exhibited greater dispersion in multivariate hormone space, consistent with the diversity of reproductive states represented within this group. Females approaching ovulation, newly pregnant females, and individuals in the luteal phase would be expected to differ substantially in the relative production of progesterone, androgens, and estrogens (Robeck et al., 2017; Steinman et al., 2016), producing greater variability in endocrine profiles. The greater decline in PC2 scores among females without calves suggests that multihormone differences between reproductive classes were most pronounced early in the breeding season and became less distinct over time. Together, these multivariate patterns

indicate that reproductive state is reflected by coordinated endocrine variation that is not apparent from individual hormones alone.

2.5.5 Social group comparison

Despite long-standing assumptions that female social behavior reflects reproductive state (Clapham, 1992; Darling et al., 1983), group composition was not a significant predictor of any hormone examined. Previous work in Australian humpback whales reported elevated blubber estradiol concentrations in escorted females relative to solitary females and interpreted this pattern as evidence that escorted females were more likely to be in estrus (Mingramm et al., 2020b). The lack of a similar relationship in the present study suggests that social associations do not consistently reflect underlying reproductive physiology. In Hawai‘i, social associations are typically short-lived due to frequent changes in group composition driven by male–male competition and variable female responses to escorting males (Baker and Herman, 1984; Jones, 2010; Mobley and Herman, 1985). Although multi-day escort associations have been documented (Henderson et al., 2022), they appear to be uncommon. With males outnumbering females on the breeding grounds (operational sex ratio 1.8:1; Herman et al., 2011), competition for access to females may be intense regardless of reproductive state, and males may preferentially associate with larger females independent of hormonal profile (Pack et al., 2009). Furthermore, births have been observed within multiple-escort groups (Ransome et al., 2022), demonstrating that group composition does not reliably indicate reproductive state.

Collectively, these observations suggest that social associations on the breeding grounds are influenced more by male competition and mating behavior than by female endocrine state, highlighting the value of blubber hormone profiling for assessing reproductive state.

2.5.6 Limitations and future directions

Although this study provides new insight into reproductive endocrinology in female humpback whales, interpreting blubber hormone profiles remains limited by our ability to independently verify reproductive state. Advances in analytical methods now allow multiple reproductive hormones to be quantified simultaneously, yet reproductive state for most females must still be inferred from indirect observations such as calf presence or absence. Consequently, many of the endocrine patterns identified in this study represent interpretations based on inferred reproductive states rather than independently validated reproductive outcomes. Future work should therefore prioritize obtaining hormone profiles from females with known reproductive outcomes, including confirmed pregnancies, known postpartum females, and, where feasible, repeated samples collected from the same individuals across the breeding season. This is particularly important on the Alaska feeding grounds, where relatively few pregnant females have been sampled, limiting characterization of gestational endocrine variation and validation against subsequent reproductive outcomes. Such datasets will provide the critical validation needed to interpret endocrine variation across reproductive states with greater confidence.

Long-term monitoring, continued resighting of known individuals, and paired unmanned aircraft system (UAS) photogrammetry with biopsy sampling offer practical opportunities to strengthen endocrine validation in humpback whales. Emerging identification approaches, such as tubercle identification (Evans et al., 2026), will further improve the ability to resight females across breeding and feeding grounds and retrospectively confirm reproductive outcomes. Integrating hormone profiles with UAS-derived body condition measurements, particularly girth, may help identify females approaching parturition, while estimating calf age from body size or developmental characteristics (Cartwright and Sullivan, 2009; van Aswegen et al., 2025a) could help approximate time since parturition, providing important context for interpreting postpartum endocrine profiles. Together, these complementary approaches would improve reproductive-state assignment and strengthen interpretation of endocrine variation during the transition from late pregnancy to lactation.

Perhaps the most important mechanistic question raised by this study is whether local steroid metabolism within blubber contributes to the endocrine patterns observed in humpback whales. Blubber is metabolically active and capable of interconverting glucocorticoids in cetaceans (Galligan et al., 2018), while adipose tissue in other mammals expresses enzymes involved in steroidogenesis, including aromatase and 17β-HSD (Corbould et al., 1998; Stocco, 2012). Whether these pathways are similarly active in cetacean blubber remains unknown. Resolving this question is particularly important because the unexpected estradiol patterns observed in Alaska raise the possibility that local steroid metabolism contributes to blubber hormone profiles.

Future studies should quantify the expression and activity of steroidogenic enzymes within humpback whale blubber across reproductive states and seasons to determine the extent to which blubber hormone concentrations reflect circulating endocrine activity, local steroid metabolism, or a combination of both.

2.6 Conclusions

General conclusions. This study shows that reproductive endocrinology in female humpback whales is more complex than can be captured by a single hormone or behavioral observation. While progesterone provided a useful marker of pregnancy among females sampled on the Alaska feeding grounds, it could not distinguish the diverse reproductive states represented by females without calves on the Hawai‘i breeding grounds. Instead, reproductive state was better reflected by the combined profile of multiple steroid hormones. Furthermore, neither social group composition nor escort status consistently reflected underlying endocrine profiles, demonstrating that behavioral observations alone provide limited insight into female reproductive state.

Conservation implications. The results of this study have important implications for the application of endocrine biomarkers in cetacean conservation and population monitoring. Progesterone is a valuable biomarker for identifying pregnancy on humpback whale feeding grounds, but reproductive-state assessment remains substantially more challenging on the breeding grounds, where females without calves represent a heterogeneous mixture of reproductive states. By demonstrating that multihormone endocrine profiles provide greater resolution of endocrine variation than

any single hormone alone, this study provides an important foundation for developing validated approaches capable of distinguishing reproductive states within breeding populations. Such approaches could ultimately allow managers to assess reproductive state directly from blubber biopsy samples, reducing reliance on behavioral observations and retrospective confirmation through subsequent calf sightings.

The seasonal hormone patterns documented here also provide practical guidance for endocrine monitoring programs. Because reproductive hormone concentrations varied across the breeding season, sampling date should be considered when interpreting hormone profiles and comparing endocrine data among studies. Furthermore, the unexpected regional differences in estradiol highlight the importance of understanding how blubber hormone dynamics vary between feeding and breeding habitats before blubber endocrine biomarkers can be broadly applied in reproductive monitoring across seasons and populations. As endocrine techniques become increasingly incorporated into long-term monitoring programs, validated multihormone profiles derived from blubber have the potential to provide managers with a powerful tool for assessing reproductive state, improving reproductive monitoring, and supporting conservation of humpback whale populations.

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2.8 Tables

Table 2.1. Reproductive class and group compositions for female humpback whales sampled in Hawai‘i.

Reproductive class	Group type	Sample size
Immature	Variable	6
Female (no calf)	Lone	1
	Male–female pair	22
	Competitive group: multiple male escorts	32
Mother	Mom–calf	32
	Mom–calf escort (male)	51
	Competitive group: multiple male escorts	12

Table 2.2. Varimax-rotated principal component (PC) loadings for $\log_{10}$-transformed reproductive hormones from female humpback whales sampled on the Hawai‘i breeding grounds. Loadings with the strongest contribution to each component are shown in bold.

Hormone	Rotated PC1	Rotated PC2
% variance	31.2%	27.2%
Progesterone (logP4)	0.222	**0.669**
Androstenedione (logA4)	**0.753**	0.17
Testosterone (logT)	−0.089	**0.798**
Estrone (logE1)	**0.784**	−0.361
Estradiol (logE2)	**0.567**	0.339

Table 2.3. Principal component (PC) loadings for $\log_{10}$-transformed reproductive hormones from female humpback whales sampled on the Alaska feeding grounds. Loadings with the strongest contribution to each component are shown in bold.

Hormone	PC1	PC2	PC3
% variance	61.3%	21.2%	9.54%
Progesterone (logP4)	**0.991**	−0.047	0.118
Androstenedione (logA4)	0.008	**−0.799**	−0.276
Testosterone (logT)	0.011	−0.037	0.227
Estrone (logE1)	0.123	0.195	**−0.925**
Estradiol (logE2)	−0.052	**−0.565**	0.047

2.9 Figures

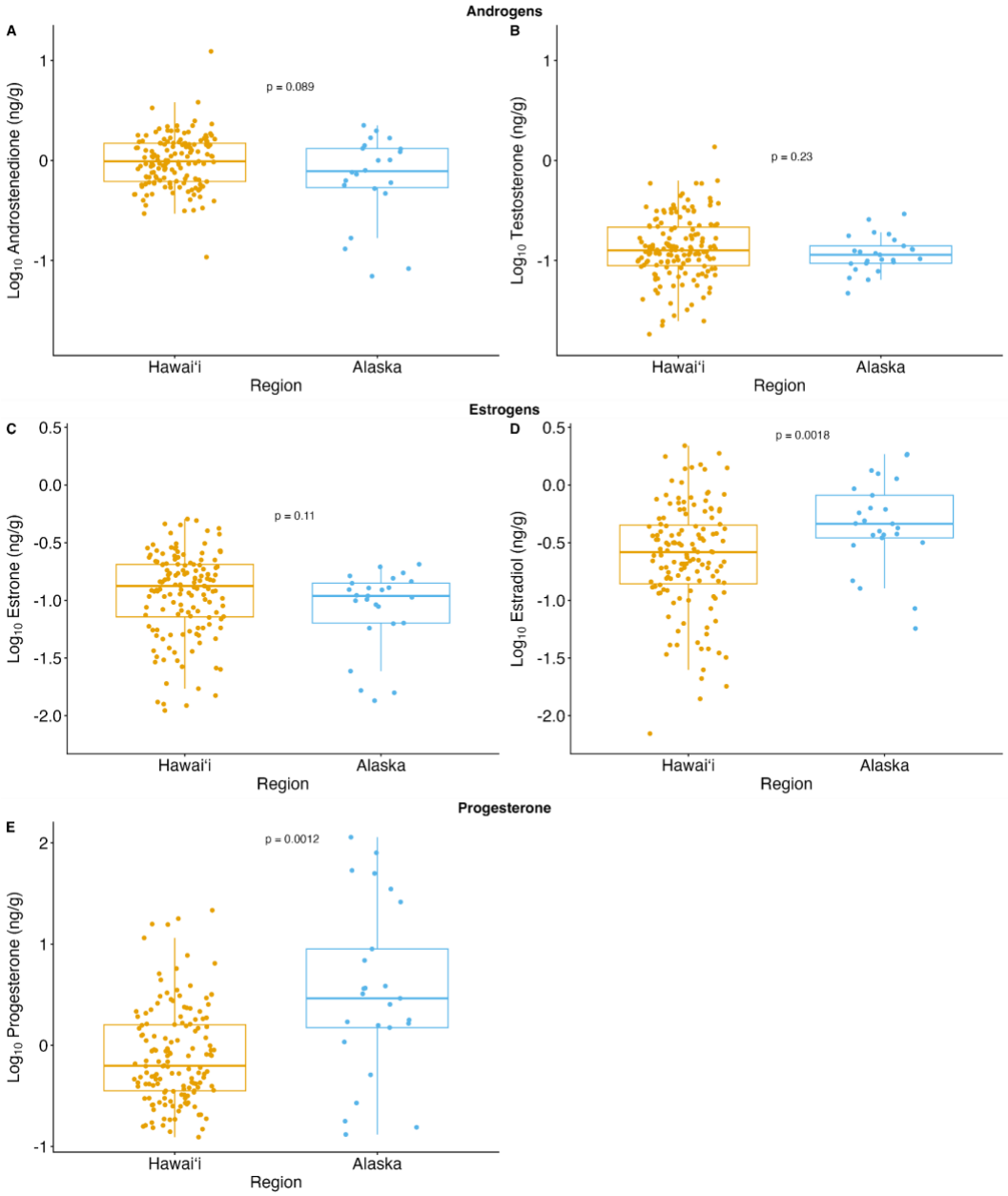

Figure 2.1. Regional differences in log₁₀-transformed reproductive hormone concentrations in mature female humpback whales sampled in Hawai'i and Alaska across all reproductive classes: (A) androstenedione, (B) testosterone, (C) estrone, (D) estradiol, and (E) progesterone. Boxes indicate the median and interquartile range, whiskers represent 1.5 × the interquartile range, and points represent individual samples.

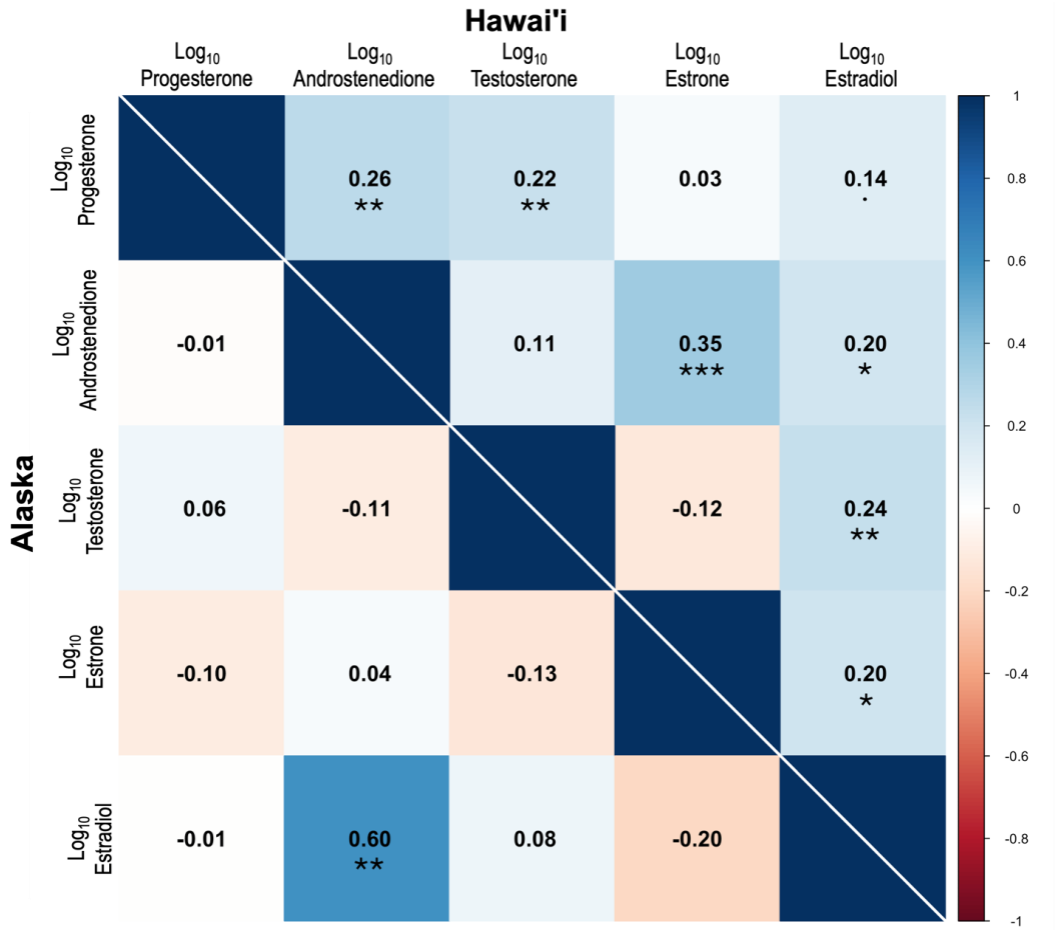

Figure 2.2. Spearman correlation heatmap showing relationships among $\log_{10}$-transformed reproductive hormone concentrations for all females sampled during the Alaska feeding season (lower triangle) and Hawai‘i breeding season (upper triangle). Values within cells represent Spearman correlation coefficients (r_s).

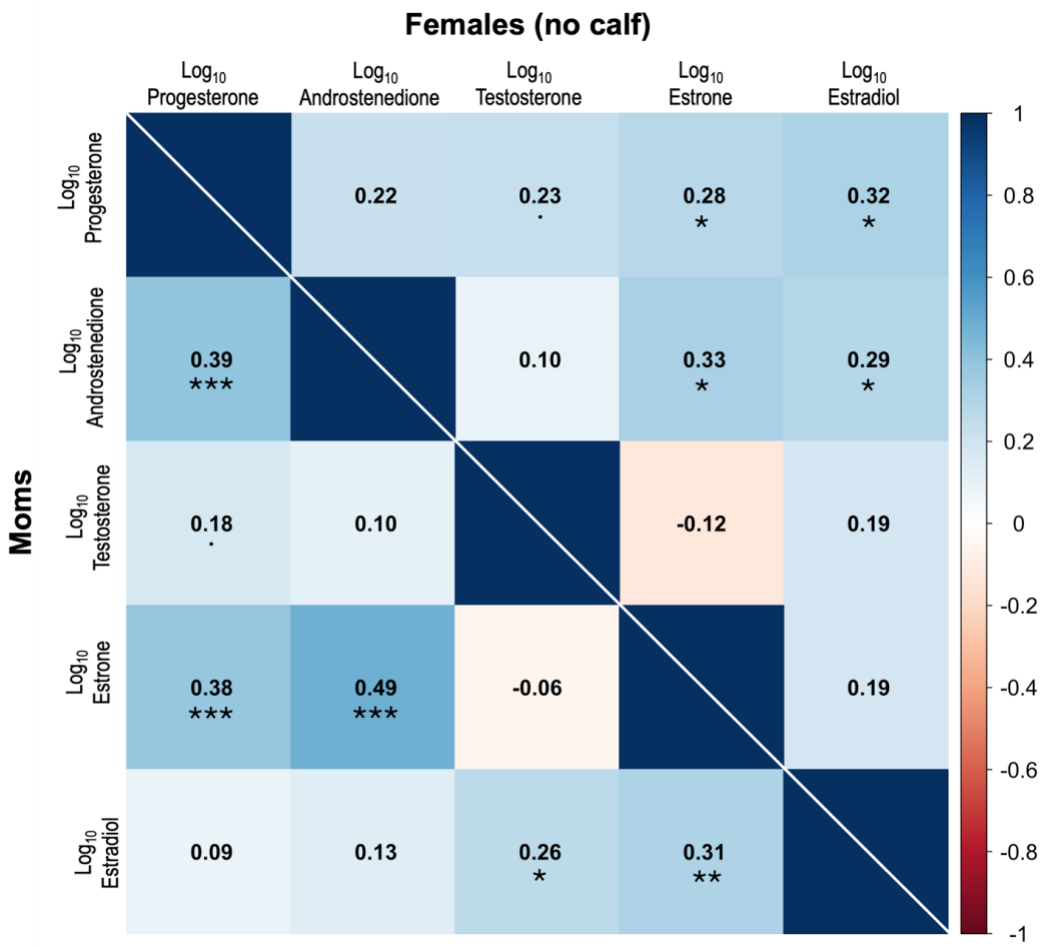

Figure 2.3. Spearman correlation heatmap showing relationships among $\log_{10}$-transformed reproductive hormone concentrations for mothers (lower triangle) and females without calves (upper triangle) sampled within the Hawai‘i breeding grounds. Values within cells represent Spearman correlation coefficients (r_s).

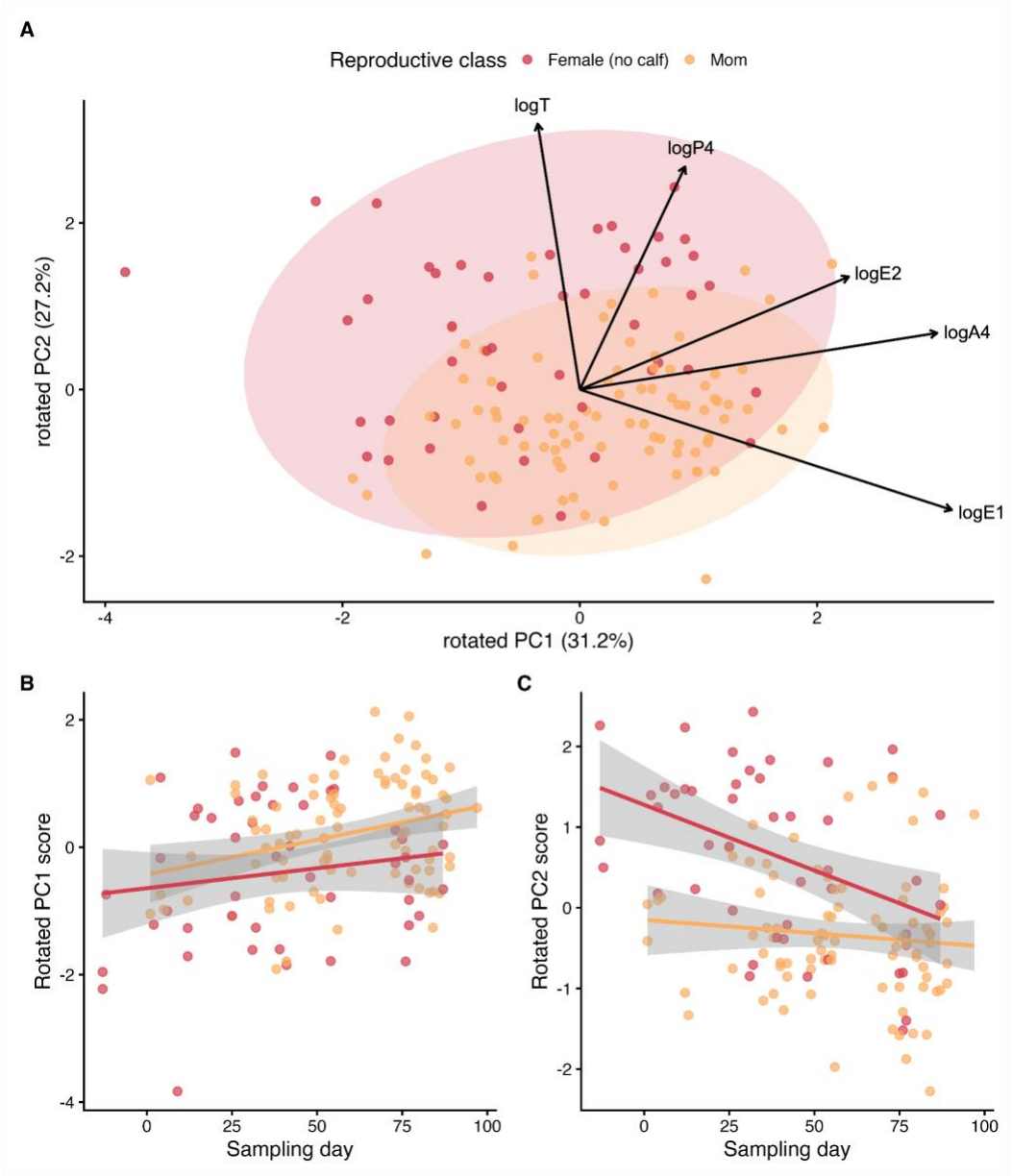

Figure 2.4. (A) Rotated principal component analysis (PCA) scores and hormone loading vectors of reproductive hormone concentrations for female humpback whales sampled on the Hawai‘i breeding grounds, colored by reproductive class, with shaded ellipses representing 95% confidence intervals around group centroids. Panels (B) and (C) show seasonal trends in PC1 and PC2 scores, respectively, with solid lines representing linear model fits and shaded 95% confidence intervals.

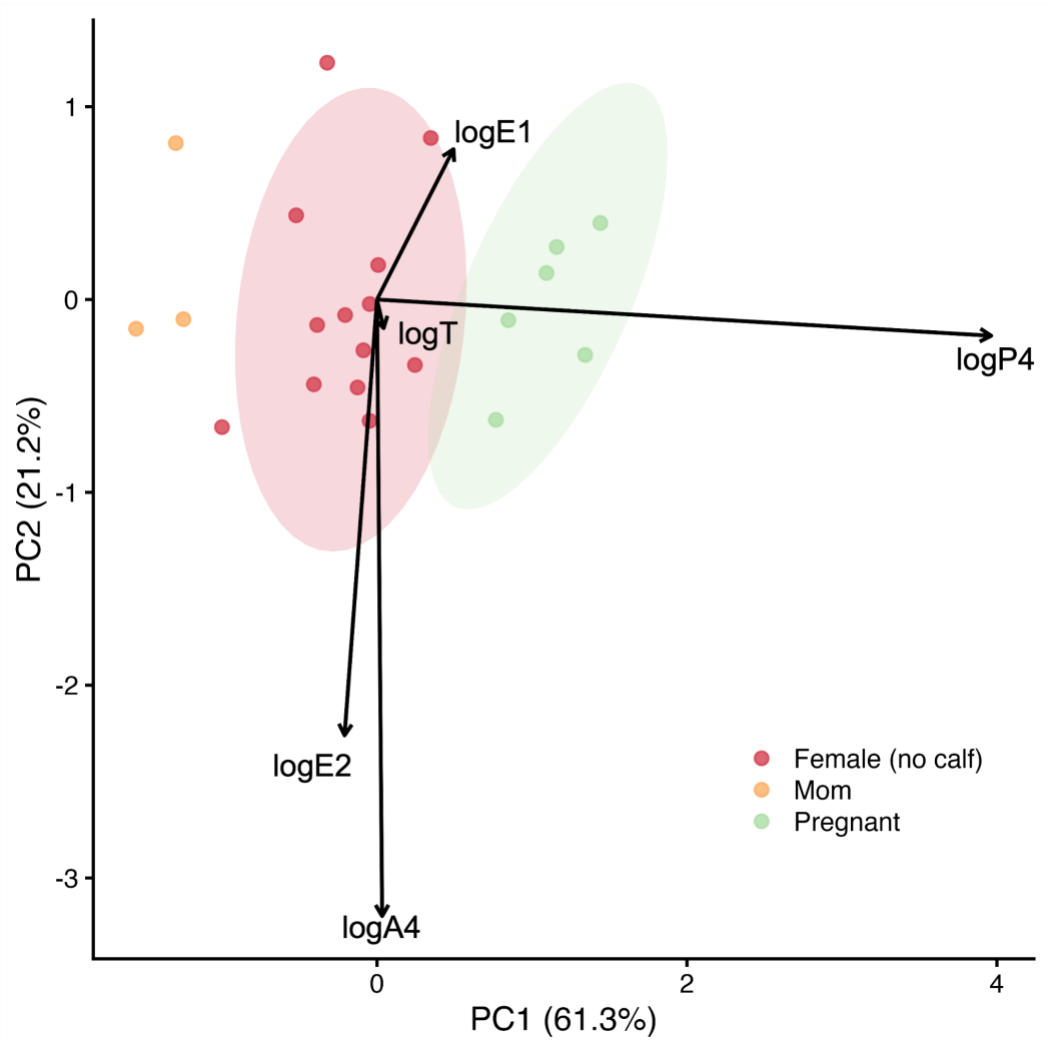

Figure 2.5. Principal component analysis (PCA) scores and hormone loading vectors of reproductive hormone concentrations for female humpback whales sampled in Alaska, colored by reproductive class, with shaded ellipses representing 95% confidence intervals around group centroids.

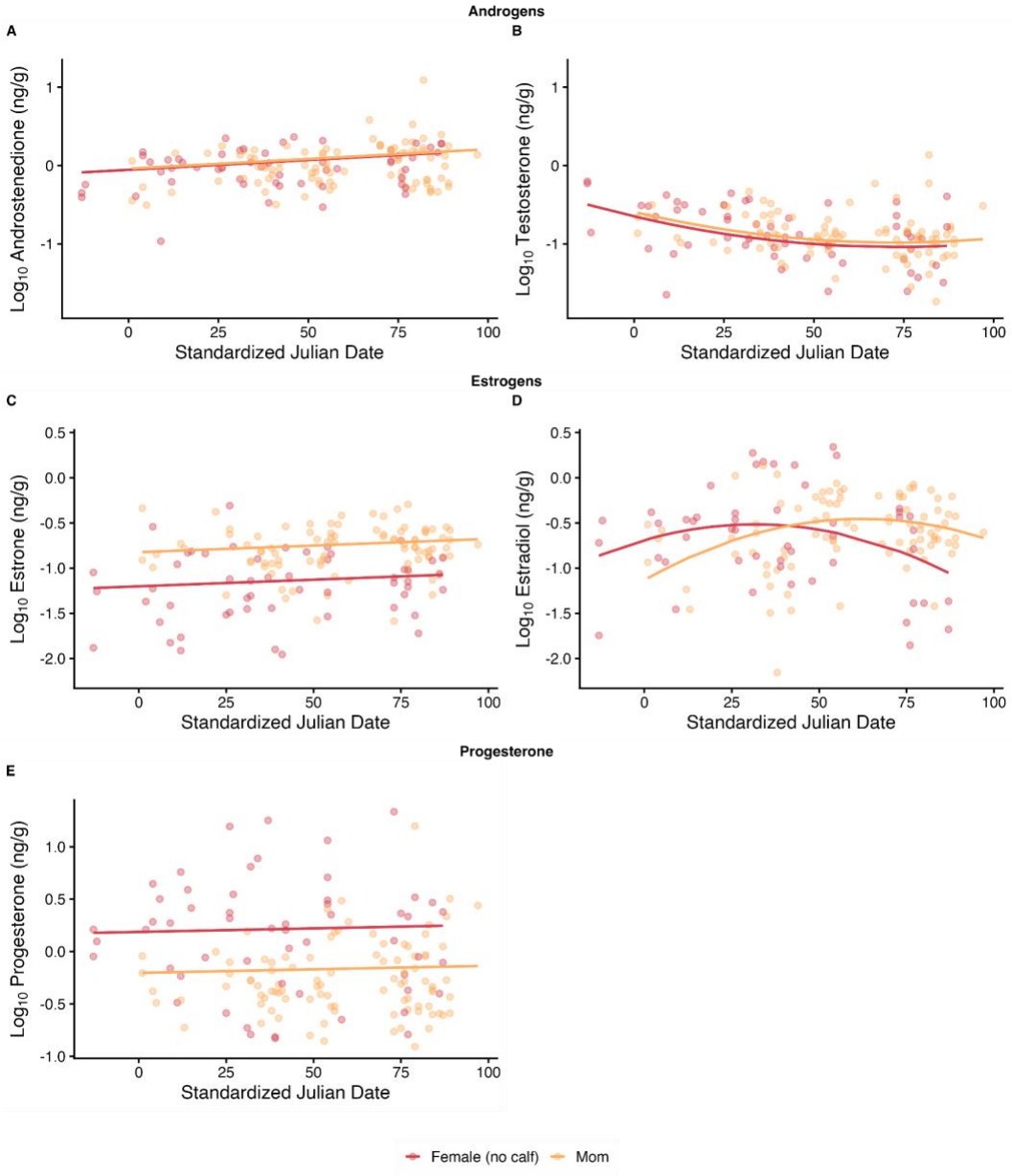

Figure 2.6. Intraseasonal hormone trends for mothers and mature females without calves sampled during the Hawai‘i breeding season from 2014–2025. Lines represent predicted hormone trajectories from final linear mixed-effects models with year included as a random intercept for $\log_{10}$-transformed concentrations of (A) androstenedione, (B) testosterone, (C) estrone, (D) estradiol, and (E) progesterone. Points represent individual biopsy samples.

2.10 Supplemental Materials

Table S2.1. EIA kit descriptions for target hormones.

Target hormone	EIA kit	Manufacturer	Reactivity to hormone	Detection limit
Androstenedione (A4)	11-ANRHU-E01	ALPCO	100%	0.1–10 ng/mL
Estrone (E1)	K031-H1	ARBOR Assays	100%	31.25–2,000 pg/mL
17β-Estradiol (E2)	900-008	ENZO Life Sciences	100%	29.3–30,000 pg/mL
Progesterone (P4)	900-011	ENZO Life Sciences	100%	15.62–500 pg/mL
Testosterone (T)	900-065	ENZO Life Sciences	100%	7.81–2,000 pg/mL

Table S2.2. Mean (± SD), range (italics), and sample sizes of reproductive hormone concentrations in female humpback whales sampled on the Hawai‘i breeding grounds (HI; 2014–2025) and Alaska feeding grounds (AK; 2023–2025), separated by reproductive class. Sample sizes vary among hormones because not all assays could be performed for every individual.

Reproductive class	Region	A4	T	E1	E2	P4
Immature	HI	0.994 ± 0.507 *0.57–1.95* n = 6	0.257 ± 0.159 *0.11–0.54* n = 6	0.027 ± 0.013 *0.02–0.05* n = 6	0.289 ± 0.241 *0.15–0.78* n = 6	0.282 ± 0.159 *0.02–0.51* n = 6
Female (no calf)	AK	1.04 ± 0.645 *0.07–2.25* n = 13	0.123 ± 0.053 *0.05–0.26* n = 15	0.111 ± 0.041 *0.01–0.17* n = 15	0.664 ± 0.513 *0.06–1.82* n = 15	3.03 ± 2.26 *0.51–8.95* n = 15
Female (no calf)	HI	1.06 ± 0.531 *0.11–2.31* n = 55	0.184 ± 0.142 *0.02–0.63* n = 55	0.088 ± 0.081 *0.01–0.49* n = 53	0.433 ± 0.538 *0.01–2.19* n = 50	2.94 ± 4.33 *0.15–21.61* n = 55
Mother	AK	0.496 ± 0.320 *0.13–0.73* n = 3	0.117 ± 0.046 *0.07–0.18* n = 4	0.102 ± 0.096 *0.02–0.21* n = 4	0.509 ± 0.292 *0.3–0.93* n = 4	0.183 ± 0.060 *0.13–0.27* n = 4
Mother	HI	1.26 ± 1.33 *0.31–12.35* n = 91	0.164 ± 0.163 *0.02–1.37* n = 95	0.187 ± 0.010 *0.03–0.51* n = 91	0.347 ± 0.271 *0.01–1.37* n = 89	0.870 ± 1.67 *0.12–15.78* n = 95
Pregnant	AK	0.890 ± 0.651 *0.17–1.98* n = 6	0.142 ± 0.080 *0.08–0.29* n = 6	0.096 ± 0.06 *0.02–0.19* n = 6	0.615 ± 0.621 *0.13–1.85* n = 6	59.7 ± 32.3 *26.07–113.9* n = 6

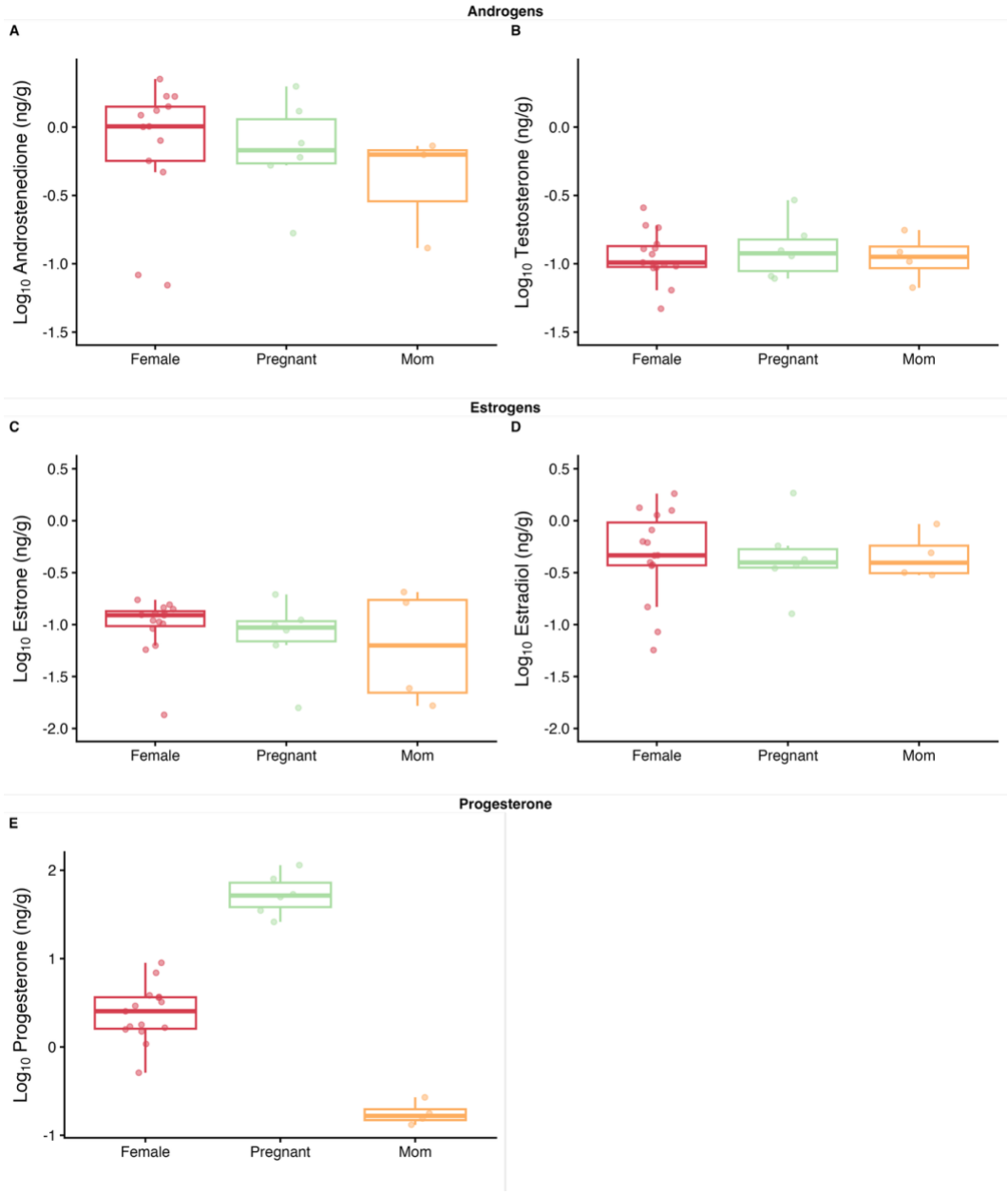

Figure S2.1. Log_{10}-transformed reproductive hormone concentrations by reproductive class for females sampled in Alaska. Panels show (A) androstenedione, (B) testosterone, (C) estrone, (D) estradiol, and (E) progesterone for females without calves, pregnant females, and mothers. Boxes represent the interquartile range, horizontal lines indicate the median, whiskers extend to 1.5 × the interquartile range, and points represent individual samples.

Minimum age relationships

Relationships between minimum age and reproductive hormone concentrations were evaluated for a subset of females sampled in Hawai‘i with extensive sighting histories (≥10 sightings across breeding or feeding grounds). Minimum age was estimated from first sighting records, with mothers assigned a minimum age of 8 years based on the estimated age of first calving for this population (Gabriele et al., 2007). Spearman rank correlations were used to assess relationships between minimum age and $\log_{10}$-transformed hormone concentrations.

Minimum age showed limited relationships with reproductive hormone concentrations in adult female humpback whales sampled in Hawai‘i (Figure S2.2). Among the five hormones examined, only androstenedione showed evidence of a positive relationship with minimum age ($r_s = 0.383$, $p = 0.023$), indicating that older females tended to exhibit higher androstenedione concentrations. There was little or no evidence for relationships between minimum age and testosterone ($r_s = 0.262$, $p = 0.129$), estrone ($r_s = 0.074$, $p = 0.672$), estradiol ($r_s = 0.150$, $p = 0.388$), or progesterone ($r_s = -0.041$, $p = 0.817$).

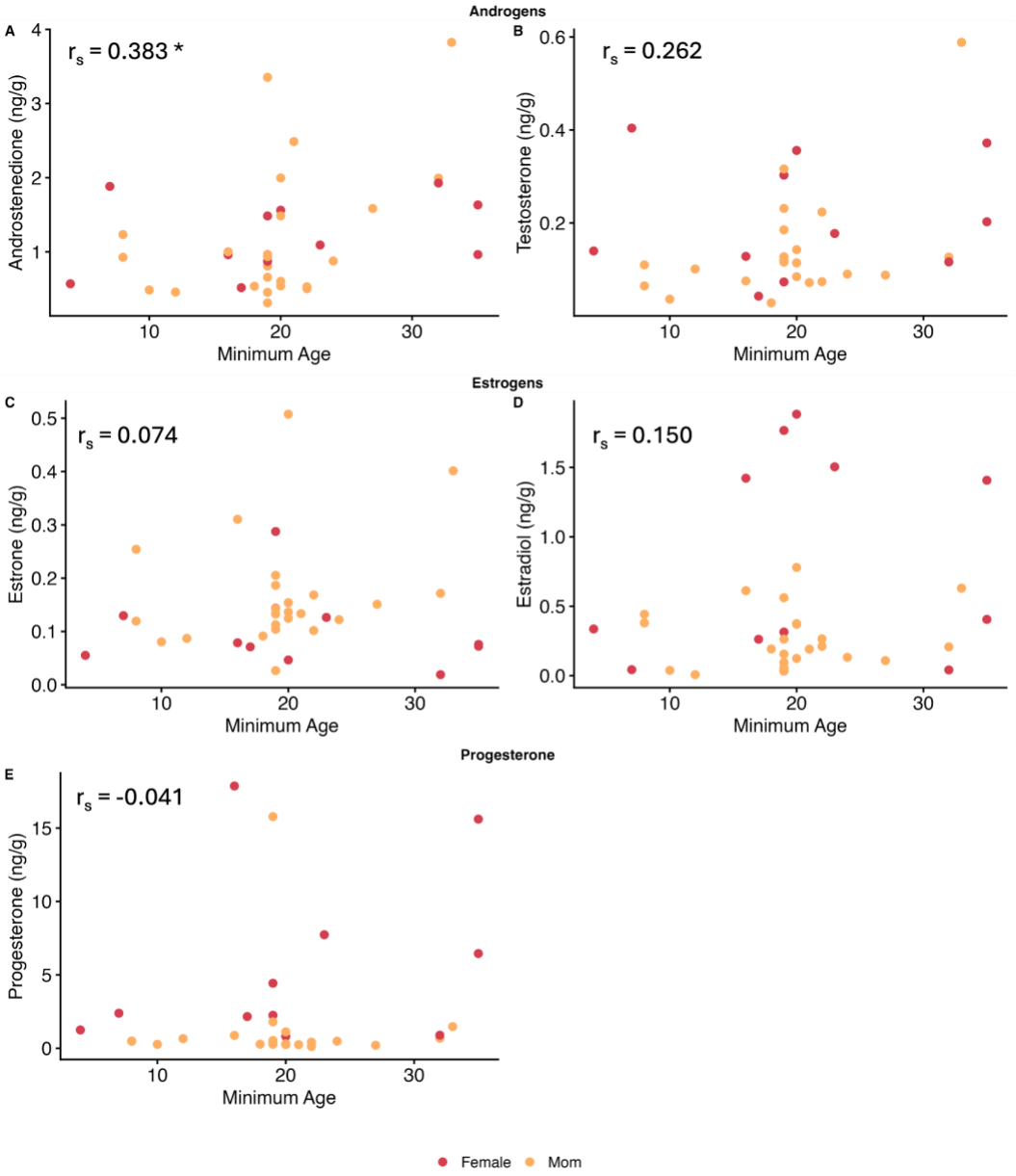

Figure S2.2. Relationships between minimum age and reproductive hormone concentrations in adult female humpback whales sampled on the Hawai‘i breeding grounds. Panels show Spearman rank correlations between minimum age and concentrations of (A) androstenedione, (B) testosterone, (C) estrone, (D) estradiol, and (E) progesterone for mothers (orange) and females without calves (red). Spearman correlation coefficients (r_s) are shown within each panel, and the asterisk (*) in panel A denotes moderate evidence for a correlation ($p < 0.05$).

Chapter 3: Trends in glucocorticoid levels in the North Pacific humpback population

3.1 Abstract

Blubber glucocorticoids are increasingly used to assess physiological state in cetaceans, yet the biological factors underlying natural variation remain poorly understood in humpback whales (*Megaptera novaeangliae*). This study quantified blubber cortisol and corticosterone concentrations in North Pacific humpback whales sampled on the Hawai‘i breeding grounds and Alaska feeding grounds to characterize variation across season, reproductive class, and sex, while also evaluating the influence of social group composition within Hawai‘i. Cortisol and corticosterone were positively correlated, although cortisol consistently occurred at higher concentrations, indicating that cortisol is the predominant glucocorticoid measured in humpback whale blubber. Cortisol concentrations were higher in Hawai‘i than in Alaska, consistent with the increased physiological demands associated with fasting and reproduction. Within Hawai‘i, glucocorticoid concentrations varied seasonally, with males and females without calves generally declining across the breeding season, whereas mothers maintained relatively stable concentrations. On the breeding grounds, social group composition also influenced glucocorticoid concentrations. Cortisol concentrations were highest in competitive groups and males escorting females without calves, whereas corticosterone concentrations were lowest in lone males. Cortisol was positively correlated with testosterone in male humpback whales, supporting an association between glucocorticoid concentrations and reproductive activity. In

contrast, glucocorticoid variation in Alaska was comparatively limited, with little evidence of differences among reproductive classes.

These findings demonstrate that glucocorticoid concentrations reflect the combined influence of energetic state, reproductive activity, and social behavior, improving the interpretation of endocrine responses to environmental change and other stressors in humpback whales.

3.2 Introduction

Physiological measurements are increasingly used to understand how wild animals respond to changing environmental and ecological conditions while maintaining homeostasis. Because physiological responses often precede observable changes in behavior, survival, or reproduction, endocrine biomarkers provide valuable insight into the internal state of individuals and populations. However, directly assessing physiology in large marine mammals remains challenging, particularly in cetaceans (Hunt *et al.*, 2013). Consequently, hormone measurements have become an important tool for evaluating physiological condition, energetic state, and reproductive function in whales (Atkinson *et al.*, 2018; Maia *et al.*, 2025).

Among these hormones, glucocorticoids regulate energy balance during periods of increased physiological demand through activation of the hypothalamic–pituitary–adrenal (HPA) axis (Norris & Carr, 2020; Reeder & Kramer, 2005). In addition to mediating stress responses, they promote gluconeogenesis, lipolysis, and protein catabolism, and vary with reproductive state, nutritional condition, life-history stage, and other demographic factors (Atkinson *et al.*, 2015; Pallin *et al.*, 2022; Sapolsky *et*

al., 2000). In cetaceans, glucocorticoids can be measured from blood, feces, respiratory vapor, baleen, and blubber, with blubber becoming particularly valuable because remote biopsy sampling provides a minimally invasive method for assessing hormone concentrations throughout the annual cycle of large whales (Hunt *et al.*, 2013). Blubber glucocorticoids have now been measured in numerous cetacean species and have been associated with physiological stress responses and changing energetic demands (Agusti *et al.*, 2022; Boggs *et al.*, 2019; Champagne *et al.*, 2018; Cates *et al.*, 2020; Dalle Luche *et al.*, 2019, 2021; Mingramm *et al.*, 2020a; Pallin *et al.*, 2022). However, because glucocorticoid concentrations also vary naturally across life-history stages and ecological contexts, interpreting these hormones in wild cetaceans requires an understanding of natural physiological variation.

North Pacific humpback whales are migratory capital breeders that experience changing energetic demands between feeding and breeding seasons (van Aswegen *et al.*, 2025a). During the summer feeding season in high-latitude regions such as Alaska, humpback whales accumulate energy reserves that are subsequently relied upon during the winter breeding season in Hawai‘i, where feeding is limited or absent (Baker *et al.*, 1986; Chittleborough, 1965; Dawbin, 1966; Nishiwaki, 1966). As a result, whales on the Hawai‘i breeding grounds must balance the energetic demands of reproduction while fasting. Whales occupying the Hawai‘i breeding grounds represent multiple reproductive and behavioral contexts, including males, females without calves, and mothers with calves (Darling *et al.*, 1983). Social group composition and energetic demands vary among these reproductive classes. Females without calves are commonly

observed in male–female pairs or multi-male competitive groups and are thought to primarily use the breeding grounds for mating (Darling, 1983; Glockner-Ferrari & Ferrari, 1985; Mobley & Herman, 1985). Mothers may occur alone with a calf or accompanied by one or more male escorts while simultaneously supporting the energetic demands of lactation and calf growth (Glockner & Venus, 1983; Mobley & Herman, 1985; van Aswegen *et al.*, 2025a). Males engage in a range of energetically demanding breeding behaviors, including singing, escorting, and competition for access to females (Baker & Herman, 1984; Darling, 1983; Darling & Bérubé, 2001). Collectively, these differences in reproductive state, behavior, and energetic demand are expected to contribute to natural variation in glucocorticoid concentrations.

Despite these advances, gaps remain in understanding how glucocorticoid concentrations vary across the annual cycle of humpback whales. Most previous studies have focused on a single glucocorticoid, geographic region, or demographic group, limiting our understanding of how intrinsic biological factors influence endocrine variation (Cates *et al.*, 2020; Dalle Luche *et al.*, 2021; Mingramm *et al.*, 2020a; Pallin *et al.*, 2022). In particular, relatively few studies have simultaneously examined both cortisol and corticosterone or evaluated how reproductive class, seasonal timing, and social interactions influence glucocorticoid concentrations within the same population. Improving our understanding of this natural variability is critical for interpreting glucocorticoids as biomarkers of physiological condition, energetic demand, and disturbance in humpback whales.

This study aimed to (1) assess the relationship between cortisol and corticosterone concentrations in humpback whale blubber, (2) characterize natural glucocorticoid variation between the Alaska feeding grounds and Hawai‘i breeding grounds across sex and reproductive classes, and (3) evaluate how glucocorticoid concentrations varied among reproductive classes and social group types within the Hawai‘i breeding grounds, while also examining reproductive-class differences within Alaska. Glucocorticoid concentrations were expected to be higher during the Hawai‘i breeding season, reflecting the increased energetic demands associated with reproduction and fasting. Additionally, glucocorticoid concentrations were expected to differ among reproductive classes and social group types, with the highest concentrations occurring in competitive groups, which were expected to exhibit the greatest energetic demands. Together, these findings improve our understanding of natural glucocorticoid variation in humpback whales and provide important physiological context for interpreting glucocorticoids as biomarkers of energetic state and population health in conservation and monitoring applications.

3.3 Methods

3.3.1 Field observations and classifications

Biopsy samples from humpback whales (*Megaptera novaeangliae*) were collected in Hawai‘i during the boreal winter breeding season (December–April) from 2014–2025 in the Au‘au, Pailolo, and Kalohi channels (20.88°N, 156.89°W; Figure I.2). Additional biopsy samples were collected from humpback whales in Southeast Alaska

during the boreal feeding season (July) from 2023–2025 in Chatham Strait (56.95°N, 134.62°W).

Each whale was assigned to an age class (immature or mature) based on relative body size. Immature whales were defined as individuals larger than a calf but appreciably smaller than full-sized adults. Whales in Hawai‘i were further classified into one of three reproductive classes: male, female without a calf, or mother–calf. Whales sampled in Alaska were assigned to one of four reproductive classes: male, female without a calf, pregnant female, or mother. Pregnancy status in Alaska was determined using the validated progesterone classification model described by Pallin *et al.* (2018). Social group composition was also assigned for whales sampled in Hawai‘i, with group categories differing slightly among reproductive classes (Table 3.1). Photographs of the ventral flukes of sampled whales were taken for individual identification and uploaded to Happywhale.

3.3.2 Biopsy sample collection

Skin/blubber biopsies were collected using a crossbow system fitted with a modified bolt and 40-mm barbed hollow tip (CetaDart). Samples were taken from the mid-lateral region beneath the dorsal fin. Following collection, the biopsy dart was retrieved from the water within approximately three minutes, and the sample was immediately placed into a sterile vial or bag before being stored on ice until returning to shore. For each biopsy, the sample location, time, GPS coordinates, and whale reaction (scored 0–3 following Weinrich *et al.*, 1992) were recorded. Upon return to

shore, samples were stored at −20 °C and transferred to −80 °C at the end of each field season.

3.3.3 Genetic sex determination

Skin subsamples (~25 mg) were used for genetic sex determination. Samples collected before 2022 were processed at the NOAA Southwest Fisheries Science Center (SWFSC; La Jolla, CA). DNA from samples collected in 2022 or later was extracted at the University of California, Santa Cruz, using a Qiagen DNEasy kit and subsequently sent to the Cetacean Conservation and Genetics Laboratory at Oregon State University for sex assignment.

3.3.4 Hormone extraction and quantification

Steroid hormone extraction followed a protocol adapted by N. Kellar (SWFSC; pers. comm.) from Kellar *et al.* (2006). Blubber samples were cut perpendicular to the skin into 0.05–0.1 g subsamples, placed in stainless-steel microvials with a ¼" ceramic bead, and homogenized in ethanol. A negative control and a positive control spiked with known concentrations of cortisol and corticosterone were prepared from stranded humpback whale blubber.

Following homogenization, samples underwent sequential washes with ethanol and a 4:1 ethanol:acetone solution and centrifugation. The resulting supernatant was evaporated in a dry bath, and the remaining lipid pellet was weighed. Acetonitrile and hexane were added to separate steroids from remaining lipids; the hexane (lipid) layer

was removed, and the steroid-containing acetonitrile phase was dried and reconstituted in PBS buffer with BSA.

Commercial enzyme immunoassay (EIA) kits were used to quantify cortisol (F) and corticosterone (B) (Table S3.1). To improve detection at low hormone concentrations, two additional standards (25 and 12.5 pg/mL) were added to the cortisol assay, and one additional standard (9.766 pg/mL) was added to the corticosterone assay. Extraction efficiency was calculated by subtracting the measured negative-control concentration from the positive control, with efficiencies >60% considered acceptable. Samples exceeding kit detection limits were diluted and reanalyzed; samples below detection limits were assigned a value equal to one-half of the lowest standard to avoid censoring.

To avoid pseudoreplication, only the first biopsy collected from each individual was retained for analysis. Juveniles were excluded from the main statistical analyses but were summarized relative to mature males in the Supplemental Materials (Table S3.2).

3.3.5 Assay Validation

Parallelism and accuracy of the corticosterone enzyme immunoassay (EIA) were evaluated to assess its suitability for humpback whale blubber extracts. Assay validation for cortisol has been previously established and is described elsewhere (Pallin *et al.*, 2022). For the parallelism assessment, blubber extracts from 10 individuals were pooled and serially diluted (neat to 1:64, twofold dilutions). Serial dilutions of pooled blubber extract were compared to assay standards to evaluate

parallelism. Accuracy was assessed by comparing observed concentrations to expected values across the dilution series using linear regression, with the slope and coefficient of determination (R^2) used to assess model fit.

3.3.6 Statistical analysis

All statistical analyses were performed in R (R Core Team, 2025). Statistical support for effects was interpreted using the evidence framework outlined by Muff *et al.* (2022), where p-values were categorized as little or no evidence ($p > 0.1$), weak evidence (0.05–0.1), moderate evidence (0.01–0.05), strong evidence (0.001–0.01), and very strong evidence (≤ 0.001). Glucocorticoid concentrations were analyzed using generalized linear mixed-effects models (GLMMs) with a Gamma error distribution and log link. Candidate models were evaluated using Akaike's Information Criterion (AIC), Type II ANOVA, residual diagnostics, and biological interpretability. Fixed effects and interaction terms showing little or no evidence of an effect ($p > 0.1$) were removed during model simplification unless retained for biological relevance to the study objectives. Sampling day was standardized within each region such that the first sampling date across all years was assigned as day 1 (Hawai‘i: December 17; Alaska: July 16).

Correlations among hormones. Relationships between cortisol and corticosterone were first evaluated across the combined dataset using Spearman's rank correlation because the overall relationship was non-linear and violated assumptions of normality. Hawai‘i and Alaska were then analyzed separately using Pearson

correlations on log-transformed concentrations because within-region relationships were approximately linear. Females sampled in Alaska were analyzed using Spearman's rank correlation due to the small sample size and weaker linear relationship between hormones.

Regional comparisons. Regional differences in blubber glucocorticoid concentrations between the Hawai‘i breeding grounds and the Alaska feeding grounds were modeled separately for cortisol and corticosterone using GLMMs. Year was included as a random intercept in all models. Hawai‘i samples included cortisol concentrations from 2014–2025 and corticosterone concentrations from 2024–2025, whereas Alaska samples included both cortisol and corticosterone concentrations from 2023–2025.

For each hormone, full models included region, sex, adjusted sampling day, a quadratic sampling day term, and all two-way interactions. Region and sex were retained in all candidate models to facilitate direct comparisons between seasons and sexes. Additional models replaced sex with reproductive class (male, female without a calf, mother) to evaluate variation among reproductive groups.

Estimated marginal means for each region were obtained from the final models, back-transformed to the response scale, and calculated at the mean adjusted sampling day across the combined Hawai‘i and Alaska dataset.

Hawai‘i trends. Variation in glucocorticoid concentrations across the Hawai‘i breeding season was evaluated separately for cortisol and corticosterone using

GLMMs with year included as a random intercept. Fixed effects included reproductive class, adjusted sampling day, and a quadratic sampling-day term to test for nonlinear seasonal trends. Interactions between reproductive class and sampling-day terms were retained when supported.

Separate models were fit within each reproductive class to evaluate whether social group composition influenced glucocorticoid concentrations. Because social group categories differed among reproductive classes, each class was analyzed independently (Table 3.1). Separate models using sampling month as a categorical predictor were also fit within each reproductive class to further evaluate seasonal trends. In males, the relationship between cortisol and testosterone concentrations was evaluated using a Pearson correlation test.

To examine age-related variation in glucocorticoid concentrations in Hawai‘i, minimum age estimates were obtained for a subset of individuals from long-term sighting histories. Minimum age was assigned to whales that had been sighted at least 10 times across the breeding and/or feeding grounds. For females classified as mothers, a minimum age of eight years was assigned, consistent with the earliest reported age at first reproduction in humpback whales (Gabriele *et al*., 2007). Spearman correlation tests were conducted separately within each reproductive class to assess relationships between minimum age and glucocorticoid concentrations.

Alaska trends. Cortisol and corticosterone trends within Alaska were evaluated using samples collected during five sampling days spanning 16–30 July across three

years (2023–2025). Sampling date was standardized so that the first sampling day across all years was day 1.

Hormone concentrations were analyzed using GLMMs. Fixed effects included adjusted sampling day, reproductive class, and their interaction. Year was included as a random intercept to account for repeated sampling across years. Reproductive classes were defined as males, females without calves, pregnant females, and mothers. Pregnancy status was assigned using the validated binomial logistic regression model from Pallin *et al.* (2018) based on blubber progesterone concentrations.

3.4 Results

3.4.1 Assay Validation

Corticosterone demonstrated strong parallelism and accuracy in humpback whale blubber extracts. Serial dilutions of pooled extract produced binding curves parallel to assay standards (Figure S3.3.1a), and observed concentrations closely matched expected concentrations across dilutions ($R^2 = 0.9995$, slope = 1.0007; Figure S3.3.1b).

3.4.2 Correlations among hormones

Cortisol and corticosterone exhibited a consistent positive correlation across both regions combined (Spearman's $\rho = 0.33$, $p < 0.001$) and within each region separately (Hawai‘i: $r = 0.31$, $R^2 = 0.09$, $p < 0.001$; Alaska: $r = 0.33$, $R^2 = 0.11$, $p = 0.009$; Figure 3.1).

When analyzed separately by sex, positive associations between cortisol and corticosterone were generally stronger in males than in females across both regions

(Figure 3.1). Females sampled in Alaska showed little evidence of an association between the two hormones ($p = 0.20$; Figure 3.1).

3.4.3 Regional comparisons

Cortisol. Cortisol concentrations were higher during the Hawai‘i breeding season than during the Alaska feeding season (Figure 3.2). In the sex-based model, region showed moderate evidence of an effect on cortisol concentrations, with higher values in Hawai‘i than in Alaska ($p = 0.021$; Table 3.2). In contrast, cortisol concentrations did not differ between sexes ($p = 0.112$), although there was moderate support for an interaction between sex and sampling day ($p = 0.024$), indicating that temporal patterns differed between males and females.

Replacing sex with reproductive class yielded similar results, with higher cortisol concentrations in Hawai‘i than in Alaska and little evidence of differences among reproductive classes. Observed mean concentrations, ranges, and sample sizes by region and sex are provided in Table S3.3.

Corticosterone. There was little evidence that corticosterone differed between Hawai‘i and Alaska. Instead, corticosterone concentrations were primarily associated with sampling day. There was little evidence that either region ($p = 0.790$) or sex ($p = 0.735$) affected corticosterone concentrations (Table 3.2). However, adjusted sampling day showed a strong negative relationship with corticosterone concentrations ($\beta = -0.006 \pm 0.001$ SE, $p < 0.001$), indicating that corticosterone

declined over time across regions. Raw mean concentrations, ranges, and sample sizes by region and sex are provided in Table S3.3.

3.4.4 Hawai‘i trends

Cortisol across reproductive class. Within Hawai‘i, seasonal patterns in cortisol differed among reproductive classes, driven primarily by mothers, which exhibited a distinct temporal trajectory relative to males and females without calves (Figure 3.3a). The final model included linear and quadratic effects of sampling day, indicating seasonal changes in cortisol concentrations (Table 3.3). There was little evidence that overall cortisol concentrations differed among reproductive classes ($\chi^2_2 = 4.10$, $p = 0.129$), although mothers differed from males in both the linear and quadratic seasonal trajectories, whereas females without calves exhibited seasonal patterns similar to males (Table 3.3).

Corticosterone across reproductive classes. Seasonal patterns in corticosterone also differed among reproductive classes, driven primarily by mothers (Figure 3.3b). The final model included both linear and quadratic effects of sampling day, indicating seasonal changes in corticosterone concentrations (Table 3.3). There was little evidence that overall corticosterone concentrations differed among reproductive classes ($\chi^2_2 = 0.635$, $p = 0.728$). However, mothers differed from males in both the linear and quadratic seasonal trajectories, whereas females without calves exhibited seasonal patterns similar to males (Table 3.3).

Cortisol across social groups. The effect of social group composition on cortisol differed among reproductive classes. Social group composition influenced cortisol concentrations only in males ($\chi^2_3 = 13.3$, $p = 0.004$; Figure 3.4). Relative to lone males, males in competitive groups exhibited higher cortisol concentrations, whereas males escorting females without calves showed a more moderate increase in cortisol. Males escorting mother–calf pairs did not differ from lone males (Figure 3.4). Male cortisol also changed across the breeding season, with both linear and quadratic effects of sampling day (Adjusted Day: $\beta = -0.030 \pm 0.009$ SE, $p = 0.001$; Adjusted Day^2: $\beta = -0.0002 \pm 0.00008$ SE, $p = 0.007$). Additionally, male humpback whales in Hawai‘i exhibited very strong evidence of a positive correlation between log-transformed cortisol and testosterone concentrations (Pearson's $r = 0.272$, $R^2 = 0.074$, $p < 0.001$).

In contrast, females without calves showed weak evidence of cortisol differences among social group types ($\chi^2_1 = 3.08$, $p = 0.079$), whereas there was little evidence that cortisol differed among social group types in mothers ($\chi^2_2 = 2.56$, $p = 0.278$). Females without calves also exhibited nonlinear seasonal patterns (Adjusted Day^2: $p = 0.006$), whereas mothers showed little evidence of seasonal variation (Adjusted Day: $p = 0.977$).

Corticosterone across social groups. The effect of social group composition on corticosterone also differed among reproductive classes. Social group composition influenced corticosterone concentrations only in males ($\chi^2_3 = 11.2$, $p = 0.010$; Figure 3.4). Relative to lone males, males in competitive groups, males escorting females

without calves, and males escorting mother–calf pairs all exhibited higher corticosterone concentrations. Male corticosterone also changed across the breeding season, with both linear and quadratic effects of sampling day (Adjusted Day: $\beta = -0.029 \pm 0.007$ SE, $p < 0.001$; Adjusted Day^2: $\beta = -0.0002 \pm 0.00005$ SE, $p < 0.001$).

In contrast, females without calves and mothers showed little evidence of group-related differences in corticosterone concentrations (females without calves: $\chi^2_1 = 0.540$, $p = 0.462$; mothers: $\chi^2_2 = 1.17$, $p = 0.556$). Females without calves exhibited seasonal changes in corticosterone concentrations (Adjusted Day^2: $p = 0.034$), whereas mothers showed little evidence of an overall seasonal pattern (Adjusted Day: $p = 0.854$).

3.4.5 Alaska trends

Cortisol. In Alaska, cortisol concentrations showed limited overall differentiation among reproductive classes (males, females without calves, pregnant females, and mothers; $\chi^2_3 = 5.35$, $p = 0.148$; Figure 3.5). However, there was moderate evidence that seasonal patterns differed among groups (Adjusted Day × Reproductive Class: $p = 0.048$), and sampling day showed a moderate overall effect on cortisol concentrations ($\chi^2_1 = 6.18$, $p = 0.013$). In the final model, females without calves exhibited higher cortisol concentrations than males (0.730 ± 0.253 SE, $p = 0.004$).

Corticosterone. In contrast to cortisol, there was little evidence that corticosterone concentrations in Alaska varied among reproductive classes (χ^2_3 = 2.17, p = 0.538) or across sampling day (χ^2_1 = 2.42, p = 0.120; Figure 3.5).

3.5 Discussion

Glucocorticoid concentrations in North Pacific humpback whales vary across seasonal, reproductive, and social contexts, likely reflecting multiple physiological processes associated with fasting, reproduction, and breeding behavior. Cortisol concentrations were consistently higher than corticosterone concentrations and were elevated during the Hawai‘i breeding season, suggesting that cortisol is the dominant glucocorticoid expressed in humpback whale blubber. Within the breeding season, endocrine profiles further differed among reproductive classes and male social group types, highlighting additional sources of natural glucocorticoid variation. Together, these findings provide a framework for interpreting glucocorticoid variation across different biological contexts in humpback whales.

3.5.1 Correlations among hormones

Cortisol and corticosterone were positively correlated in both Hawai‘i and Alaska, indicating that the two glucocorticoids generally covary in humpback whale blubber. However, cortisol concentrations consistently exceeded corticosterone concentrations across both regions and sexes, indicating that cortisol is the predominant glucocorticoid measured in humpback whale blubber. This finding is consistent with previous studies of cetacean blubber reporting higher cortisol than corticosterone concentrations (Boggs

et al., 2019; Dalle Luche *et al.*, 2019, 2021), but differs from the rationale underlying Cates *et al.* (2020), which focused exclusively on corticosterone in males based on unpublished observations suggesting sex-specific glucocorticoid predominance. By quantifying both hormones in males and females, the present study demonstrates that cortisol predominates in both sexes while corticosterone exhibits broadly similar patterns of variation. The relationship between cortisol and corticosterone appeared stronger in male humpbacks, although this was weakly supported (Figure 3.1). The weaker relationship observed in females, particularly in Alaska, may reflect greater endocrine variability associated with reproductive state, although the relatively small sample size makes it difficult to distinguish biological patterns from sampling effects.

3.5.2 Regional comparisons

Cortisol concentrations were higher in Hawai‘i compared to Alaska, whereas corticosterone did not differ between regions. Glucocorticoids promote lipolysis, protein catabolism, and gluconeogenesis, thereby mobilizing energy reserves to support increased metabolic demands (Atkinson *et al.*, 2015; Norris & Carr, 2020; Reeder & Kramer, 2005). The role of glucocorticoids in mobilizing energy reserves is highly conserved across vertebrates (Atkinson *et al.*, 2015; Romero, 2002). However, seasonal changes in glucocorticoid concentrations during reproduction are more variable among mammals and appear to depend on species-specific life-history strategies and energetic demands (Romero, 2002). Elevated glucocorticoid concentrations during periods of fasting and reproduction have been documented in several seasonally breeding marine mammals and are generally associated with the

increased metabolic demands of reproduction (Burgess *et al.*, 2013; Hunt *et al.*, 2018). In fasting capital breeders, elevated glucocorticoid concentrations are thought to facilitate prolonged fasting while supporting reproductive investment, as demonstrated in northern elephant seals and harbor seals (Champagne *et al.*, 2015; Ensminger *et al.*, 2014; Kershaw & Hall, 2016). As migratory capital breeders, humpback whales similarly rely on stored energy reserves while fasting on the breeding grounds and engaging in energetically demanding activities such as mate competition and, for females, lactation (Christiansen *et al.*, 2016; Darling *et al.*, 1983; Irvine *et al.*, 2017). Seasonal variation in glucocorticoid concentrations has also been reported in humpback whales, including higher cortisol concentrations during migration toward the breeding grounds than during the return migration to feeding grounds (Cates *et al*., 2020; Mingramm *et al*., 2020a). The elevated cortisol concentrations observed in Hawai‘i in the present study are therefore consistent with the increased physiological demands associated with fasting and reproduction.

Despite higher cortisol concentrations in Hawai‘i than in Alaska, the regional models provided little evidence that glucocorticoid concentrations differed among reproductive classes or between sexes. Sex differences in baseline glucocorticoid concentrations have been reported across mammals, although both the magnitude and direction of these differences vary among species (Reeder & Kramer, 2005; Romero, 2002). Likewise, glucocorticoid concentrations associated with female reproductive states, including pregnancy and lactation, vary among species, reproductive stages, and biological matrices, reflecting differences in reproductive strategy and life-history

rather than a universal mammalian pattern (Brown & Lehnhardt, 1995; Burgess *et al.*, 2013; Edwards & Boonstra, 2018; Reeder & Kramer, 2005). Within cetaceans, studies of other rorqual whales have also reported little evidence of sex differences in blubber glucocorticoid concentrations (Atkinson *et al.*, 2020; Melica *et al.*, 2022). Previous studies of humpback whales have likewise reported little overall variation in blubber glucocorticoid concentrations among demographic groups. Mingramm *et al.* (2020a) found no differences in cortisol among Southern Hemisphere humpback demographic groups, and Pallin *et al.* (2022) reported little overall sex-related variation during the Antarctic feeding season, despite some differences among female reproductive groups. Because pregnancy status was not included in the regional models, additional variability associated with female reproductive state may have obscured differences among females in the present study. Although overall glucocorticoid concentrations did not differ between sexes, the moderate interaction between sampling day and sex for cortisol indicates that males and females followed different seasonal trajectories, suggesting that sex-specific physiological demands may become more apparent through seasonal changes than through differences in overall hormone concentrations.

3.5.3 Hawai‘i trends

Cortisol and corticosterone exhibited broadly similar seasonal patterns across the Hawai‘i breeding season. Seasonal trajectories differed among reproductive classes, with males and females without calves generally showing declining concentrations through much of the breeding season, whereas mothers maintained relatively stable glucocorticoid concentrations. Although the fitted models included a modest quadratic

component for males and females without calves, the predominant pattern was an early-season decline followed by comparatively little change later in the season. Consistent with this interpretation, separate analyses of mothers provided little evidence of seasonal variation in either cortisol or corticosterone concentrations.

The relatively stable glucocorticoid concentrations observed in mothers likely reflect the sustained energetic demands of lactation during prolonged fasting. Across mammals, gestation and lactation represent the most metabolically expensive periods of a female's life history (Gittleman & Thompson, 1988), and these energetic demands are accompanied by shifts in glucocorticoid regulation, although patterns vary among species (Reeder & Kramer, 2005). In fasting marine mammals, glucocorticoids are thought to facilitate the mobilization of endogenous energy reserves during lactation, and elevated cortisol concentrations have been associated with increased milk lipid content in northern elephant seals (Houser *et al.*, 2007; Fowler *et al.*, 2016). However, glucocorticoid patterns during lactation differ among species. For example, southern right whales exhibit declining blubber cortisol concentrations toward the end of the calving season, a pattern thought to reflect reduced maternal energetic demands as calf growth slows (Shuttleworth *et al.*, 2026). In contrast, calf body volume in North Pacific humpback whales increases steadily throughout the breeding season (van Aswegen *et al.*, 2025a), suggesting that mothers continue to invest substantial energy in calf growth while fasting. These continued energetic demands may help explain the relatively stable glucocorticoid profiles observed in mothers throughout the breeding season.

Interpretation of seasonal glucocorticoid patterns in females without calves is complicated by the heterogeneous composition of this reproductive class. Females without calves may include individuals occupying multiple reproductive states, including estrous and pregnant females, each of which may exhibit distinct endocrine profiles and seasonal trajectories. In addition, females without calves are thought to have shorter residence times on the breeding grounds than either mature males or mothers, reflecting their primary role in mating rather than prolonged breeding-ground residency (Craig *et al.*, 2001; Darling *et al.*, 1983). Consequently, variation in reproductive state, residency time, and asynchronous arrival and departure from the breeding grounds may have contributed to the greater glucocorticoid variability observed within this group and obscured broader seasonal patterns.

In contrast to females, seasonal glucocorticoid variation in males may be shaped by both temporal changes and the energetic demands associated with breeding behavior and male–male competition. Males exhibited a more pronounced decline in glucocorticoid concentrations through the first half of the breeding season than females without calves. Elevated glucocorticoid concentrations early in the breeding season are consistent with previous studies showing higher cortisol concentrations in humpback whales migrating toward breeding grounds than during the return migration to feeding areas (Dalle Luche *et al.*, 2021; Mingramm *et al.*, 2020a), suggesting greater physiological demands during the onset of the breeding season. However, those studies compared different phases of the annual migration and did not examine temporal changes within a breeding ground. Similarly, Cates *et al.* (2020) reported seasonal

variation in blubber corticosterone concentrations among male humpback whales in Hawai‘i, although the strength of this relationship varied among years, highlighting substantial interannual variability within this population. These observations are consistent with seasonal changes in reproductive activity and breeding behavior among males.

Social group composition explained variation in glucocorticoid concentrations only among males, suggesting that endocrine profiles are associated with behavioral context during the breeding season. Males engaged in competitive groups, and those escorting females without a calf, exhibited the highest cortisol concentrations, whereas males observed alone or escorting mothers had comparatively lower concentrations. Similarly, corticosterone concentrations were lowest in lone males and elevated across all social group types involving interactions with other whales. Elevated glucocorticoid concentrations associated with aggression and reproductive competition have been reported across a variety of mammals (Creel, 2005; Goymann & Wingfield, 2004; Pavitt *et al.*, 2015). Comparable patterns have also been observed in marine mammals, where glucocorticoids increase during periods of social competition and reproductive activity (Burgess *et al.*, 2013; Hunt *et al.*, 2006; Mashburn & Atkinson, 2007). For male humpback whales, these findings indicate that social interactions are an important source of glucocorticoid variation during the breeding season, consistent with the increased physiological demands associated with reproductive competition.

Male humpback whales employ flexible breeding strategies that include singing, escorting females, and competing in multi-male groups for access to females (Darling

& Bérubé, 2001; Dunlop & Frere, 2023; Pack *et al.*, 2009; Tyack & Whitehead, 1983). Competitive interactions among males are energetically demanding and frequently involve aggressive displays and physical interactions (Baker & Herman, 1984; Darling *et al.*, 1983; Tyack & Whitehead, 1983), providing a plausible explanation for the elevated cortisol concentrations observed in competitive groups. Similarly, males escorting females without calves may have recently engaged in competitive interactions, as competitive groups frequently form and dissolve around male–female pairs (Darling *et al.*, 1983; Glockner-Ferrari & Ferrari, 1985; Tyack & Whitehead, 1983). In contrast, lone males, which were commonly observed traveling, singing, or resting, exhibited lower glucocorticoid concentrations than males engaged in social interactions, consistent with reduced physiological demands associated with these behavioral contexts.

The positive association between cortisol and testosterone provides additional evidence that glucocorticoid concentrations are associated with reproductive activity in male humpback whales. Similar positive relationships between glucocorticoids and testosterone have been reported in male cetaceans and other mammals during periods of reproductive activity and social competition (Dalle Luche *et al.*, 2021; Hunt *et al.*, 2006, 2018; O'Brien *et al.*, 2017). Although glucocorticoids can suppress testosterone during periods of prolonged or severe stress (Champagne *et al.*, 2018; Ensminger *et al.*, 2014), elevated concentrations of both hormones may occur simultaneously during periods of increased reproductive effort and social competition. The positive

association observed here is therefore consistent with glucocorticoids supporting the physiological demands of reproduction rather than reflecting chronic stress alone.

3.5.4 Alaska trends

Compared to Hawai‘i, glucocorticoid patterns in Alaska showed weaker temporal trends and less differentiation among reproductive classes. This likely reflects the fundamentally different physiological demands associated with the feeding and breeding seasons. Unlike whales on the Hawai‘i breeding grounds, which experience prolonged fasting while investing in reproduction, whales sampled in Alaska were actively replenishing energy reserves during the feeding season. Consequently, energetic demands may have been more similar across reproductive classes, resulting in less pronounced glucocorticoid variation overall.

Consistent with this interpretation, cortisol and corticosterone concentrations showed little evidence of overall differences among reproductive classes in Alaska. Although pregnancy-associated variation in glucocorticoids has been reported in some marine mammals, particularly when measured in feces and baleen (Burgess *et al.*, 2013; Hunt *et al.*, 2006, 2014, 2019; Valenzuela-Molina *et al.*, 2018), cetacean blubber studies generally report comparatively little differentiation among reproductive classes (Atkinson *et al.*, 2020; Kellar *et al.*, 2015; Melica *et al.*, 2022), including humpback whales (Mingramm *et al.*, 2020a; Pallin *et al.*, 2022). The absence of strong reproductive-class differences observed here is therefore consistent with previous studies of cetacean blubber.

Despite the lack of overall reproductive-class differences, there was evidence that cortisol varied with sampling day among reproductive classes. However, because sampling occurred over a relatively short period of the feeding season, this interaction should be interpreted cautiously and may reflect a combination of temporal change and individual variability. Limited repeated sampling of four individuals supports the latter interpretation. Three whales exhibited slight increases in cortisol between successive biopsies collected within 0–2 days, whereas one individual showed a larger decrease over four days (Figure S3.3.2). Corticosterone concentrations were even more variable within individuals, consistent with previous observations of corticosterone variability in male humpback whales (Cates *et al.*, 2020). These observations suggest that glucocorticoid variation in Alaska was not fully explained by reproductive class alone and likely reflects additional biological and ecological factors not captured in this analysis.

3.5.5 Limitations and future work

Several factors likely contributed to the glucocorticoid variability observed in this study and should be considered when interpreting these findings. Cortisol and corticosterone concentrations are linked to energetic state and are influenced by body condition, age, environmental conditions, and responses to stressors (Atkinson *et al.*, 2015). Previous studies across marine mammals suggest that glucocorticoid concentrations can vary with age and body condition, although these relationships differ among species, tissue type, and life-history context (Burgess *et al.*, 2013; Cates *et al.*, 2020; Dalle Luche *et al.*, 2021; Melica *et al.*, 2022; O'Brien *et al.*, 2017;

Shuttleworth *et al.*, 2026; Trana *et al.*, 2016). In this study, calves and individuals in visibly poor body condition were excluded from sampling, and analyses of minimum age within Hawai‘i showed little evidence of age-related glucocorticoid patterns across reproductive classes. However, females without calves showed some evidence of declining corticosterone concentrations with increasing age, although sample sizes for this group were limited (Figure S3.3.2).

Vessel presence should also be considered, as elevated glucocorticoids in response to vessel traffic and associated noise have been reported in some cetacean populations (Pallin *et al.*, 2022; Rolland *et al.*, 2012). However, responses may differ among populations depending on habituation to human activity (Teerlink *et al.*, 2018), as may also be the case in Hawai‘i. Although efforts were made to minimize disturbance during sampling, the perfusion rate of circulating hormones into humpback whale blubber remains poorly understood. This complicates the interpretation of whether blubber hormone concentrations reflect acute responses to vessel presence or longer-term physiological processes. Studies in cetaceans have demonstrated positive relationships between circulating and blubber cortisol concentrations (Agusti *et al.*, 2022; Champagne *et al.*, 2017, 2018), supporting the use of blubber as a matrix for assessing physiological state. However, many of these studies were conducted under acute stress conditions or experimental HPA-axis stimulation, and the temporal dynamics of glucocorticoid incorporation into blubber under natural conditions remain poorly understood. Temperature and tissue perfusion differences may further influence hormone deposition and interpretation (Boggs *et al.*, 2019). Nevertheless, Mingramm

et al. (2020a) found no effect of encounter time or number of biopsy attempts on blubber cortisol concentrations in humpback whales, suggesting that blubber glucocorticoids may be less sensitive to short-term vessel interactions.

Future studies should integrate glucocorticoid analyses with known-age individuals, body condition measurements, and other physiological markers to better understand the sources of glucocorticoid variability and the role these hormones play in maintaining homeostasis in humpback whales. Greater emphasis on individual variability would also strengthen the interpretation of endocrine patterns, although the large population size in Hawai‘i (Cheeseman *et al.*, 2023) and low within-season resight rates observed in the present study (2.2–5.6% of individuals between 2021 and 2025) make repeated sampling of individuals challenging. Increasing use of UAS-based methods may help address some of these limitations by increasing resight rates while also providing body condition measurements (Christiansen *et al.*, 2016; Evans *et al.*, 2026; van Aswegen *et al.*, 2025a). Coupling glucocorticoid analyses with complementary metabolic and physiological markers, such as thyroid hormones or heart rate measurements, may further clarify energetic state and improve the interpretation of glucocorticoids as indicators of health.

3.6 Conclusions

This study provides the first characterization of blubber cortisol and corticosterone variation across seasonal, reproductive, and social contexts in North Pacific humpback whales. Cortisol was the predominant glucocorticoid measured in blubber and varied more strongly than corticosterone across biological contexts, particularly between the

Alaska feeding grounds and the Hawai‘i breeding grounds. Within Hawai‘i, glucocorticoid concentrations also varied with reproductive class, seasonal timing, and male social behavior, demonstrating that endocrine profiles reflect the combined influence of energetic state, reproduction, and breeding behavior rather than any single physiological process.

These findings highlight the importance of establishing natural endocrine variation before glucocorticoids can be interpreted as indicators of disturbance or population health. Because glucocorticoid concentrations are influenced by multiple intrinsic and extrinsic factors, this biological context must be considered when evaluating responses to environmental change or anthropogenic stressors. This framework provides a stronger foundation for applying glucocorticoids as biomarkers of physiological state in conservation and long-term monitoring of wild marine mammal populations.

3.7 References

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3.8 Tables

Table 3.1. Group type descriptions for humpback whales in Hawai‘i by reproductive class.

Reproductive class	Group type	Description
Male	Lone	Lone male either singing or traveling
	Pair	Male–female pair
	Mother–calf escort	Escort to a mother–calf pair
	Competitive group	A male in a multi-male competitive group with a single nuclear female
Female (no calf)	Pair	Male–female pair
	Competitive group	Female as the nuclear animal in a multi-male competitive group
Mother	Mother–calf	Mother with calf less than 1 year old
	Mother–calf escort	Mother with calf less than 1 year old and a male escort
	Competitive group	Mother as the nuclear animal in a multi-male competitive group

Table 3.2. Fixed-effect parameter estimates for final regional glucocorticoid models. Estimates (log scale), standard errors (SE), z-values, and p-values from GLMMs evaluating cortisol and corticosterone variation by region, sex, and adjusted sampling day, with year included as a random effect.

Hormone	Term	Estimate	Std. error	z value	p value
Cortisol	Region (HI)	0.353500	0.152956	2.311	0.0208
	Sex (F)	−0.278107	0.174744	−1.591	0.1115
	Adjusted Day	−0.004913	0.001981	−2.480	0.0132
	Sex (F) × Adjusted Day	0.005988	0.002658	2.252	0.0243
Corticosterone	Region (HI)	−0.034489	0.129498	−0.266	0.790
	Sex (F)	0.026038	0.077043	0.338	0.735
	Adjusted Day	−0.006087	0.001413	−4.308	1.65e−05

Table 3.3. Fixed-effect parameter estimates for the final Hawai‘i cortisol and corticosterone model by reproductive class. Estimates are shown on the log scale from a GLMM, with standard errors (SE), z-values, and p-values. Fixed effects included adjusted sampling day, a quadratic day term, reproductive class, and their interactions. Males were the reference class, and Adjusted Day 1 = December 17.

Hormone	Term	Estimate	Std. error	z value	p value
Cortisol	Intercept	6.510e−01	2.709e−01	2.403	0.016
	Adjusted Day	−3.784e−02	9.537e−03	−3.968	7.26e−05
	Repro Class (Female – no calf)	−5.921e−01	5.104e−01	−1.160	0.246
	Repro Class (Mother)	−2.086	8.061e−01	−2.587	0.010
	Adjusted Day^2	2.909e−04	8.426e−05	3.452	0.001
	Adjusted Day × Repro Class (Female – no calf)	3.472e−03	1.976e−02	0.176	0.861
	Adjusted Day × Repro Class (Mother)	8.228e−02	2.689e−02	3.060	0.002
	Adjusted Day^2 × Repro Class (Female – no calf)	7.847e−05	1.709e−04	0.459	0.646
	Adjusted Day^2 × Repro Class (Mother)	−6.270e−04	2.053e−04	−3.055	0.002
Corticosterone	Intercept	−3.771e−01	1.918e−01	−1.965	0.049
	Adjusted Day	−3.317e−02	7.842e−03	−4.230	2.33e−05
	Repro Class (Female – no calf)	4.030e−02	4.167e−01	0.097	0.923
	Repro Class (Mother)	−1.209e+00	5.382e−01	−2.247	0.025
	Adjusted Day^2	2.437e−04	7.008e−05	3.477	0.001
	Adjusted Day × Repro Class (Female – no calf)	−4.932e−03	1.688e−02	−0.292	0.770
	Adjusted Day × Repro Class (Mother)	4.705e−02	1.874e−02	2.510	0.012
	Adjusted Day^2 × Repro Class (Female – no calf)	4.542e−05	1.474e−04	0.308	0.758
	Adjusted Day^2 × Repro Class (Mother)	−3.713e−04	1.494e−04	−2.486	0.013

3.9 Figures

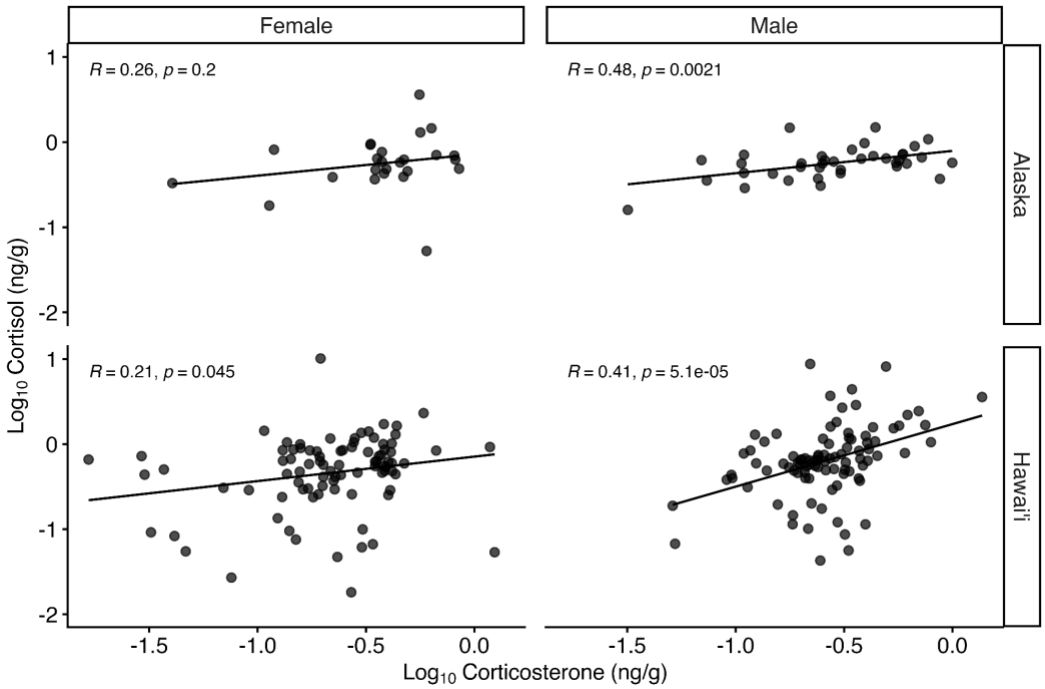

Figure 3.1. Relationship between $\log_{10}$ cortisol and $\log_{10}$ corticosterone concentrations in humpback whale blubber samples from Hawai‘i and Alaska. Panels are separated by region (rows) and sex (columns). Points represent individual samples, and solid lines indicate linear regression fits. Correlation coefficients are shown within each panel.

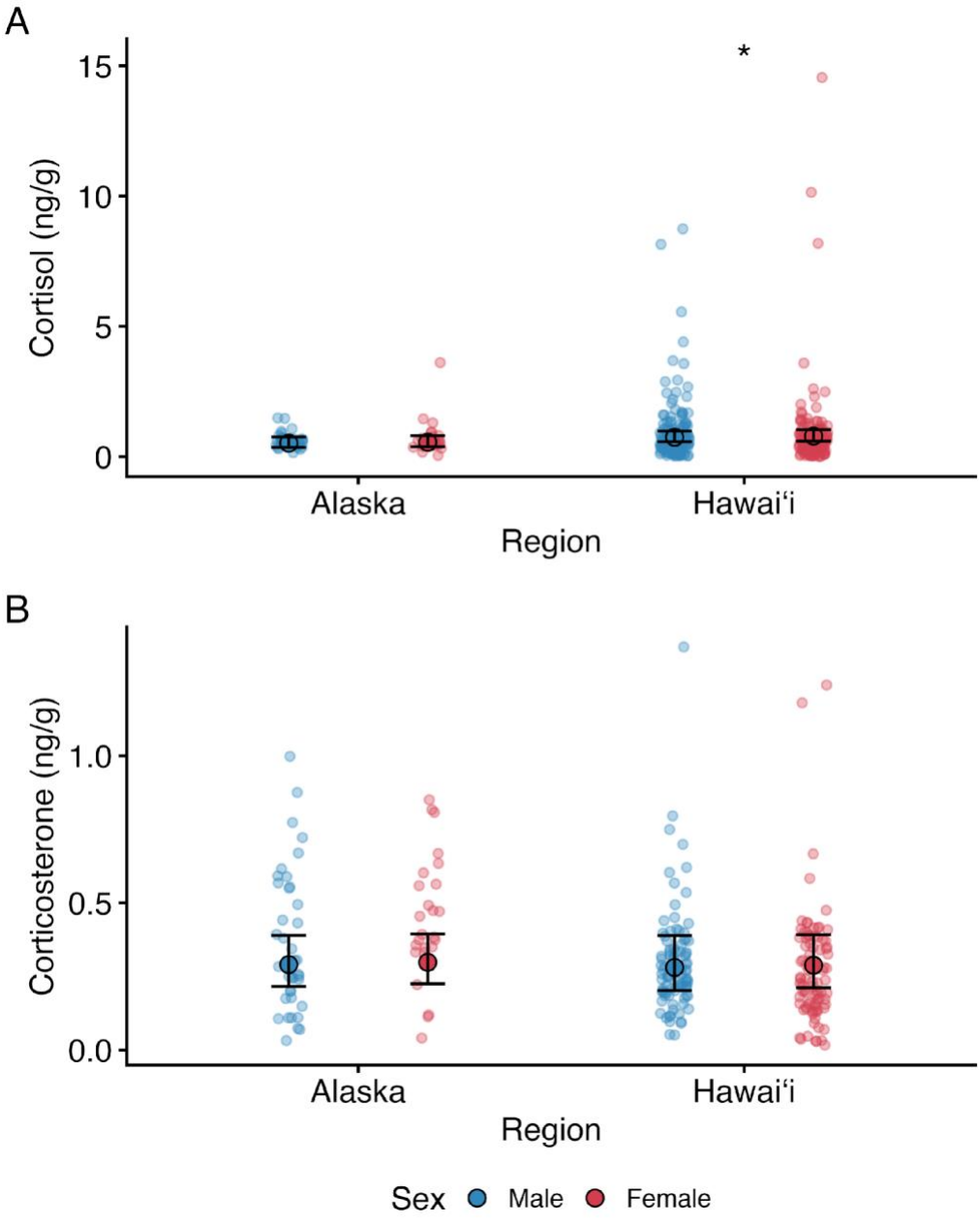

Figure 3.2. Model-predicted glucocorticoid concentrations in adult humpback whales sampled on the Hawai'i breeding grounds and Alaska feeding grounds, shown separately for males and females. Panels show (A) cortisol and (B) corticosterone concentrations. Large points and error bars represent estimated marginal means (±95% CI) predicted from the final generalized linear mixed-effects models (GLMMs) after accounting for sampling day and year. Smaller points represent individual hormone concentrations. An asterisk (*) denotes moderate or stronger statistical evidence for a regional difference ($p < 0.05$).

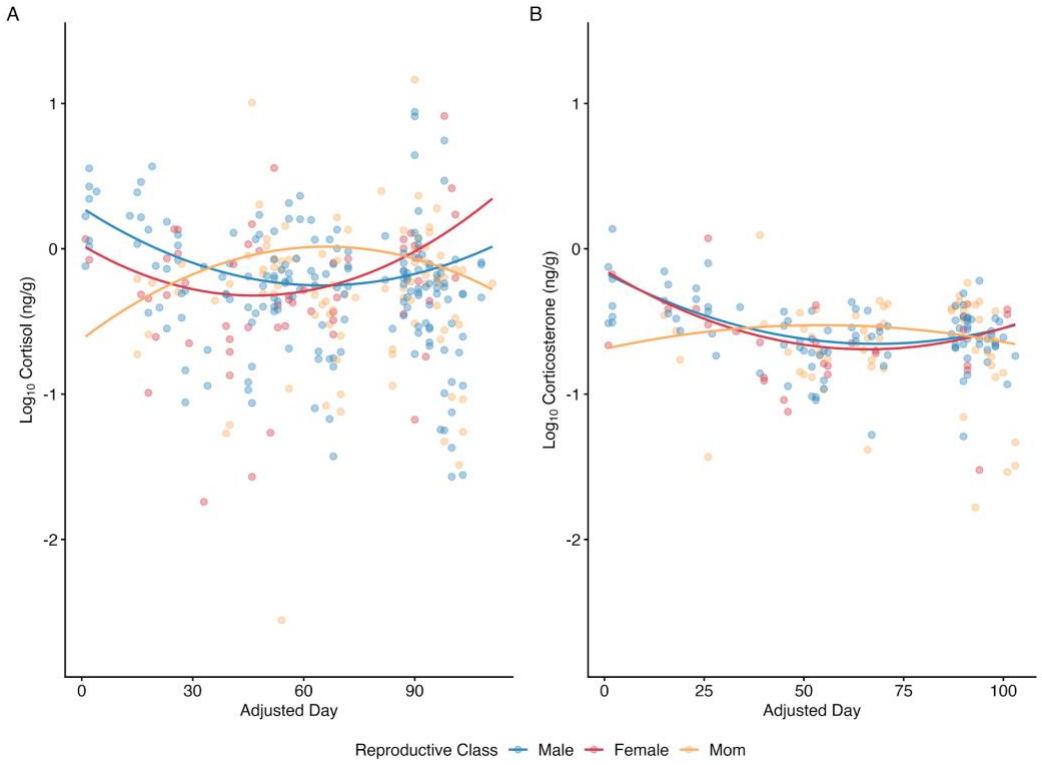

Figure 3.3. (A) $\log_{10}$ cortisol and (B) $\log_{10}$ corticosterone concentrations in Hawai‘i by reproductive class. Points represent individual samples, and lines represent model-predicted seasonal trajectories from the final GLMMs for males (blue), females without calves (red), and mothers (orange). Adjusted Day 1 = December 17.

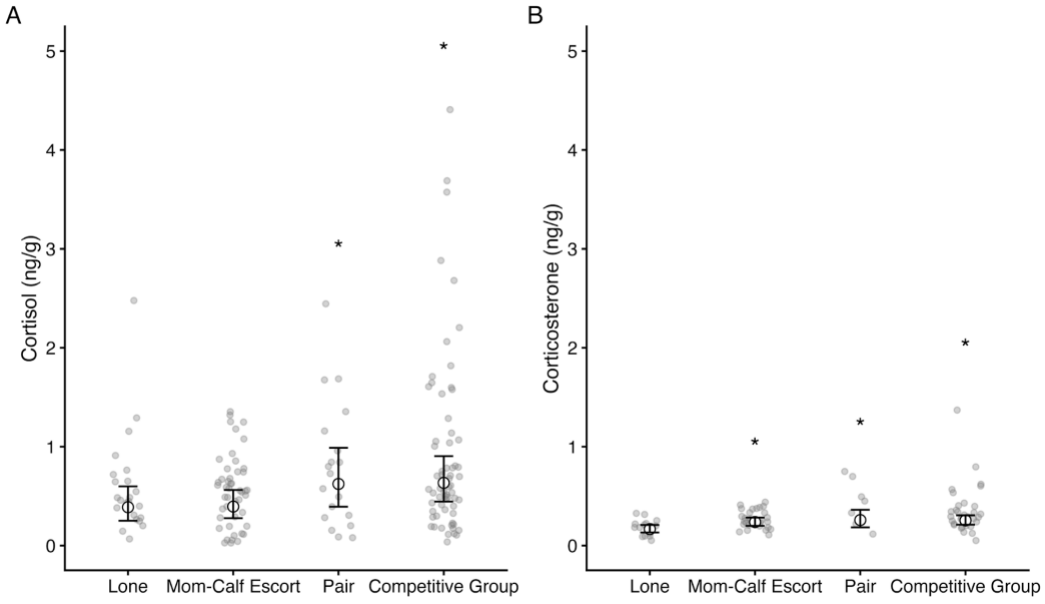

Figure 3.4. Model-predicted glucocorticoid concentrations among male social group types in Hawai‘i. Panels show (A) cortisol and (B) corticosterone concentrations. Large points and error bars represent estimated marginal means (±95% CI) predicted from the final GLMMs after accounting for sampling day and year. Smaller points represent individual hormone concentrations. Two cortisol values exceeded the plotted range and are not shown.

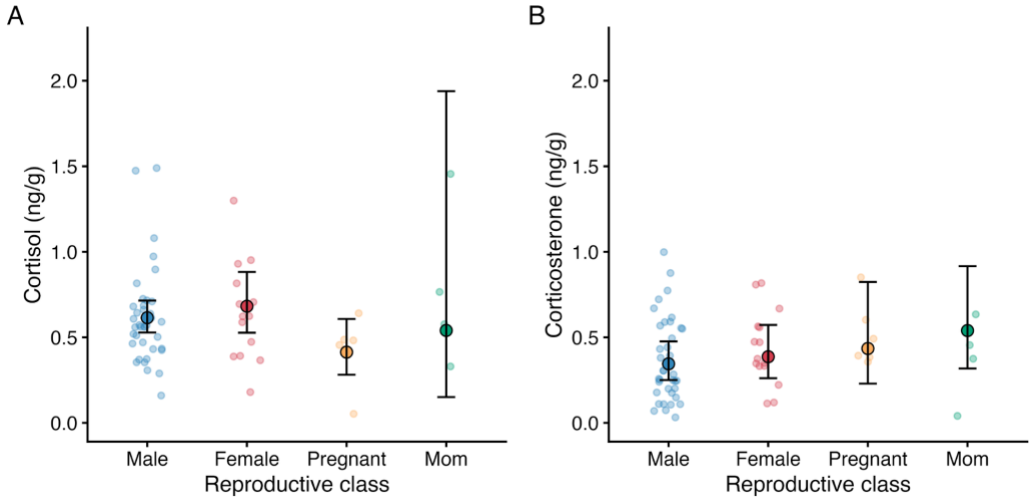

Figure 3.5. Model-predicted glucocorticoid concentrations among reproductive classes in adult humpback whales sampled on the Alaska feeding grounds. Panels show (A) cortisol and (B) corticosterone concentrations. Large points and error bars represent estimated marginal means (±95% CI) predicted from the final GLMMs after accounting for sampling day and year. Smaller points represent individual hormone concentrations. One cortisol value exceeded the plotted range and is not shown.

3.10 Supplemental Materials

Table S3.1. EIA kit description for target hormones.

Target hormone	EIA kit	Manufacturer	Reactivity to hormone	Detection limit
Cortisol (F)	K003-H	ARBOR Assays	100%	45.5–3200 pg/mL
Corticosterone (B)	K014-H1	ARBOR Assays	100%	17.5–10,000 pg/mL

Table S3.2. Mean (± SD) and range of cortisol and corticosterone for juvenile and adult humpback whales within Hawai‘i (2014–2025). No known juvenile samples were collected from Alaska.

Age class	Cortisol (ng/g)	Corticosterone (ng/g)
Juvenile (n = 6)	0.716 ± 0.436 *0.085–1.53*	0.403 ± 0.192 *0.254–0.671*
Adult (n = 324)	0.871 ± 1.36 *0.003–14.6*	0.286 ± 0.188 *0.017–1.37*

Table S3.3. Raw cortisol and corticosterone concentrations (mean ± SD), concentration ranges (*min–max*), and sample sizes (n) for humpback whales in Hawai‘i and Alaska by sex. Cortisol values include samples collected in Hawai‘i from 2014–2025 and in Alaska from 2023–2025. Corticosterone values include samples collected in Hawai‘i from 2024–2025 and in Alaska from 2023–2025.

Region	Cortisol (ng/g) Male	Cortisol (ng/g) Female	Corticosterone (ng/g) Male	Corticosterone (ng/g) Female
Alaska feeding season	0.614 ± 0.275 *0.160–1.49* n = 39	0.733 ± 0.676 *0.053–3.61* n = 25	0.365 ± 0.242 *0.032–0.998* n = 37	0.445 ± 0.212 *0.040–0.851* n = 25
Hawai‘i breeding season	0.869 ± 1.13 *0.027–8.74* n = 177	0.865 ± 1.57 *0.003–14.6* n = 155	0.299 ± 0.181 *0.051–1.37* n = 94	0.279 ± 0.197 *0.017–1.24* n = 91

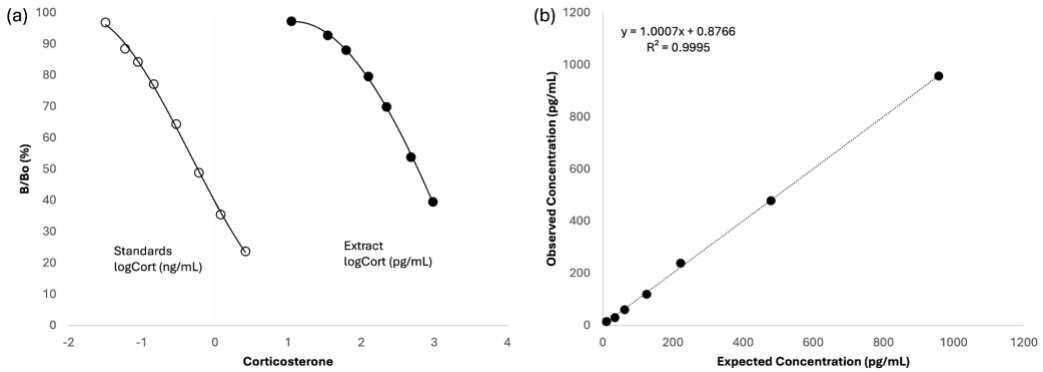

Figure S3.1. Validation of the corticosterone enzyme immunoassay (EIA) for humpback whale blubber. (a) Parallelism assessment using serial dilutions of pooled blubber extract from 10 humpback whales plotted against assay standards. The y-axis (B/B_0) represents the percent hormone bound relative to the zero standard. (b) Accuracy plot showing the relationship between observed and expected corticosterone concentrations across the dilution series.

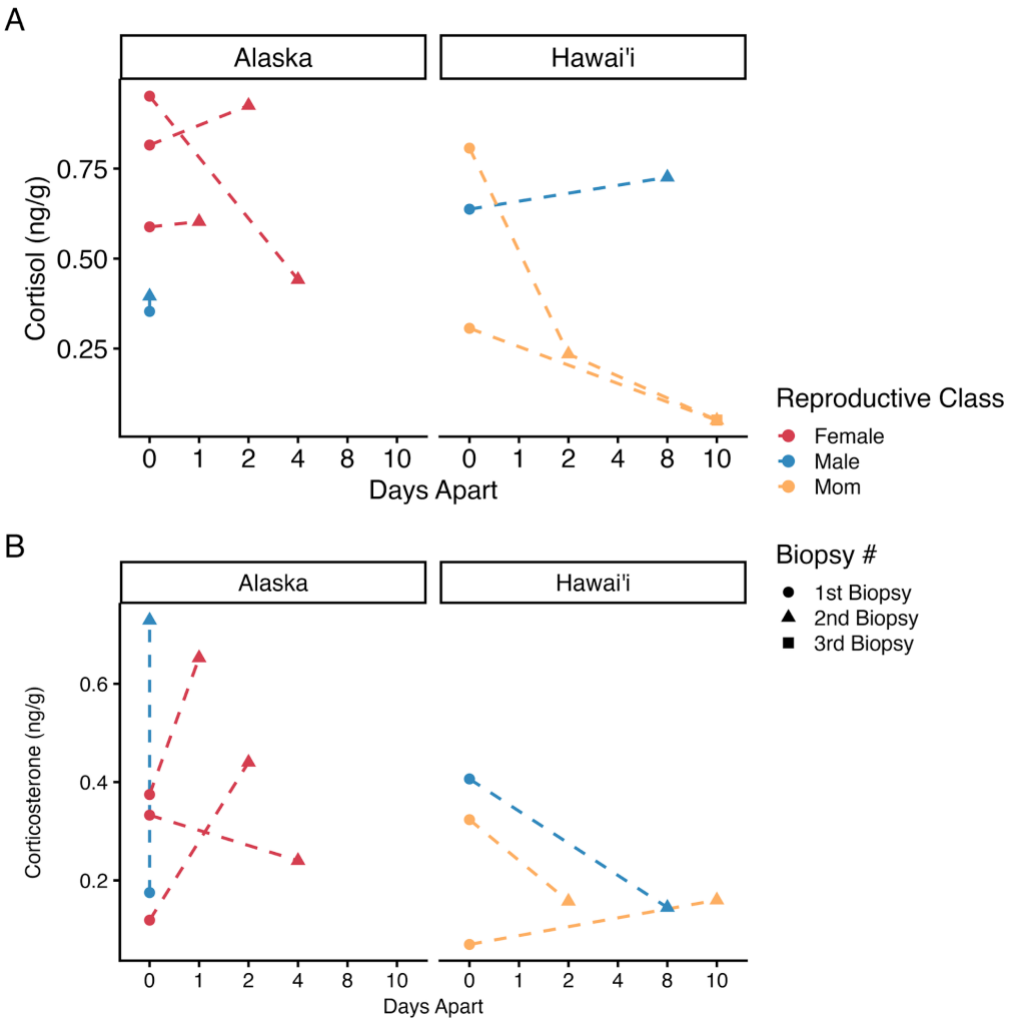

Figure S3.2. Repeated measures of (A) cortisol and (B) corticosterone concentrations in individual humpback whales sampled multiple times within the same season in Hawai‘i and Alaska. Dashed lines connect repeated samples from the same individual.

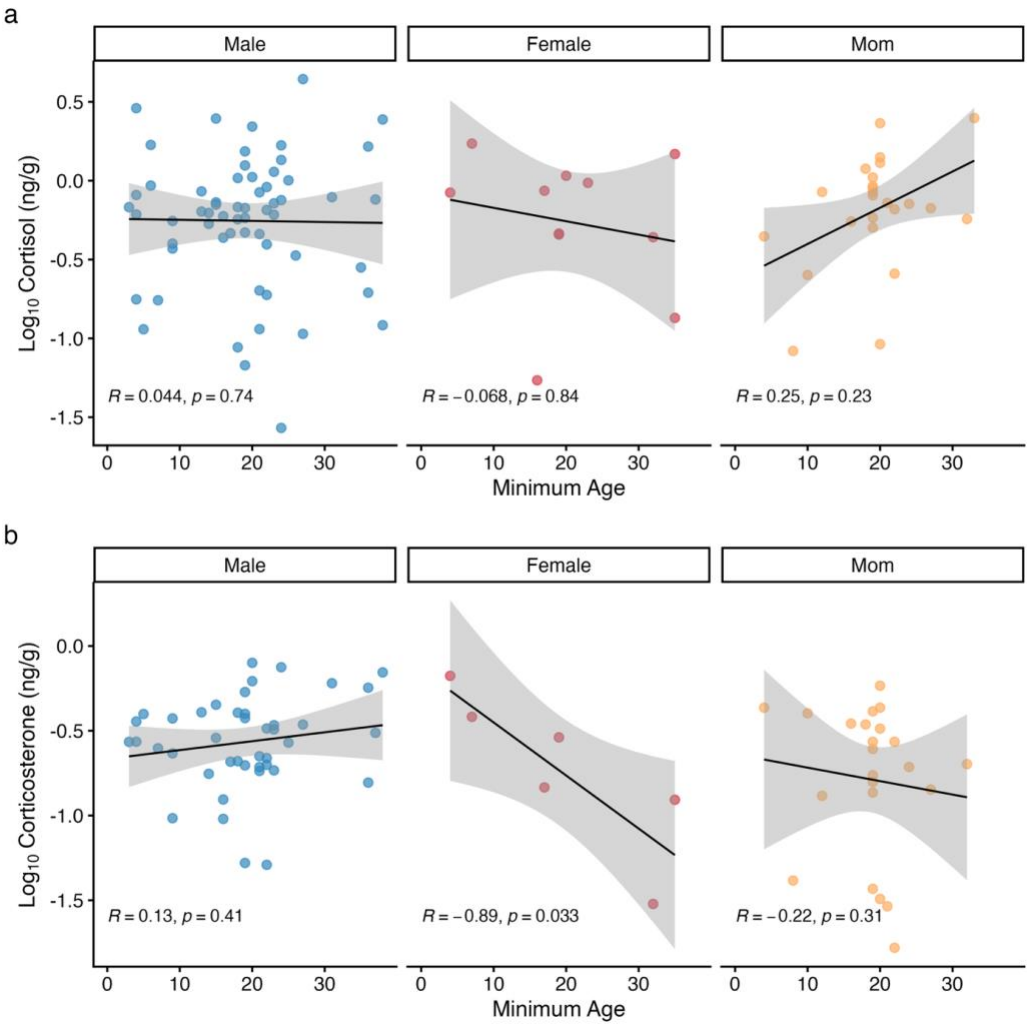

Figure S3.3. Relationship between minimum age and log-transformed glucocorticoid concentrations in Hawai‘i humpback whales by reproductive class. Panels show relationships between minimum age and (A) cortisol and (B) corticosterone concentrations.

Synthesis

Overview

This dissertation integrates reproductive steroid hormones and glucocorticoids measured in blubber biopsies to characterize endocrine variation in North Pacific humpback whales (*Megaptera novaeangliae*). Through three data chapters—male reproductive hormones, female reproductive hormones, and glucocorticoids—endocrine profiles were examined across sex, reproductive state, season, social context, and the Hawai‘i breeding and Southeast Alaska feeding grounds, providing one of the most comprehensive endocrine assessments of this population to date.

Three overarching contributions emerged from this dissertation. First, this work characterizes endocrine variation across a broad range of biological contexts, substantially expanding our understanding of reproductive and energetic physiology in North Pacific humpback whales. Second, it demonstrates that biological context shapes endocrine variation, with reproductive state, seasonal timing, and the physiological demands associated with different life-history stages accounting for much of the observed variation. Finally, this dissertation highlights the value of integrated, multihormone blubber endocrinology as a minimally invasive approach for investigating physiology and informing conservation and management.

Characterizing Endocrine Variation Across Biological Contexts

The first major contribution of this dissertation is a comprehensive characterization of endocrine variation in North Pacific humpback whales. Integrating reproductive

steroids and glucocorticoids across these contexts expands the physiological information available for this population.

In females, progesterone was a reliable indicator of reproductive state, identifying presumed pregnant females on the Alaska feeding grounds and differentiating mothers from females without calves on the Hawai‘i breeding grounds. Estrone further differentiated mothers from females without calves during the breeding season, and multihormone profiles revealed substantially greater endocrine variation within females without calves. In males, coordinated changes in progesterone, androstenedione, and testosterone characterized reproductive physiology, with elevated hormone concentrations on the breeding grounds and declining concentrations across the breeding season. Cortisol was identified as the predominant glucocorticoid in humpback whale blubber and generally reached its highest concentrations on the breeding grounds, extending endocrine characterization beyond reproductive hormones.

Collectively, characterizing multihormone endocrine profiles across these biological contexts provides a framework for investigating the physiological processes that shape endocrine variation in humpback whales.

Biological Context Shapes Endocrine Variation

Reproductive state and seasonal processes were the primary drivers of endocrine variation across this dissertation. As whales transitioned among reproductive states and life-history stages, hormone concentrations shifted accordingly and must therefore be interpreted within the biological context in which they were measured.

Within females, reproductive hormones provided insight into the physiological changes associated with pregnancy, parturition, and subsequent reproductive stages. Progesterone remained a valuable biomarker of mid-gestation pregnancy (Pallin *et al.*, 2018), reliably distinguishing presumed pregnant females sampled on the Alaska feeding grounds. However, interpreting reproductive hormones during the breeding season was more complex because endocrine profiles overlapped among reproductive states. Mothers, despite having recently given birth, exhibited elevated estrone concentrations. Because estrone has also been reported to increase before birth in other cetaceans (Steinman *et al.*, 2016), elevated estrone concentrations in mothers suggest that late-gestation endocrine signals may persist in blubber following parturition.

The complexity of interpreting endocrine profiles during the breeding season is further compounded by the physiological heterogeneity of females without calves, indicating that this reproductive class likely encompasses multiple underlying reproductive states. Distinct seasonal estradiol trajectories between mothers and females without calves further indicate that endocrine profiles are influenced by both reproductive state and the timing of sampling during the breeding season. In contrast, cortisol and corticosterone concentrations remained relatively constant in mothers while declining across the breeding season in females without calves, consistent with the sustained energetic demands of lactation.

Within males, reproductive hormones reflected changing reproductive physiology over the breeding season. Elevated androgens on the Hawai‘i breeding grounds suggest that males arrive in a heightened reproductive state, while their decline across the

season is consistent with a gradual reduction in reproductive investment. In contrast to females, estrogens remained relatively stable, indicating a more limited role in male reproductive physiology. Glucocorticoids provided complementary insight into these physiological changes. Cortisol concentrations were positively associated with testosterone and were highest in males engaged in competitive groups and males escorting females without calves, suggesting that glucocorticoids reflect the increased physiological demands associated with reproductive effort while remaining closely coordinated with reproductive physiology.

Advancing Blubber Endocrinology for Conservation

This dissertation demonstrates that blubber endocrinology has advanced beyond identifying individual hormone biomarkers toward a more integrated understanding of humpback whale physiology. By combining reproductive steroids and glucocorticoids with multihormone profiling and the analysis of endocrine relationships, this work establishes a physiological framework for interpreting hormone concentrations across biological contexts. Rather than assigning pregnancy or reproductive status alone, these profiles reveal how endocrine systems respond to different life-history stages, opening new avenues to investigate the underlying physiological mechanisms.

Historically, much of our understanding of whale reproduction has come from animals examined during the commercial whaling era (Chittleborough, 1958, 1965; Nishiwaki, 1959; Dawbin, 1966). The development of endocrine techniques capable of extracting physiological information from remotely collected blubber biopsies transformed opportunities to study living whales, and the findings presented here

further expand those possibilities (Hunt *et al.*, 2013). Integrating multiple hormone classes provided greater biological insight than individual hormones alone, revealing patterns and relationships that single biomarkers would have missed. This framework also points to clear directions for future research. Unexpected endocrine patterns, including elevated estradiol concentrations on the Alaska feeding grounds and differences in hormone relationships among reproductive classes, raise new questions regarding steroid metabolism and hormone incorporation within blubber.

Resolving these mechanisms will improve interpretation of endocrine biomarkers while expanding our understanding of cetacean reproductive physiology. Likewise, integrating endocrine measurements with long-term photo-identification, body condition assessments from unmanned aircraft systems (UAS), repeated sampling of known individuals, and reproductive histories will enable future studies to directly link endocrine profiles with reproductive success, individual fitness, and population resilience. As humpback whales continue to experience changing environmental conditions throughout the North Pacific, the physiological framework established by this dissertation provides a foundation for interpreting endocrine responses to environmental change and for integrating physiology into long-term conservation and population monitoring.

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