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Hormones and blubber: An endocrinological approach for assessing life-history states in free-ranging cetacean populations

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Marine Biology

by

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2008

The dissertation of Nicholas M. Kellar is approved, and it is acceptable in quality and form for publication on microfilm:

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DEDICATION

My dissertation is dedicated to Dr. Susan Stewart Suchocki. Leading by example, she taught me the utility of the scientific method, the strength of perseverance, and the power of compassion for all. She cultivated my love for nature, the pursuit of knowledge, and pitcher dominated baseball. She has been my motivation from the start of it all.

Mom, you will be missed…always.

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ACKNOWLEDGEMENTS

Chapter One will be submitted, in part or in full, as a manuscript for publication. I was the primary researcher and author. The co-authors listed in this publican either helped generate raw data or supervised and directed the research from which this chapter was formed. This chapter was written in collaboration with M. L. Trego and F. I., Archer.

The material found in Chapter Two is an adapted version of the text that was published in Marine Mammal Science 22:1-16. I was the primary researcher and author. The co-authors M. L. Trego and C. I. Marks helped generate replicate raw data and A. Dizon supervised the research that formed the foundation of this chapter.

The manuscript from which Chapter Three was derived has been accepted for publication in Marine Mammal Science. I was the primary researcher and author. The co-authors listed in this publican either helped generate raw data or supervised the research from which this chapter was formed. This chapter was written in collaboration with M. L. Trego, C. I. Marks, S. J. Chivers, K. Danil, and F. I., Archer.

Chapter Four will be submitted, in part or in full, as a manuscript for publication. I was the primary researcher and author. The co-authors listed in this publican either helped generate raw data or supervised and directed the research from which this chapter was formed. This chapter was written in collaboration with M. L. Trego and F. I., Archer.

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ABSTRACT OF THE DISSERTATION

Hormones and blubber: An endocrinological approach for assessing life-history state in free-ranging cetacean populations

By

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This compilation of research describes the development, validation, and application of a novel approach to assess life-history states in free-ranging cetacean populations. Two assays were developed in which reproductive hormone concentrations were quantified in readily available skin samples to assess 1) pregnancy state and 2) male maturity state, information that can be in turn used to assess reproductive output and demographic structure of dolphin groups in the wild.

Toward these ends and important for the validation of this approach, new procedures were developed to verify reproductive state in dead female specimens in which reproductive tract was at hand (Chapter 1). Here several histological characters

of the corpus luteum were used to help differentiate females undergoing ovulation from those who were pregnant.

Using these new procedures to identify reference samples (females of known pregnancy condition) a pregnancy assay for use on skin biopsies was developed and validated on three species of small delphinids (Chapter 2). Levels of blubber progesterone in dolphins of known pregnancy state were determined from carcasses collected from fishery-bycatch and local strandings. Pregnant short-beaked common dolphin blubber were found to contain dramatically more progesterone (16 times) than the blubber of non-pregnant mature and immature females. In addition, no overlap was found between the pregnancy states such that pregnancy diagnosis was unambiguous. Using these concentrations as reference we quantified the levels of progesterone in the blubber connected to projectile biopsies of wild pan-tropical spotted dolphins and found that animals exposed to higher levels of tuna-fishing effort were less likely to be pregnant (Chapter 4).

Finally, unlike with blubber progesterone and pregnancy, blubber testosterone was less precise in determining the maturity state of individual male dolphins. However, with a sufficient reference collection of blubber testosterone concentrations from male dolphins of known maturity state we developed a Bayesian method to estimate the probability of maturity of individual animals, which could be used in turn to estimate the proportion of males that were mature within any given group of biopsy samples (Chapter 3).

I. GENERAL SUMMARY

In order to develop and apply novel tools that aid the study of dolphin life-history (especially free-ranging populations), this dissertation draws from an array of biological subjects. The summary provided here highlights not only the more important findings of this dissertation but also delineates the relevant aspects of these diverse fields, including reproductive physiology, endocrinology, and life history, to provide the background foundation on which the research herein was built. In addition, given the novelty of the samples used for study (i.e., blubber biopsies), a description of the properties, composition, and physiology of cetacean blubber is also included. At the conclusion of this summary a bibliography provides a list of most of the major works used to generate this overview.

Why do we study life history?

The study of life history strives to understand the bridge that connects organismal and population biology. Its fundamental components are the probabilities that govern the birth, development, reproduction and death of an individual. These are translated into population vital rates (rates of birth, growth, maturation, reproduction and survival within a group of individuals) used to model demography and population

dynamics in attempts to forecast growth or decline in abundance (Caswell 2001). In essence this is fundamental information which we strive to obtain to manage or conserve populations of wildlife.

These rates are especially informative in long-lived mammalian species for which fecundity is low and vital rates are relatively inelastic. In the spectrum of all mammalian life-history strategies, cetaceans are the poster children for this long-lived, low-fecundity approach (Blueweiss *et al.* 1978, Sibly and Brown 2007). This is perhaps due to the fact that they are restricted to producing only one live offspring at a time[1]. In fact, Cetacea is likely the largest monophyletic clade of Mammalia with such a reproductive restriction (Charnov and Ernest 2006). Undoubtedly, the reason for this distinction stems from the obstacles inherent in being a perpetually obligate aquatic inhabitant that must breathe air within moments of birth, meaning that at birth a cetacean must be large (relative to mother) and have well developed physiological systems, especially skeletal, muscular, and respiratory, to support instant competent swimming in order to breathe (Charnov and Ernest 2006, Lefebvre *et al.* 2006). The consequences with respect to life history are that litter size, one of the four main avenues that contributes to species-level diversity in life-history strategies (the others being age of sexual maturity, reproductive frequency, and age-specific survival) is not variable for cetaceans.

All of this is important in a conservation/management perspective, in that compared to other mammals (or just about any other organism on the planet for that matter), vital rates of stable cetacean populations are relatively inelastic. Shifts in any one of these rates extended over time will more likely result in an increase or decrease

[1] Successful twinning in cetaceans is exceedingly rare if existent at all.

in abundance because this life-history inelasticity restricts compensatory reactions by each of the other rates that could offset the effects of the change of the initial and allow population levels to remain stable. In fact there are hallmark trends in vital rates that indicate whether a population is expanding or contracting (Hindell 1991, Bradbury *et al.* 2001, O'Grady *et al.* 2004). Therefore, monitoring these rates within a population can "red flag" or help forecast potential catastrophic declines in abundance, potentially well before an abundance trend can be definitely established. Moreover, because stable cetacean populations are relatively restricted in the values of these rates, monitoring just a couple can substantially increase the probability to forecast changes in abundance.

How do we study life history?

So that's why it is important to study life-history and estimate population vital rates, but how is it done? In the past, cetacean life-history information has been obtained by examining the reproductive tracts of dead animals, and thus vital rates have only been estimated from individuals collected from strandings, harvests, or incidental bycatch (Calzada *et al.* 1996, Heise 1997, Kasuya *et al.* 1997). There are two major shortcomings in data derived from these approaches. First, the resulting sample sets are frequently biased, with one or more demographic groups either over- or under-represented (George et al. 1995, Iwasaki and Kasuya 1997). The causes of

these biases are poorly understood and it is not possible to make accurate mathematical corrections to obtain the true vital rate information. Second, these approaches are limited to opportunistic sampling; harvesting activities or the conditions that create strandings dictate the timing, location, and composition of these sample sets (Read 1990, Zeh *et al.* 1995, Hohn *et al.* 1996). Moreover, with the laudable recent efforts to minimize bycatch and direct harvesting, too few specimens are currently acquired to make new, up-to-date life-history assessments.

Molecular diagnostics may hold the solution to allow the extraction of reproductive and developmental information of individuals without requiring entire carcasses for examination. Every physiological event is marked by a unique molecular signature as different cellular and organ systems communicate with each other to coordinate their roles in each event (Reimers 2003). The process to measure these signals for diagnostic proposes is familiar to us all, as it is similar to when blood or urine is sampled by medical health providers to diagnose ailments or assess physiological condition in human patients.

Steroid reproductive hormones

Endocrine hormones are some of the most familiar molecules that are assessed for diagnostic purposes, and of these, probably the most well known are steroid

hormones such as progesterone and testosterone. Wildlife researchers have for a while been using measurements of progesterone and androgens (e.g., testosterone) to assess reproductive condition and cycling of many wild terrestrial mammals (Monfort *et al.* 1993, Stoops *et al.* 1999, Maher 2000, Walter *et al.* 2002, Ostrowski *et al.* 2005, Pereira *et al.* 2006). These molecules are quantified from fecal and blood samples and condition is assessed by comparing the levels to those found in reference animals of known reproductive or developmental condition (Stoops *et al.* 1999, Ostrowski *et al.* 2005). A recent study has shown that this approach can be also used when studying cetaceans. Despite the obstacles of sample collection in the aquatic environment, reproductive hormones have been quantified from fecal samples of free-ranging right whales, and they were found to be diagnostic of pregnancy and male maturity state (Rolland *et al.* 2005). What makes these molecules so desirable to use for different applications in many different animals is that they have been exceedingly well studied and, unlike protein hormones, they are molecularly identical across mammalian taxa (Thornton 2001).

Known as the "hormone of pregnancy", progesterone is a primary regulator of estrous cycling and pregnancy (Pineda 2003a). After ovulation, it signals the uterine lining to prepare for fertilization and implantation, and then during pregnancy it maintains this environment for successful fetal development (Wuttke *et al.* 1998). Progesterone is predominately produced by the corpus luteum (CL) in the ovary and

for many mammals by the placenta[2] during the later stages of pregnancy (Bedford *et al.* 1972, Pineda 2003a). In the CL, lutein cells uptake cholesterol and other precursors and return synthesized progesterone into the circulatory system, where the majority of it is bound to albumin or trancortin to protect it from kidney and liver metabolism (Pineda 2003a). Only about 5-10% is unbound, and it is in this form that progesterone enters cells throughout the organism, including its target cells (i.e., those containing the appropriate molecular receptors) (Pineda 2003a). In these cells, it binds to cytoplasmic receptors allowing entrance into the nucleus, where it stimulates or inhibits the transcription of genes, thereby inducing its ultimate biological actions (Spencer and Bazer 2002). Progesterone has a short residence time in the blood, on the order of tens of minutes to a few hours, and is produced in frequent pulsate boluses in response to dropping concentrations regulated in punctuated homeostatic fashion (in part by the GnRF/LH pathway) (Pineda 2003a). Also, it is lipophilic and can therefore accumulate in relatively high concentrations in fatty tissues throughout the body (Deslypere *et al.* 1985, Hamudikuwanda *et al.* 1996).

Androgens, such as testosterone, are also steroid hormones, and as such they share many molecular properties with progesterone; however, they illicit very different physiological effects. They simulate the development and function of male genitalia, accessory sex glands, and secondary sexual characteristics (Pineda 2003b). Chief among these roles is their support of spermatogenesis. In the testes, luteinizing

[2] In some mammals including some cetartiodactyls, placental production of progesterone is minimal or non-detectable and therefore insufficient for pregnancy maintenance.

hormone (or Interstitial Cell Stimulating Hormone as it is sometimes referred to in males) stimulates the Leydig cells to transform cholesterol, creating testosterone (Preslock 1980). This is released locally in paracrine fashion into the seminiferous tubules, where it interacts with the germinal epithelium to promote sperm development (Pineda 2003b). Additional testosterone is taken up by the circulatory system and transported to cells throughout the body, where it exhibits many of the anabolic effects associated with general secondary sexual characteristics (e.g., increased muscular and skeletal development) (Pineda 2003b). Like progesterone, testosterone is lipophillic and disproportionately accumulates in anatomical regions that are high in fat (Deslypere *et al.* 1985). This is particularly important when studying cetaceans due to the large role, in terms of importance and total mass, that adipose tissues play in the physiology of cetaceans.

Blubber: Composition and Physiology

Cetaceans are relatively unique mammals in that they contain large quantities of fatty dermal adipose tissue in the form of blubber. Blubber is the lipid-laden dermal layer of the integument, sandwiched between the outer pigmented epidermis and the inner hypodermis and muscle. This tissue, which functions to regulate heat, store energy and aids in locomotion, has very unique physiological properties relevant to the accumulation of reproductive steroid hormones (Iverson 2002). First, blubber is high

in fat content; most areas are greater than 30% lipid by weight, and up to nearly 90% (Parry 1949, Aguilar and Borrell 1990, Dahl *et al.* 2000, Iverson 2002), plus it represents greater than 15% of the body mass of most adult cetaceans (Parry 1949, Iverson 2002). It can act as a giant sponge or reservoir, accumulating lipophillic molecules like steroids, from the circulatory system (Deslypere *et al.* 1985, Borobia *et al.* 1995). Second, the region of the blubber that abuts the epidermis is highly vascularized, as it supports some of the fastest epidermal growth rates measured in mammalian species, and consequently this area is often bathed in circulating blood (Hicks *et al.* 1985). Third, blubber is different from the subcutaneous fat observed in terrestrial mammals in that it exhibits a high degree of rigidity from the extensive fibrous network of collagen and elastin bundles seen throughout the dermal integument. They are especially high in concentration in flukes, flippers, and dorsal fins (consequently these are areas where we find the some of the lowest lipid concentrations in the dermis) (Iverson 2002). Finally, cetacean blubber is often distinctively layered in different characteristics including fatty acid composition such that often the outermost layer and the innermost are completely different, exhibiting different types and vastly different percent compositions of fatty constituents (Aguilar and Borrell 1991, Olsen and Grahl-Nielsen 2003).

Perhaps the most important characteristic of blubber, with regards to diagnostic analysis, is that it is routinely collected from free-ranging cetacean populations all over the globe. Obtained via projectile biopsy sampling, skin cores have been used for

over two decades to study genetic relationships (Escorza-Trevino and Dizon 2000, LeDuc *et al.* 2002), trophic status (Todd *et al.* 1997, Hooker *et al.* 2001), sex ratios (Escorza-Trevino and Dizon 2000), diet composition (Hooker *et al.* 2001, Olsen and Grahl-Nielsen 2003), and contaminate load (Hobbs *et al.* 2003). A small bit of blubber is contained in many of these skin cores, often greater than 50mg. Consequently, large banks of these blubber samples have been created over time, including one at the Southwest Fisheries Science Center which houses well over 8,000 of these samples collected from dozens of cetacean species.

With this large accessible bank of samples in mind, the object of this study was to develop, validate and apply an approach that estimates life-history parameters of groups of cetaceans by measuring steroid hormone concentrations in these skin biopsies. More specifically it was to investigate 1) whether reproductive steroids could be measured in cetacean blubber, 2) if so, were there relationships between these levels and different reproductive and maturity states, and 3) could concentrations of these hormones be used as diagnostics, in biopsy samples, to help estimate life-history parameters in free-ranging cetacean populations. We examined the relationships between blubber progesterone and pregnancy and blubber testosterone and male maturity.

But before describing this process I present a chapter, housekeeping in nature, germane to the validation of our approach. In this chapter, I examined the histological

development of the corpus luteum in attempts to find characters other than presence of embryo or fetus to differentiate pregnant from non-pregnant females. This was important because a pivotal point along the logical progression from the development of the molecular assays to their application in real populations was the ground-truth exercises (described in the subsequent chapters) in which we established the levels of the blubber steroids associated with each reproductive or maturity state. For these exercises, I extracted the steroids from blubber samples from specimens of known reproductive state that were often necropsied on the deck of fishing ships in conditions not ideal for examination. Consequently, at the start of my thesis research often all that was on hand were the gonads, a slab of blubber, measurements describing the morphometrics of each animal and some observations about overall condition, including for females whether a fetus was observed within the reproductive tract. As I conducted the exercise that would eventually link progesterone concentrations with pregnancy states, I was repeatedly asked, "How can you be certain that a female was truly not pregnant? Could a fetus have been overlooked, especially during ship-board examination?" These questions prompted me to search for an independent diagnostic of pregnancy to verify the reproductive status of our 'non-pregnant' reference specimens for which we have ovaries without the remaining reproductive tract. The results of this search became the first chapter in this dissertation.

We can be certain that if no fetus is found and no CL is present then the dolphin is not pregnant. So CL presence is a helpful diagnostic. But the question arises when

no fetus is found but a CL is present: was the female pregnant and the fetus or embryo overlooked, or was this perhaps a CL associated with an infertile ovulation or recently terminated pregnancy?

In attempts to help answer this question, my co-authors and I examined CLs of pantropical spotted dolphins caught in the eastern tropical Pacific purse-seine tuna fishery. In females where no fetus was present, we found a clear bimodal distribution in CL volume and hypothesized that the smaller volume mode was primarily composed of CLs of ovulation and the larger one was largely composed of CLs of pregnancy in which the fetus was either overlooked or had been aborted or birthed prior to sampling. That lead us to search for potential independent histological characters to evaluate the veracity our hypothesis, and we found that the fraction of vasculature and luteinized tissue were characters that changed rapidly during the first weeks of pregnancy (fetal lengths 1-10mm) and were therefore potentially useful for CL differentiation. Using these two characters, a comparison with CLs not associated with a fetus, found that the smaller of these CLs were more similar in these characters to those of very early pregnancy and the larger ones more similar to those of later pregnancy, though there was considerable overlap between these cluster groups. Our interpretation of these results is that in many cases CLs of ovulation and pregnancy can be distinguished morphologically; the former are less vascularized, have more unluteinized area, and are on average one-third smaller in volume. These results allowed us to better determine the pregnancy status of reference specimen (those

which we assume pregnancy state is known) that were used to evaluate the accuracy of blubber progesterone as a diagnostic of pregnancy.

Using our new CL histology method to determine reproductive state in reference animals, the next chapter investigated and validated the use of blubber progesterone as a diagnostic of dolphin pregnancy. Employing an organic solvent extraction procedure, which isolates steroid hormones from other blubber constituents based on differential polarity, progesterone was isolated and then quantified in 110 blubber samples from dolphins of known reproductive status. The samples were collected from fishery-bycaught and stranded dolphins representing three species (*Delphinus delphis*, *Lissodelphis borealis*, and *Lagenorhynchus obliquidens*). It was found that in this sample set, blubber progesterone concentrations could unambiguously discriminate pregnant from non-pregnant mature and immature females. In all three species, average concentrations were on the order of 12 to 20 times greater in pregnant females, and more importantly there was no observed overlap in concentration between pregnant and non-pregnant animals. In fact the lowest concentration measured in a pregnant female was approximately 4-fold higher than in the highest level of a non-pregnant female. Important for the future application of this procedure with biopsies, these results were consistent across various blubber sampling depths and anatomical sampling locations on the body, suggesting that progesterone levels are relatively homogenous throughout the blubber, at least compared to the large differences between pregnancy states. Also, variation in "storage" conditions had

little effect on blubber progesterone levels. Two situations were examined: blubber stored at -20° C for various periods up to 10 years and blubber left at ambient conditions for over two days, attempting to mimic decay conditions associated with stranded carcasses. In neither case did we find evidence of a trend in progesterone concentration relative to time, suggesting that progesterone is relatively stable in blubber tissue. It was also thought that blubber progesterone concentration would increase throughout gestation, so we might be able to approximate stage of pregnancy. However, no trend was found in progesterone concentration with respect to fetal length, indicating that pregnancy staging was infeasible. Despite the inability to stage pregnancy, the results from this validation investigation signify that blubber progesterone has all the hallmarks of a useful informative diagnostic for use with biopsies. The assay demonstrated: 1) a robust signal in that in the specimens sets examined it could definitively differentiate pregnancy states; 2) consistent results across species, which suggests it can be used in other species not yet tested; and 3) insensitivity to the vagaries of sampling (i.e., tissue depth, body location, and storage), which had little effect on measurement values and therefore limited-to-no effect on diagnostic accuracy.

The third chapter was intended to develop a diagnostic, similar to the one presented in chapter two, but in this case to assess sexual maturity in male cetaceans. By quantifying testosterone in the blubber of skin biopsies, androgen level was assessed and then evaluated as an indicator of seasonality and reproductive maturity.

We measured testosterone in the blubber of 116 male short-beaked common dolphins collected from fishery bycatch or strandings. When these concentrations were compared between maturity states (determined independently) we found average ($\mu \pm$ SEM) blubber testosterone levels of mature common dolphins (14.3 ± 3.0ng/g) were significantly higher those of pubertal (2.5 ± 0.5ng/g; $p = 0.006$) and immature animals (2.2 ± 0.3ng/g; $p < 0.0001$). We also found that testosterone concentrations in the blubber of mature males were much higher in summer months than during the rest of the year, indicating seasonality in mating. However, in non-summer months, though mature males had higher testosterone concentrations than immature ones, the levels overlapped so substantially that it was clear that blubber testosterone would not be an effective diagnostic for sexual maturity state for individual common dolphins sampled during this period. In spite of this, we realized that the data could nevertheless be used to estimate the proportion of mature individuals within a sample set even, with blubber samples collected during non-summer months. Using the data collected from the fishery and stranding specimens for which maturity status was known, we modeled the probability of maturity as a function of blubber testosterone concentration and ordinal date. Then we employed this model to estimate the proportion of mature males found in 299 biopsied common dolphins that were sampled off California. From two resulting models, we estimated that approximately one-third of the biopsied males were sexually mature.

The last chapter describes the first application of our pregnancy diagnostic to investigate an ongoing conservation/management issue affecting dolphin populations. Here we examined the current reproductive patterns of pantropical spotted dolphins using the blubber progesterone assay to measure hormone levels in biopsy samples. The background for this issue begins in the eastern tropical Pacific, where pantropical spotted dolphins of the northeastern population are closely associated with yellowfin tuna (Perrin 1969). This dolphin population has been decimated from extensive bycatch mortality associated with tuna harvesting from the mid 1950s through the early 1990s (Smith and Lo 1983, DeMaster 1992, Dizon *et al.* 1994, Wade 1994). The population shows no indications of recovery even though mortality has dropped to negeliable levels in the last two decades (Wade *et al.* 2002, Gerrodette and Forcada 2005, Wade *et al.* 2007). One possible explanation for the lack of recovery is that continued chase and encirclement of these dolphins by the tuna fishery to harvest the yellowfin tuna swimming beneath, negatively affects dolphin reproduction (Archer *et al.* 2004, Wade *et al.* 2007). However, insufficient life history sampling in this region over the last two decades due to reduced fishery mortality makes traditional estimation of population pregnancy rates impossible.

To examine the current reproductive patterns of these dolphins, blubber progesterone was quantified in 212 spotted dolphins biopsied between 1998 and 2003. The results showed that concentrations were sharply bimodal with a gap in values between 50 and 100 ng/g; a finding consistent with the concentration gap between

known pregnant and non-pregnant dolphins. Assuming that high blubber progesterone is diagnostic of pregnancy, we found that roughly twelve percent of the biopsied females were pregnant. This is substantially lower than an estimate of the proportion pregnant found in the fishery kill over the same region (22.3%) between 1973 and 1992. We believe the most likely reason for this large disparity is difference in sample biases relative to age/demographic groups; specifically, there is evidence that immature dolphins are under represented in the fishery kill. If true this might lead to an overestimation of the proportion pregnant based on data from the kill. However, if and what biases are associated with biopsy sampling are unknown.

Other possible factors contributing to this disparity may include effects of the fishery on pregnancy. When examining this further, we found a significant negative relationship between fishery exposure and pregnancy rate, suggesting that the chase and encirclement of dolphins may be interfering with successful reproduction. Moreover, a subsequent spatial pattern analysis indicated that pregnant females were significantly aggregated away from areas where fishery activity was greatest. Though additional investigation is necessary to determine the actual cause of this relationship, we suggest that perhaps the physiological stress associated with frequent chase and encirclement by the fishery is a primary contributor of retarded reproductive rate in regions with high fishery activity.

In total, this dissertation attempts to lay the foundation for a non-lethal approach to collect life-history data from free-ranging cetacean populations. Again, the concept on which this approach tries to capitalize is that molecular signals can be measured in routinely collected skin biopsies, and these signals are diagnostic of specific developmental and reproductive conditions. The two assays that we developed toward this end show promise in their utility and in fact were each employed to measure life-history parameters in dolphin populations. These are to our knowledge the first applications of such a molecular approach to study cetacean life-history in the wild. There is likely more life-history information that can be extracted from these skin samples, especially as the technical advances from the growing biomedical field trickle down for use in wildlife biology. In the future, there will be more identified markers or even marker patterns determined in other mammals, which we can then attempt to measure in the skin of cetaceans, that will be associated with other important developmental processes such as lactation, aging, and estrus. Beyond steroids, the markers will likely include other hormone types, fatty acids, and nucleic acids.

However, there are limitations to this approach. First it is not likely that all physiological events pertinent to life-history studies will produce signals in the skin that we will be able to measure or discern. The skin is peripheral in its signaling contact with other organ systems, so that the "amplitude" of some signals has diminished by the time they reach the skin. Moreover the skin has relatively little

known physiological role in many of the conditions that we want to detect. We would not necessarily predict, for instance, that prolactin receptors would be up-regulated in cetacean skin cells at the onset of lactation. We might have been fortunate by starting with steroid hormones; the robust signal of progesterone and testosterone may have merely been a byproduct of their hydrophobicity and their generalized circulation that carried them passed lipid rich blubber cells. Nonetheless, I imagine there are molecular changes within cetacean skin cells associated with each of the physiological events that we are interested in detecting but whether these changes can be identified, detected, and differentiated from all the other molecular information that is associated with skin physiology not related to these life-history characteristics is obviously the important question when trying to determine the extent of the utility that this approach offers.

Another possible limitation stems from our reliance on biopsy sampling and whether the samples obtained from this process are representative of the groups of animals we study. Biopsy samples come almost exclusively from dolphins that are willingly to come and ride the bow wave pushed by the research vessels from which we sample. It is not likely that all dolphins participate in this behavior equally, but it is unknown whether certain demographic groups (e.g., juvenile males) would be more or less likely to bowride. If there is a bowriding behavior bias with respect to age or reproductive state, then the values we report from this approach would not be the same as the vital rates which we are trying to obtain, thus constraining the utility of the data

derived from biopsy samples. Future studies focused on quantifying the presence and level of any biases related to biopsy sampling are needed to fully understand how these data relate to the true biological rates which we are attempting to estimate.

However, even if there is such a bias, as long as this bias is relatively constant through time then this approach can be used to derive indices to help estimate trends in these vital rates, which when combined with serial abundance estimates are useful to detect or forecast population changes. The idea is that certain demographic groups, like calves and newly weaned juveniles are much more vulnerable compared with other demographic groups to the majority of hazards and environmental perturbations cetaceans are likely to face. Given this assumption there are clear trend-patterns to these parameters (i.e., proportion pregnant and proportion mature) which would help indicate the change in population abundance. For instance, if there were a possible, yet non-significant, downward trend in abundance and we found that the proportion immature were declining while pregnancy rates were increasing, that would raise a red flag indicating a higher probability that the population was experiencing or likely going to experience a sharp decline. This approach is also helpful when investigating in populations which already have strong evidence of decline. Trends in the proportion mature and pregnant can help investigators focus on which demographic groups are being affected, and that in turn can help determine or rule out possible causes.

In the end, in spite of the potential limitations delineated above, to our knowledge there are few other practical means to obtain non-lethal estimates of life-history information in wild cetacean populations. The two assays developed and validated here indicate that measuring molecular signals in skin samples is a promising approach to study cetacean life history. They are relatively robust in signal strength, largely insensitive to many of the vagaries of sampling, consistent across different species, and accurate, especially in the case of detecting pregnancy from blubber progesterone concentrations, for which we have yet to find empirical evidence of misdiagnosis.

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II. CHAPTER ONE

Growth and development of the *Stenella attenuata* corpus luteum: identifying potential characters to differentiate CLs of ovulation from those of pregnancy

Abstract

The morphological development of the corpus luteum (CL) was examined in pantropical spotted dolphins (*Stenella attenuata*) in attempts to delineate characters to differentiate CLs of ovulation from those of pregnancy. We found a distinctive bimodal distribution in CL volume in females where no embryo/fetus was found and speculated that the smaller-volume mode was mostly composed of CLs of ovulation and the larger-volume mode was predominately made up of CLs of pregnancy in which the embryo/fetus was either overlooked or had been aborted or birthed prior to sampling. Consequently, we searched for potential independent histological characters to confirm or refute this idea and found that the fraction of vasculature and luteinized tissue were characters that changed rapidly during the first weeks of pregnancy (fetal lengths 1-20mm) and were therefore potentially useful for CL differentiation. A subsequent statistical comparison of "small" and "large" CLs not associated with an embryo/fetus against CLs of known pregnancy, found that the "small" unassociated CLs were statistically different in these characters to CLs of known pregnancy, but near what we would expect for ovulation based on the trend analysis and therefore, likely represent CLs of ovulation. The larger CLs (akin in volume to CLs associated with pregnancy) not associated with an embryo/fetus were not statistically different from those of pregnancy, and therefore likely represent missed embryo/fetuses or recent abortion/parturition. Although there was considerable overlap between these groups, our interpretation of these results is that in many cases CLs of ovulation and

pregnancy can be distinguished morphologically; the former are less vascularized, have more unluteinized tissue, and are on average one-third smaller in volume.

Introduction

The corpus luteum (CL) is a transient ovarian endocrine gland that is responsible for producing progesterone, which in turn maintains the uterine lining during pregnancy. In cetaceans, the CL is particularly important because it is possibly the primary producer of progesterone throughout gestation as it is in other cetartiodactyls (Mills and Morrissette 1970, Sangha *et al.* 2002, Asano *et al.* 2003). The cetartiodactyl CL is formed, as it is in all mammals, from the Graaffian follicle after ovulation in response to luteinizing hormone. The luteinized follicular tissue hypertrophies and proliferates rapidly as it undergoes dramatic histological changes as it develops into a mature CL (Balboni 1983). Maturation is completed within 3-6 days, and if fertilization does not occur within the following 10-12 days, the CL (a.k.a. the CL of ovulation or CLO) degrades and the events that lead to the next ovulation commence (Zalud 1994, Pineda 2003). If fertilization does occur, the CLO becomes a CL of pregnancy (CLP) and is maintained throughout pregnancy until the weeks or days before parturition when it starts degrading as part of the calving process (Niswender *et al.* 2000, Sangha *et al.* 2002).

The structural changes that occur as the follicle becomes a CLO and ultimately a CLP are driven by extremely rapid cell growth (among the fastest cellular growth rates in mammalian physiology) (Zheng *et al.* 1994, Reynolds *et al.* 2000). Many of the

steps involved in this maturation process are similar for most mammals. After ovulation, the collapsed walls of the follicle are left involuted about a fluid-filled void (Zalud 1994). The newly luteinized granulosa and theca cells invade the void from the folds of the former follicle wall in finger-like projections with branching blood vessels (derived from luteinized theca cells) at their core, fueling growth (Reynolds *et al.* 2000). As these projections meet, the entire tissue simultaneously expands outward, increasing the total volume of the CL. In most mature CLOs, an acellular fluid-filled cavity, lined by a layer of connective tissue, remains in the center of the luteinized tissue (Smith *et al.* 1994, Sangha *et al.* 2002). In the absence of fertilization, the CLO regresses (an event marked by autolysis and fibrosis of the luteal zone) and becomes a corpus albicans (CA) (Niswender *et al.* 2000). If the ovum is fertilized, the CLO is "rescued" (degradation is averted), and additional growth and morphological transformations occur (Fields and Fields 1996). Three common and conspicuous changes are 1) a dramatic expansion in total volume via cell proliferation, hypertrophy, and cystic distention, 2) an exaggerated proliferation of endothelia cells and an overall increase in vasculature, and 3) a continued invasion of luteinized and connective tissue into the central cavity (Fields and Fields 1996).

Classifying CLs into CLOs or CLPs can be difficult when examining the reproductive tract of cetaceans. Discovery of an embryo/fetus clearly shows that the associated CL is a CLP; however, if no embryo/fetus if found, proper classification is ambiguous without additional information. Several events can generate a CL not associated with an observed embryo: first, ovulation without fertilization (CLO);

second, fertilization with a conceptus or embryo undetected, (CLP); third, pregnancy ending in parturition or abortion just prior to acquisition of examined specimen (CLP, degrading).

Due to the strong sampling effort of dolphin bycatch in the eastern Pacific purse seine tuna fishery from the early 1970s through beginning of the 1990s, NOAA's Southwest Fisheries Science Center has amassed large collections of reproductive organs of several dolphin species. The data generated from this collection have provided large amounts of reproductive and life history information for one of the most fishery-impacted dolphin species, the pantropical spotted dolphin, (*Stenella attenuata*). The information includes age at maturity, length of gestation, calving interval, length at birth, and corpus acquisition rate ("ovulation rate") (Perrin *et al.* 1976, Perrin *et al.* 1977a, Perrin and Henderson 1984, Perrin and Reilly 1984, Hohn and Hammond 1985, Myrick *et al.* 1986). In these animals, we find persistent CAs, the scars of past CLs that arguably remain throughout an individual's lifetime. Although much effort has focused on studying these corpora, questions remain about the interpretation of these scars, namely whether they are representative of past pregnancies, past ovulations, or both (Perrin and Donovan 1984, Perrin and Reilly 1984). Understanding how the CL develops during estrus and after fertilization and estimating the relative proportions of the different CLs can help us better interpret the origin and presence of these scars.

In this study, we compare the average volume of CLs associated with found embryos/fetuses against those that are not, using spotted dolphin specimens collected

as bycatch by the purse seine tuna fishery. We examine structural and histological characteristics (cell luteinization and tissue vascularization) that change during early pregnancy in attempts to find morphological indicators that might us allow us to better differentiate the two CL types. These indicators are then used in a statistical comparison to test whether CLs from 83 spotted dolphins for which an embryo/fetus was not found were more similar those of pregnancy or ovulation.

Methods

Samples

Spotted dolphin ovary pairs were collected during commercial tuna fishing operations in the eastern tropical Pacific by National Marine Fishery Service (NMFS) scientific observers following the procedures delineated by Perrin et al. (1976). The ovaries were fixed in 10% formalin and stored in 70% isopropanol. Various observations were recorded during collection and specimen processing ashore, including total length, pregnancy state, and fetal length. For previous studies (Perrin *et al.* 1976, Benirschke *et al.* 1980, Myrick *et al.* 1986), these ovaries had been measured and sliced into 1mm sections, and data regarding the presence and size of each corpus luteum (min, mid, and max diameters) and corpus albicantium (max diameter) were recorded. For this study, we included data only from specimens with CLs representing two different groups: those for which an embryo/fetus was found (n = 2048) and those where none was found (n = 469). A randomized subset of these CLs (61 with embryo/fetus, 83 without) was further examined to estimate the relative level of

lutienization and vasculature in each CL. In order to focus on the early CL development, the subset of CLs associated with an embryo/fetus were stratified in such a way that a disproportionate number of CLs were selected to be associated with embryos/fetuses less than 100mm (n=43), the rest were randomly selected from CL lengths 100 to 838mm (850mm is the approximate average length at birth (Perrin *et al.* 1977b, Hohn and Hammond 1985)).

CL volume

CL diameter measurements made previously were used to estimate CL volume by modeling the corpora as ellipsoids using the following equation:

$$CL_{vol} = D_{\max} * D_{\mathrm{mid}} * D_{\min} * (\pi / 6)$$

The frequency distribution of volume was plotted and compared for CLs with and without observed embryo/fetuses.

Fraction of luteinized tissue: Gross examination

The fraction of luteinized tissue as a proportion of total CL volume was estimated by imaging transverse cross-sections at each CL's maximum diameter. Images were captured by an optic mounted Nikon CoolPix P5000 digital camera through a Wild M32 dissecting scope (Leica Microsystems, Wetzlar, Germany) at 3-5x magnification

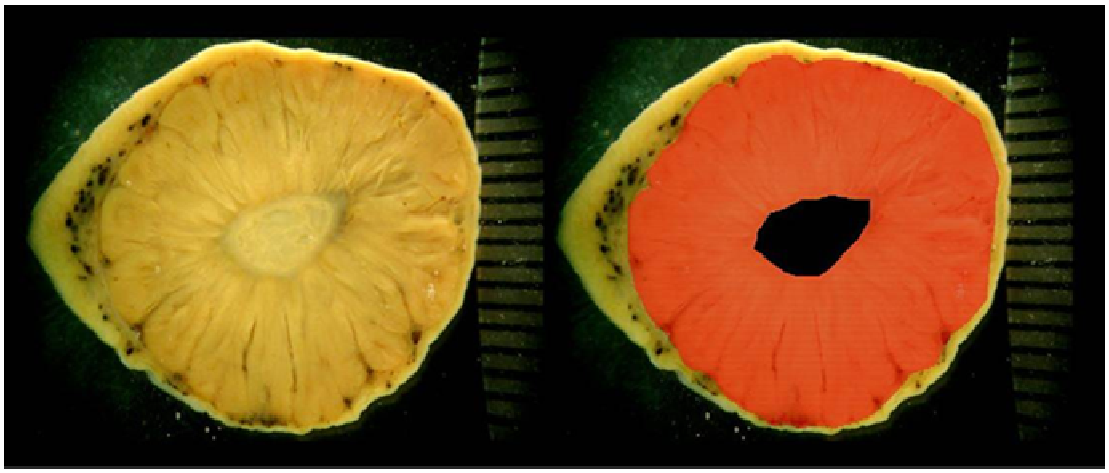

Figure 1. Quantification of luteinized CL area using computerized image analysis (CIA). The cross sectional of the entire CL was outlined manually (orange overlay) to exclude the rest of the ovary and the area measured using a size standard seen on the right of each image (one tick mark equals 1 mm).Then the same was done for its non-lutienized central cavity (NLCC: masked in black). The fractional lutienized area was calculated as (1- NLCC)/ total CL area. All three measurements were recorded (see text).

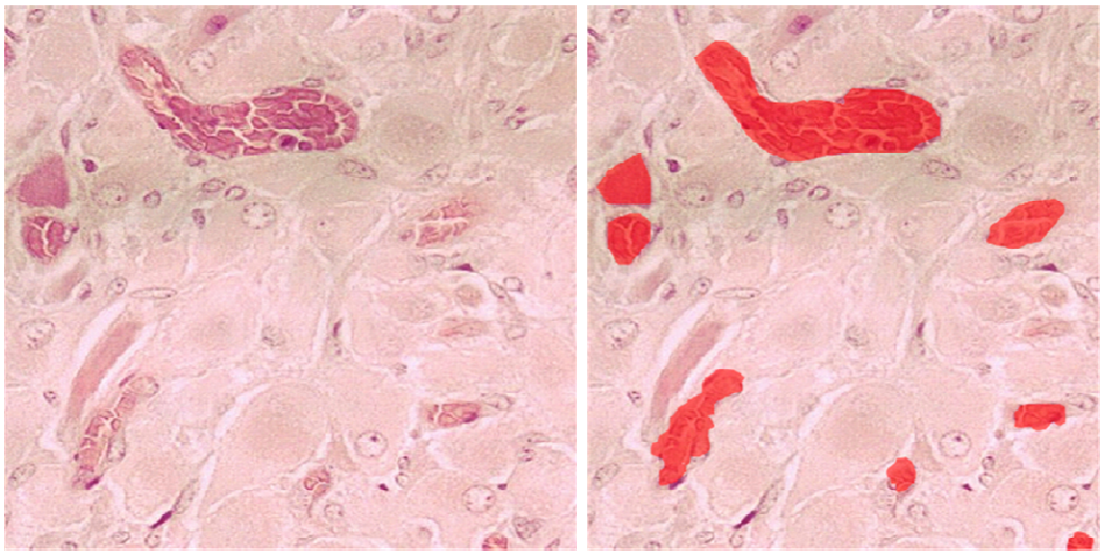

Figure 2. Determination of fractional vascularized area using computerized image analysis (CIA). Five images where taken at the margin adjacent to the un-luteinized CL core (central cavity or connective tissue). The perimeter of each blood vessel within an image was outlined manually using image analysis software. The area within the outlines (red masked) as a proportion of the total area of the image comprised the estimate of the fractional vascularized area. These values were averaged across the five images to obtain a relative level of vascularization for each CL examined.

with a size standard adjacent to the tissue. Using image analysis software, Image ProPlus 4.1 (Media Cybernetics, Bethesda, MD), the total cross-sectional area of the CL and the proportion of unluteinized area at the organ's core were outlined and measured. Figure 1 shows an example of these images and their measurements.

Vascularization: Microscopy exanimation

Tissue sections (4-5 μm) were sliced onto microscopy slides (Super-frost plus Sigma, Poole, Dorset, UK) for standard hematoxylin and eosin staining. The sections were examined under a 40x objective lens, and images were captured using a Micropublisher 5.0 RTV scope-mounted camera (QImaging, Surrey, Canada). Endothelia cells were identified according to morphologic appearance; vessel perimeter was determined visually, and cross-sectional area was measured using our image analysis software. Five microscopy images at internal cavity margins were generated, blood vessels were outlined (masked in red) manually, and their cumulative area as a proportion of the total image was determined (Fig. 2). The average vascular density of the five images was recorded

Classification Function: Statistical analysis

Data analysis was conducted in two stages with values of vasculature and lutienization log-odds transformed, a standard procedure when using fractional data in parametric tests. The first stage tested the significance of trends in the proportion of luteinized tissue and vasculature during early pregnancy. We quantified these

characters as a function of fetal length in early pregnant animals (fetal lengths: 1-100mm; or approximately the first three months of a 10- to 11-month gestation). Based on trends in these characteristics, we qualitatively approximated their likely state during ovulation.

In the second stage, we conducted a series of two-sample t-tests comparing the proportion of lutienized tissue and vasculature in "small" and "large" CLs without known fetuses/embryos against these same characteristics in CLs of known pregnant females to test the hypothesis that smaller CLs are associated with unfertilized ovulations and larger CLs associated with pregnancies. Our interpretation of these results was informed by the results of the trend analyses.

Results

Upon visual examination of the 83 CLs not associated with embryos/fetuses, it was apparent that five of these corpora were misclassified CA scars; they had high levels of connective tissue and displayed prominent hyalinization. An additional 24 were degraded (likely from postmortem storage) such that histological evaluation was impaired; these were excluded from further analysis.

Estimated CL volume was on average much higher in females with observed embryos/fetuses than those without. The size frequency distribution of CLs not associated with embryos/fetuses was bimodal with the larger volume (right-hand) peak

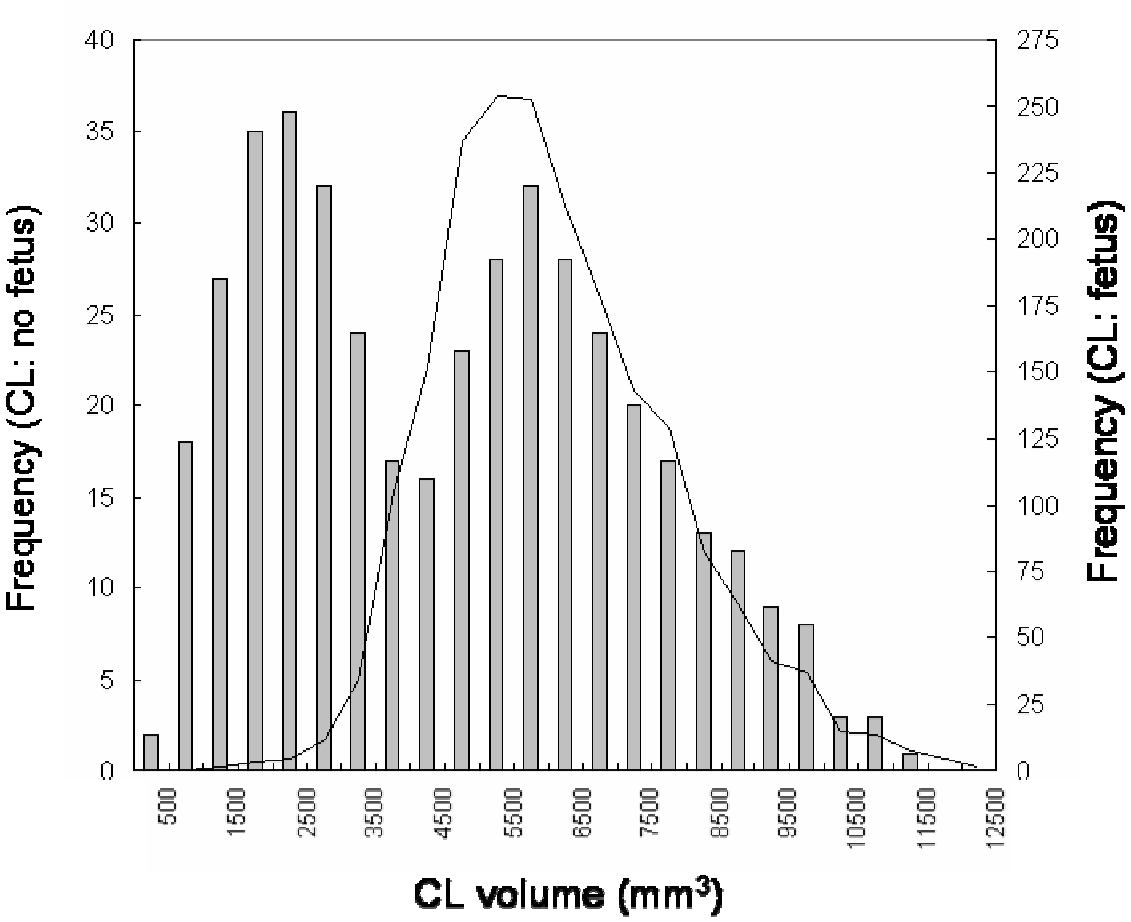

Figure 3. Relative frequency distribution of CL volume in spotted dolphin with (line) and without (bar) observed fetuses/embryos.

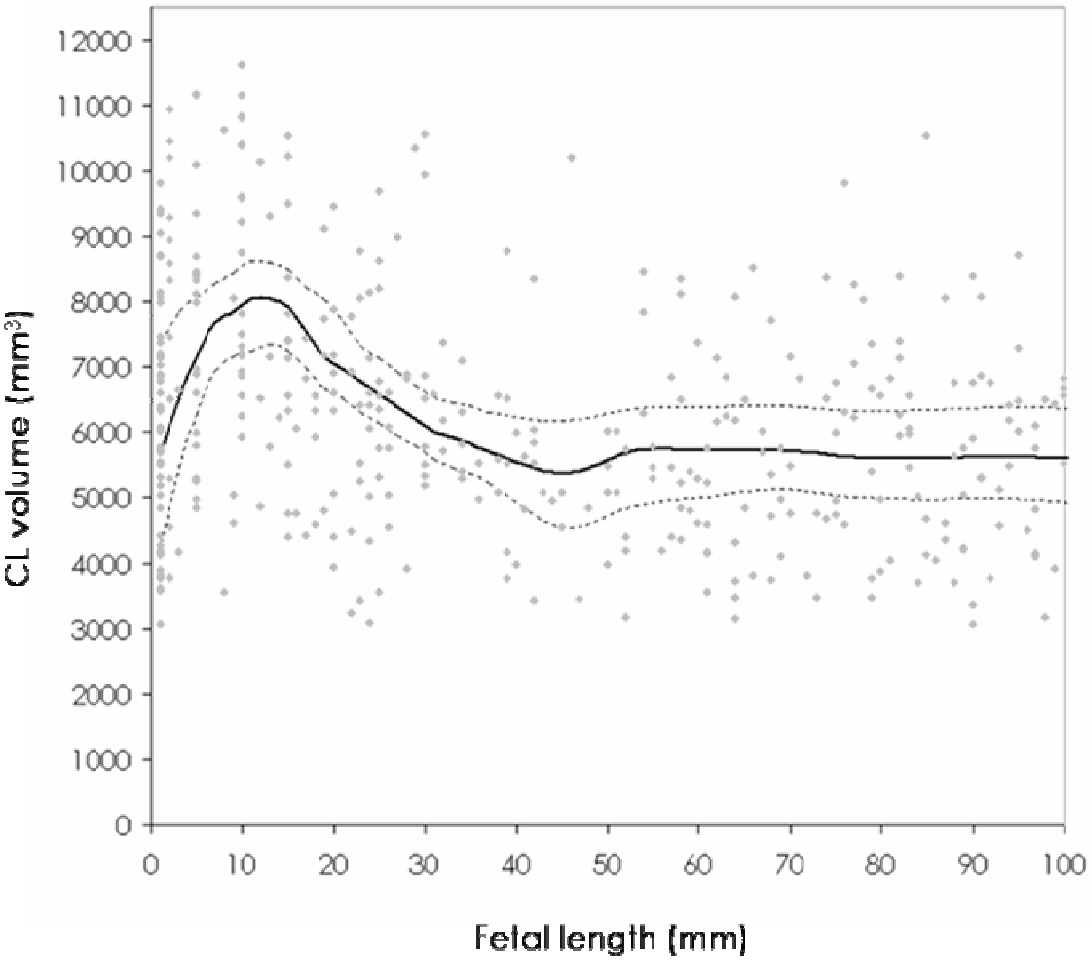

Figure 4. CL volume (mm^3), as a function of fetal length (mm), *S. attenuata* of early pregnancy. The dark line represents a 25 sample (period) moving average at 2mm intervals while the dotted lines delineate the 95% bootstrap confidence envelop of this measurement.

occurring at the same value as the mode of the CLs with associated embryos/fetuses; the smaller volume (left-hand) peak occurred below all but the smallest volumes seen in the CLs with embryos/fetuses (Fig. 3). The local minimum separating the large-volume and small-volume modes occurs at approximately 4000 mm^3. Only 6.3% of CLs associated with an embryo/fetus had volumes smaller than this local minimum. Thirty-eight percent of the CLs without an associated embryo/fetus had volumes within the lower range.

A sharp increase in CL volume is evident during the first weeks of gestation (from embryo lengths of 0-20mm), and then volume steadily decreases as embryos grow beyond 20mm (Fig. 4).

The relationship between the fraction of luteinized tissue and fetal length is shown in Figure 5. Though the relative luteinized area of the central cavity varies greatly within fetal length classes, the average proportion of the CL that is luteinized appears to increase rapidly during the first weeks of gestation (fetal lengths: 1-20mm) and continues to rise slowly during the subsequent months. The average proportion of cross-sectional area containing luteinized tissue grows from 0.950 (95% bootstrap CI 0.930 – 0.963) for embryo lengths of 1-10 mm to 0.981 (95% CI 0.973 – 0.987) for those greater than 10mm. The average proportion first increases inward as the luteinized tissue continues to invade this acellular area and then continues as the CL grows outward, making the unluteinized core a smaller proportion of the total cross-sectional area.

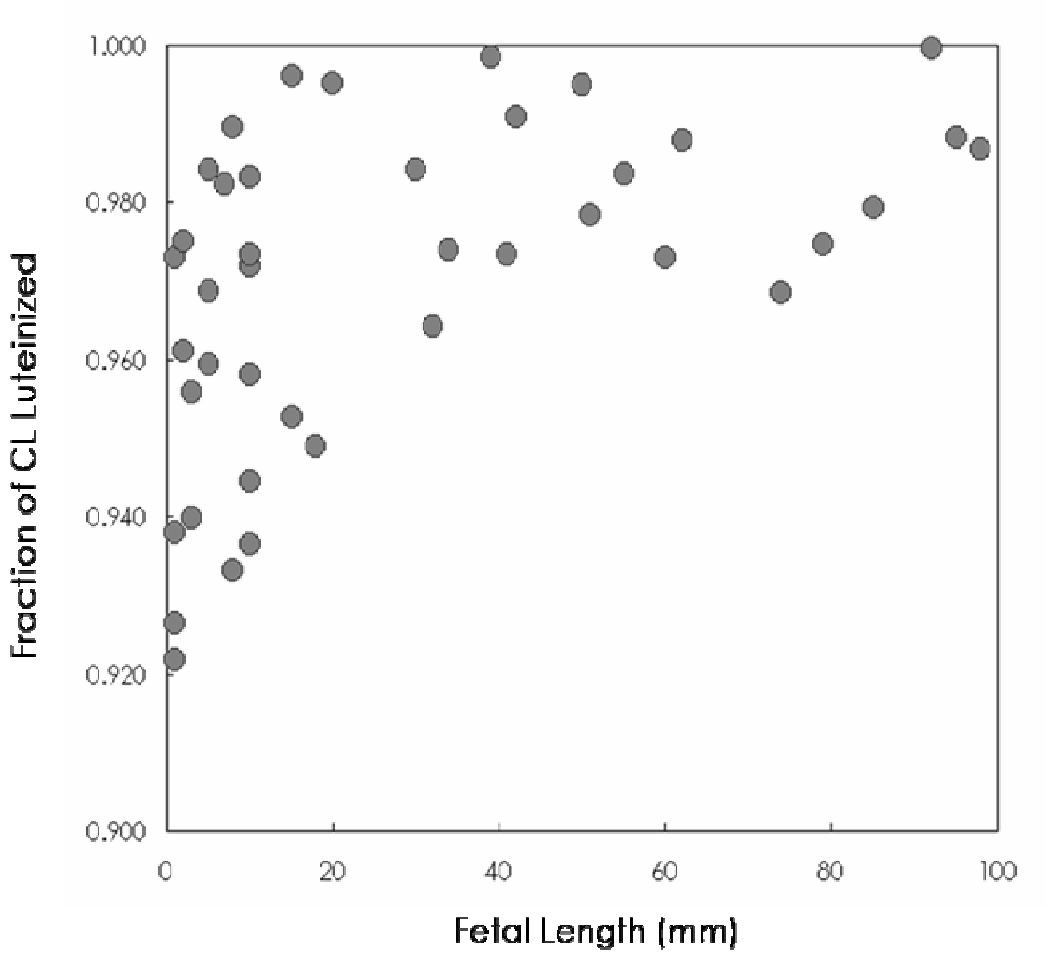

Figure 5. Fraction of corpus luteum that was lutienized as a function of fetal length (mm) during early pregnancy (fetal length < 100mm).

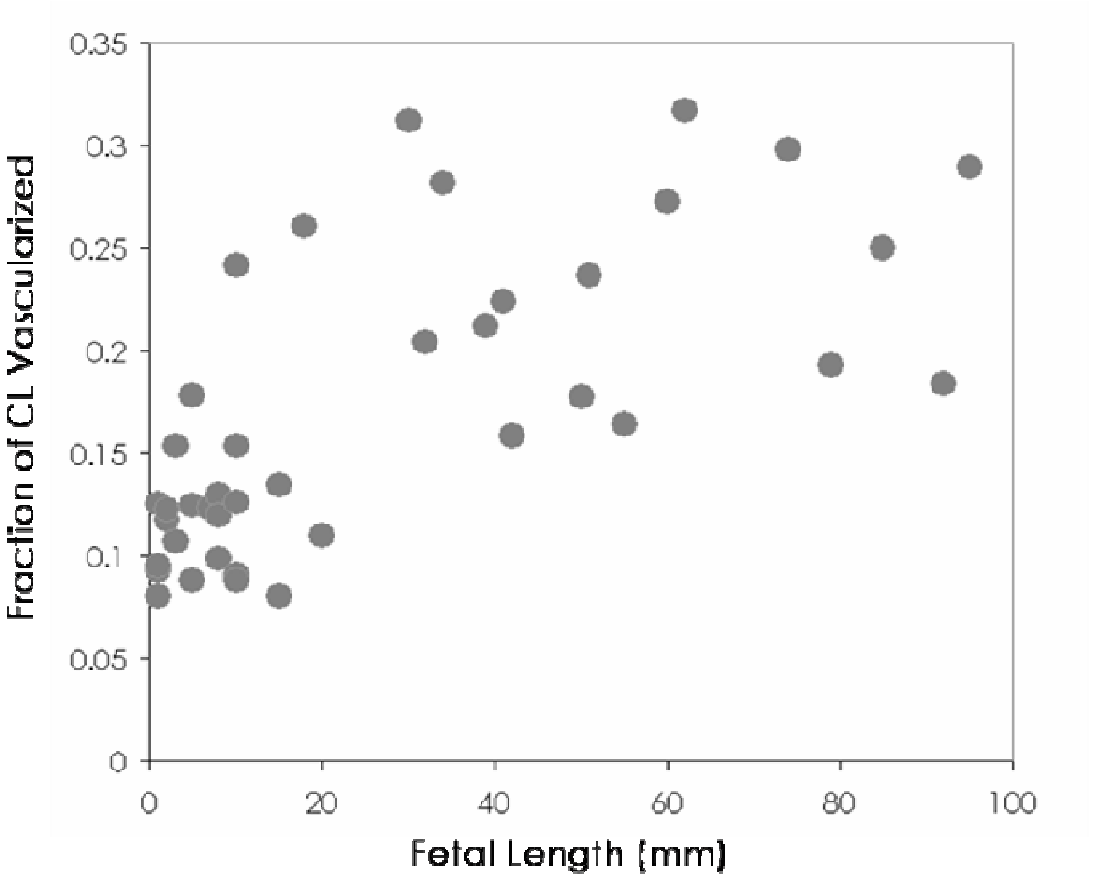

Figure 6. Fractional area of lutienized corpus luteum that was vascularized as a function of fetal length during early pregnancy (fetal length < 100mm).

The fraction of vascularized tissue also increases during early embryonic development (Fig. 6). The average proportional cross-sectional area that contains vasculature rises from 0.144 (95% bootstrap CI 0.138 – 0.149) for embryo lengths of 1-10 mm to 0.241 (95% CI 0.222 – 0.259) for those greater than 10mm. The increase appears to be driven more by growth in blood-vessel size than an increase in the number of vessels per unit area of tissue, though both were observed.

CLs with volumes less than 4000mm^3 where no embryo/fetus were found had significantly less vasculature and on average a significantly lower fraction of luteinized tissue compared to CLs of known pregnancy (*P*-values- vascularization: <0.001, luteinization: <0.001) (Table 1) and larger CLs, those greater than 4000mm^3, not associated with an embryo/fetus (*P*-values- vascularization: <0.001, luteinization: <0.001) (Table 1). On the other hand, the larger CLs without an associated embryo/fetus, showed no significant differences in either vasculature or luteinization when compare to CLs of known pregnancy (*P*-values- vascularization: 0.443, luteinization: 0.141). There is overlap in both characteristics between each of the three CL types. However, most of the overlap between the "small" CLs not associated with an embryo/fetus and those of known pregnancies occurs with those CLPs associated with small embryos less then 10mm in length (Figure 7), in essence CLPs representing the first days/weeks of pregnancy.

Table 1. T-statistics and associated P-values for comparisons of vascularization and lutienization measurement of CLs with ("Known Pregnant") and without observed embryos/fetuses in female *Stenella attenuata* caught in the ETP pursue-seine tuna fishery. CLs without observed embryos/fetuses were further separated into two categories based in volume, using the local minimum of the frequency distribution (Fig. 3) at 4000mm^3 that separates the two modes that are denoted here as "Small" (<4000mm^3) and "Large" (≥ 4000mm^3).

Variable	t-stat	*P*-value
Vascularization		
Small vs. Large	-4.41	< 0.001
Small vs. Known Pregnant	-5.38	< 0.001
Large vs. Known Pregnant	0.784	0.443
Luteinization		
Small vs. Large	-8.13	< 0.001
Small vs. Known Pregnant	-6.52	< 0.001
Large vs. Known Pregnant	1.51	0.141

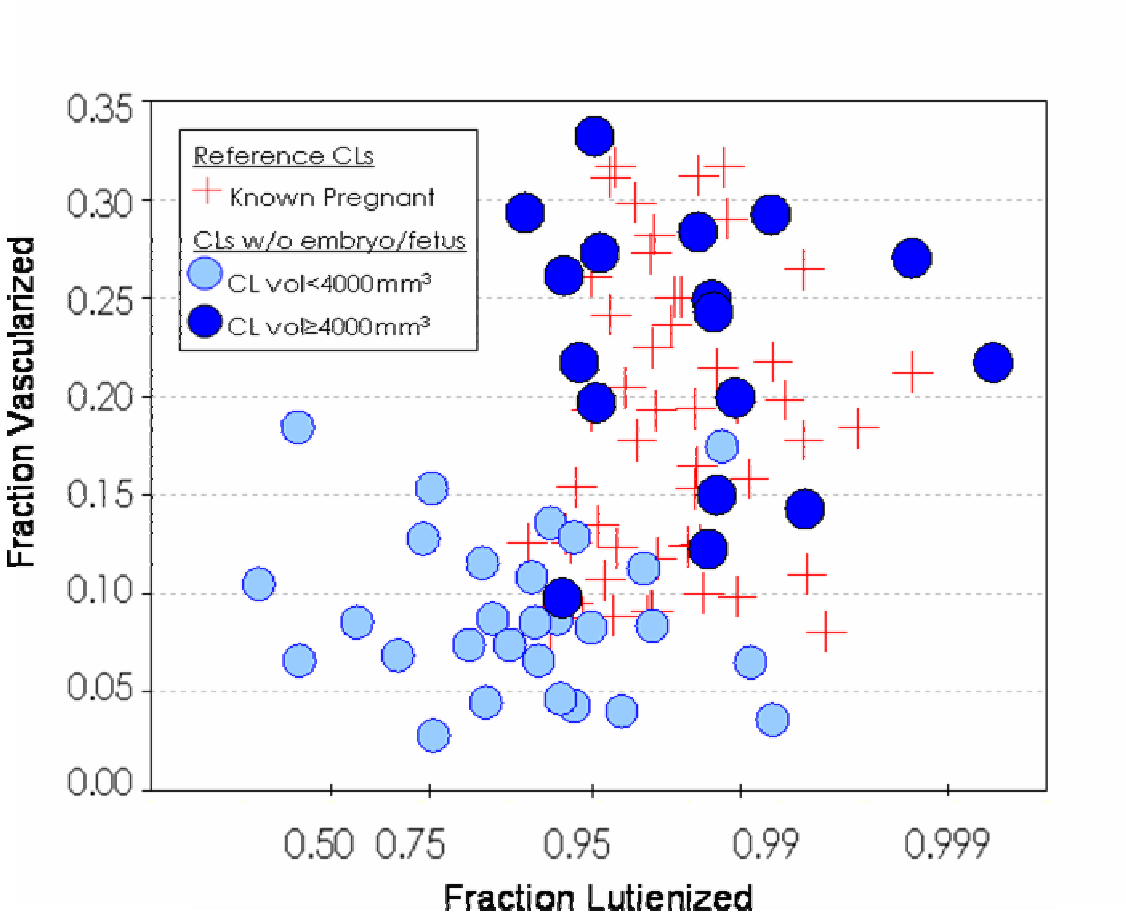

Figure 7. Scatterplot showing the relationship between vascularization and luteinization in copra lutea associated with and without embryos/fetuses. Displayed are the values for two groups of CLs where no embryos/fetus was found; one CL volumes of < 4000mm3 (light blue) and one for CL volumes of ≥ 4000mm3 (dark blue) over laid on values found in CLs of known pregnant females, where an embryo/fetus was found (red crosses). Fraction of corpus luteum that was lutienized as a function of fetal length (mm) during early pregnancy (fetal length < 100mm)

Conclusions

The results of this study suggest that the morphological and histological changes within spotted dolphin CLs after fertilization are dramatic, quantifiable, and useful in diagnosing pregnancy. We find that those CLs we suspect of being associated with ovulation only are smaller in volume, have disproportionately larger unluteinized central cavities, and exhibit less vasculature per unit volume than CLs associated with pregnancy. However, it should be noted that these conclusions are based on predominately circumstantial, albeit strong, evidence. Our study had no independent confirmation of whether the absence of a fetus was due to lack of fertilization, oversight, or abortion/parturition. However, we did find a striking bimodal distribution of CL volume in apparently non-gravid females, which indicates that there were a least two kinds of CLs not associated with a fetus. We hypothesized that the left peak (consisting of lower-volume corpora) contained predominately CLOs, and the right peak consisted of predominately CLPs in which an embryo/fetus was overlooked or birth/abortion occurred immediately prior to specimen collection.

To test this hypothesis, we looked for morphological and histological characters that would differentiate CL types. Because little has been written about the development of CLs in cetaceans, we chose to track changes in two characters (those the literature indicated might be most informative: proportion luteinized and degree of vascularization) during very early pregnancy. We presumed that establishing trends in the development of these characters would allow us to qualitatively back-extrapolate

and identify the approximate state of these characteristics in CLOs. This analysis showed that CLPs of very small embryos (<10mm) had on average larger luteal cavities (unluteinized cores) and smaller blood vessels than CLPs of larger fetuses; we therefore identified these as characters to identify CLOs.

The statistical comparison of the CLs not associated with an embryo/fetus with CLs associated with known pregnancies yielded two important findings. First, of the CLs not associated with either an embryo or fetus, the larger volume CLs had characteristics similar to those found in CLs associated with pregnancies. And second, many of the smaller CLs, had unique characteristics, i.e., characteristics not found in CLs associated with pregnancy. Our interpretation of the data is that smaller CLs could be associated with missed embryos but are more likely to be those of an infertile or not yet fertile ovulation.

Another valid interpretation is that the proportion of vasculature and fraction of luteinized tissue are traits merely proportional to CL volume regardless of ovulation or pregnancy state. Unfortunately, without CLs known to be associated with ovulation only, it would difficult to definitively refute this alternate explanation. However, besides ovulation the only other likely events to generate corpora of similar small volume are during the degradation of the CL as it becomes a CA. In this case, the histological characteristics of a degrading CL have been well delineated in many other mammals and unambiguous. In particular, the extensive hyalinization of the CL as it begins degrading is readily identifiable even in unstained sections.

CLP changes during early pregnancy

Beyond the practical utility of assisting in the determination of pregnancy, we found this information useful to elucidate several aspects of cetacean CL development during early pregnancy.

The rise in the average proportion of luteinized tissue with increasing fetal length in CLPs appears to be driven by further expansion of luteinized tissue into the acellular core region. In this respect, the growth is similar to that during CLO maturation. However, the absolute volume of the CLP non-luteinized central cavity does not decrease dramatically; instead the relative size decreases rapidly because the CL is expanding outward, driven primarily by the proliferation and hypertrophy of the luteinized cells. That is, although growth appears to occur from the outside inward (ex., we observe the extension of vascularization from the former follicular wall inward), we speculate this is true only during the first period of growth, perhaps until the finger-like cellular growth fronts meet (often leaving a more persistent acellular cavity). After that, even in the later phases of CLO maturation and during the first weeks of gestation, it appears that general proliferation throughout the CL and not growth at a particular margin drives the expansion of the total volume of the CL. Following the first weeks of gestation, both actual and proportional cavity size appear to continually decline. This later more gradual decline occurs as connective tissue proliferates from the edge of the luteinized tissue and invades any remaining acellular area.

Blood vessel growth appears less gradual than connective tissue growth during early gestation. The proportion of the CL that is vascularized increases dramatically during the first weeks of pregnancy and then plateaus approximately when average CL volume is at its greatest. However, the rise in the density of endothelial cells appears to be driven by the increase in blood vessel size more than by an increase in the number of vessels per unit of cross-sectional area. The increase in blood flow due to this vasculature expansion likely serves two functions: first it provides additional oxygen and nutrients to fuel the rapid luteinized tissue growth; and second it increases the influx of cholesterol (a precursor of steroid hormones) into the steroidogenic lutein cells while simultaneously exporting newly-produced progesterone. Other cetartiodactyls demonstrate this same pronounced vascularization after "CL rescue" (Zheng *et al.* 1994, Fields and Fields 1996, Sangha *et al.* 2002, Duggavathi *et al.* 2003) though whether this is primarily due to addition of blood vessels or, as in our case, larger blood vessel size, is unclear and inconsistent across species. Nonetheless, it appears that percent vascularization is a useful character to distinguish CLOs from CLPs.

Differentiating CLOs from CLPs

The two characters that we thought could potentially distinguish CLOs from CLPs (fraction of luteinized tissue and vasculature density), showed sharp quantifiable changes during early pregnancy. Consequently, we used them as potentially predictive variables in our statistical comparisons.

This analysis identified two groups. As predicted, the larger volume CLs had characteristics similar to CLPs of fetal lengths greater than 10mm and the smaller ones were more similar to very early pregnancies (fetal lengths < 10mm). Though not definitive evidence that CLOs are smaller than CLPs, these results coupled with the Brook et al. (2002) ultrasound study, which showed a 30% increase in CL diameter (estimated 3-fold increase in volume) in a *Tursiops aduncus* during the week after confirmed conception, forms a more persuasive argument.

If we conclude that the anomalously small CLs (where no fetus is found) are predominately those of ovulation, then it is also reasonable to conclude that CL volume increases approximately three-fold during the first weeks of pregnancy. This indicates that after fertilization, a large increase in volume as the CL transitions from a CLO to a CLP. Though the magnitude of this volume increase is similar to that occurring in other mammals, the average resulting CL mass, as a proportion to overall ovary mass is quite large; ovaries with CLs were approximately seven times larger than the corresponding ovary without a CL.

Although we cannot definitively differentiate every CL into that of ovulation or pregnancy, we have identified characteristics that can help to make this distinction. Two phenomena prevent clear classification of every CL. First, we found high individual variation in the characters we measured even at the same stage of development (e.g., all CL associated with 1mm fetuses), which limited the effectiveness of these characters as differentiation markers. For instance, within known CLPs having associated fetal lengths, we found some CLs of very early

pregnancies (fetal length < 5mm) that had similar vascularization or relative cavity size with CLs associated with much larger fetuses (> 50mm). We expect similar individual variation within CLOs at the same development stage, though in this study we had no independent way to determine CLO age. Greater variation of a character within a class creates overlap between classes and thus minimizes the effectiveness of that character to differentiate classes. The second phenomena likely contributing to misclassification is the transition state between CLO and CLP. After maternal recognition of pregnancy, CLOs develop into CLPs; though this metamorphosis is fairly rapid (on the order of tens of hours to several days) (Duncan *et al.* 1998, Brook *et al.* 2002), these transitional CLs undoubtedly have intermediate characteristics (i.e., between those of CLO and CLP) and thus would be difficult to classify.

Ramifications for CA Interpretation

By identifying likely CLOs and estimating their ratio to likely/known CLPs we can better understand the origin of CAs and the ramifications that different interpretations of their presence has on our understanding of past reproduction. It this study, the ratio of estimated CLOs to CLPs is 0.068 (95% bootstrap CI: 0.057 – 0.091). The expected ratio if all CLOs became CLPs equals the average lifespan of a CLO over the average lifespan of a CLP. Ovulations in some small dolphins have been tracked ultrasonically (Brook 2001, Brook *et al.* 2002, Brook *et al.* 2004). These studies find that a CLO is active (mature) between 12-25 days. Gestation estimates (a proxy for CLP lifespan) for *S. attenuata* in the ETP range from 270-330 days (Šterba

et al. 2000). If every conception ended in a successful pregnancy (to term) then our expected CLO to CLP ratio would be between 0.036 and 0.092. However, factoring in fetal mortality, which is high (60% to 80% (Perrin *et al.* 2003) in this species, we know that the average life span of a CLP is shorter (perhaps substantially) than our estimates of gestation time. Therefore, the expected observed ratio of CLO to CLPs is higher, perhaps double of that using gestation as our estimate of CLP lifespan. Though with this data we cannot precisely estimate the number of ovulations per pregnancy, we can conclude that most ovulations result in pregnancy. This is inline with observations of highly frequent copulation behavior in spotted dolphins (Kenagy and Trombulak 1986, Perrin and Mesnick 2003).

The ramifications that this conclusion has on the interpretation of corpus scars are complex. It is tempting to view our results as evidence that corpus scars record the past pregnancy history of a female in this species and therefore they could be used to estimate past population pregnancy rates; however, there are at least two obstacles to that conclusion. First, because of the lack of precision of average gestation time, CLO lifespan, and fetal mortality, though we are confident that most ovulations become pregnancies, we cannot say with certainty whether the conception rate (% ovulations that lead to pregnancies) is closer to 50% or 100%. Therefore, any pregnancy rate estimation from CAs would be wildly imprecise, especially if scars left by both CLOs and CLPs were enduring. There is evidence from other dolphin species (*Tursiops aduncus*) that CAs derived from CLPs (Brook *et al.* 2002) are more persistent in cetacean ovaries and CLOs do not leave scars that last for very long (< year). This

finding helps support the interpretation of CAs as records of previous pregnancies. However, it is clear that as a non-fertile CLO degrades it must become a CA that remains for some length of time, therefore we know that some CAs are from CLOs. Knowing, with greater precision, the average longevity of these CAs would allow us to estimate the percentage of CAs not associated with pregnancies and consequently allow us to estimate pregnancy rates from CA counts. Studies like those done by Brook *et al.* (2002) with captive dolphins and new ultrasound technology, with better resolution to measure CL dimensions, will greatly enhance our understanding of these phenomena. Nonetheless, our findings suggest that only a minority (or at most a slim majority) of ovulations do not result in pregnancy, and if CAs from non-fertile cycles are relatively ephemeral, as studies have shown in other species, then we expect that the overwhelming majority of CAs come from pregnancies.

The most overt problem with this conclusion is that we find dolphins with more CAs than their estimated reproductive age (i.e. the number of years that they have been sexually mature). Given that gestation is about 11 months and there is typically a resting period between births (lactation has some suppressive effect on ovulation; inter-birth interval [lactation plus resting] estimated to be no shorter that 2 years and more like 3 to 4 years), it is argued that the only way that these animals could have so many CAs is that each has been produced from an ovulation whether it was successfully fertilized or not. However, it has been shown that fetal mortality in this species is high (Perrin *et al.* 2003) and if some females have an innately more difficult time caring a fetus to term, then we would expect that within a population that some

animals could be become pregnant multiple times a year especially if fetal mortality was more concentrated during early pregnancy. That may explain why we see such high CA counts in some females.

Chapter One will be submitted, in part or in full, as a manuscript for publication. I was the primary researcher and author. The co-authors listed in this publican either helped generate raw data or supervised and directed the research from which this chapter was formed. This chapter was written with M. L. Trego and F. I. Archer.

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III. CHAPTER TWO

Determining Pregnancy from blubber in three species of Delphinids

Abstract

We quantified progesterone in 110 blubber samples from dolphins of known reproductive status in order to test the accuracy of a method to determine pregnancy status in wild cetaceans. The samples were collected from fishery-bycaught delphinids of three species (*Delphinus delphis*, *Lissodelphis borealis*, and *Lagenorhynchus obliquidens*). We ascertained that blubber progesterone concentrations could clearly distinguish pregnant *D. delphis* (range 132 – 415 ng/g, mean 261 ng/g) from non-pregnant mature and immature ones (range 0.92 – 48.2 ng/g, mean 15.2 ng/g). We found similar dramatic differences in *L. borealis* and *L. obliquidens*. These results were insensitive to various blubber sampling depths and anatomical sampling locations on the body, suggesting relative homogeneity of progesterone levels throughout the blubber. However, no trend was found in blubber progesterone concentration with fetal length, indicating that although blubber progesterone appears to distinguish pregnant status, it is unlikely to differentiate pregnancy stage. Based on the findings presented here we suggest that this method, when coupled with projectile biopsy procedures, can be used to assess the pregnancy status of free-ranging cetaceans and thus provide a new tool to determine pregnancy rates of wild populations.

Key words: blubber, adipose tissue, biopsy, progesterone, pregnancy, pregnancy rate, short-beaked common dolphin, *Delphinus delphis*, northern right-whale dolphin,

Lissodelphis borealis, Pacific white-sided dolphin, *Lagenorhynchus obliquidens*, gestation, free-ranging.

Introduction

Accurate, non-lethal approaches to determine cetacean reproductive status are needed to estimate pregnancy rate, an index of recruitment, for wild populations. In the past, reproductive status has been determined by examining the reproductive tracts of dead animals, and thus pregnancy-rate estimates have only been made from individuals collected from strandings, harvests or incidental bycatch (Calzada et al. 1996, Heise 1997, Kasuya et al. 1997). There are at least two drawbacks to these approaches. First, these sample sets are frequently biased, with one or more demographic groups either over- or under-represented (George et al. 1995, Iwasaki and Kasuya 1997). The sources of these biases are generally not understood and as such, mathematical corrections cannot be made to obtain the true rates of pregnancy. Second, these approaches are restricted to opportunistic sampling; harvesting activities or the conditions that create strandings dictate the timing, location, and composition of the sample sets (Hohn et al. 1996, Read 1990, Zeh et al. 1995).

At least two non-lethal approaches to determine pregnancy have been previously employed. Perryman and Lynn (2002) used allometric measurements made from aerial photographs to accurately differentiate pregnancy status in gray whales.

However, this methodology is only effective for mid- to late-term pregnancies when the girth-to-length ratio of pregnant females is much greater than non-pregnant ones. Consequently, data derived from this approach are only easily interpreted in populations with strong reproductive seasonality, from which photographs can be taken immediately prior to calving season. Rolland *et al*. (2005) showed that progestins in fecal material can differentiate pregnant right whales from non-pregnant ones. Though this appears to be an effective method, in most field situations fecal collection is impractical; opportunities to collect samples are rare (exceedingly so for many smaller cetaceans) and assessing whether there are multiple contributors of a single sample or repeat samplings of single individuals is challenging.

Other non-lethal methods (i.e., those used for captive cetaceans) are not useful for estimating pregnancy rates for wild populations. In captive animals, pregnancy state has been typically monitored either through sonographic visualization of the reproductive tract or the quantification of progesterone or progesterone metabolites found in the blood and urine, which increase dramatically during the onset of pregnancy (Brook et al. 2004, Brook et al. 2002, Cornell et al. 1987, Duffield et al. 1995, Sawyer-Steffan et al. 1983, Walker et al. 1988). However, due to the innate hazards for researcher and animal, restraining wild cetaceans for ultrasound examination or blood and urine collection are only feasible in the rarest of circumstances. In addition, the concentrations of progesterone often overlap in serum of pregnant and non-pregnant cetaceans (Cornell et al. 1987, Sawyer-Steffan et al. 1983, Temte and Spielvogel 1985). This is likely caused by the variability of

progesterone production by the organ(s) that generate the majority of female reproductive steroids, the corpus luteum, and possibly the placenta[3] resulting in fluctuations of serum progesterone concentration throughout gestation. Although the average concentrations seen in pregnant females are generally much higher than those seen in non-pregnant animals, periods of low production do occur during pregnancy, resulting in concentrations similar to those of non-pregnant animals, especially those levels associated with non-fertile ovulation (i.e., ovulation not leading to conception; Robeck 1996). Consequently, when solely using endocrine markers, a captive female is deemed pregnant, only after elevated progesterone levels have been observed in a series of consecutive sampling events (Sawyer-Steffan et al. 1983). Researchers studying pregnancy status of wild cetaceans can rarely sample the same animals more than once, and to this to estimate the pregnancy rate for an entire population would be impractical.

Measuring progesterone concentrations in blubber obtained via projectile biopsy procedures may be a practical alternative to analyzing serum or urine for reproductive assessment of wild cetaceans. Biopsies are routinely acquired from many cetacean populations to collect tissue to study genetic relationships (LeDuc et al. 2002, Steeves et al. 2001), sex composition (Escorza-Trevino and Dizon 2000, Gowans et al. 2000), diet (Borobia et al. 1995, Hooker et al. 2001, Todd et al. 1997), and contaminate load

[3] To our knowledge, it has not been shown whether the cetacean placenta produces substantial levels of progesterone.

(Hobbs et al. 2003). Researchers using projectile biopsy procedures can non-lethally collect many skin/blubber samples from locations and times of their choosing.

Mammalian adipose tissue, like that found in blubber, is known to accumulate steroid hormones (Dolezel et al. 1991), and the concentrations of these hormones have been associated with different pregnancy states (Hamudikuwanda et al. 1996). The lipophilic steroids amass in high concentrations in adipose tissue because they can passively diffuse from capillaries into this mostly lipid environment (Deslypere et al. 1985, Mead et al. 1986) where metabolic and physiological processes that would break down or remove the steroids are slow relative to those occurring in the blood.

Mansour *et al.* (2002) first suggested using projectile biopsy to examine cetacean pregnancy status. The authors, while not actually employing samples collected in this manner, showed that blubber, which is attached to most biopsies, might be used to assess reproductive status. They found that blubber of pregnant minke whales (*Balaenaptera acutorostrata*) killed in Norwegian whaling operations, contained, on average, 60 times more progesterone than blubber of immature minkes. However, due to lack of samples they were unable to compare progesterone concentrations between pregnant and non-pregnant, mature females. All of the non-pregnant minkes used in the study had no corpora lutea or corpora albicantia (indicators of maturity in cetaceans) thus leaving the results ambiguous as to whether the high blubber progesterone concentrations were associated with sexual maturation or pregnancy. Given what is known about the dramatic increase in production of progesterone by the

corpus luteum during pregnancy, the authors reasonably concluded that endocrine activity during pregnancy was the cause of the higher blubber progesterone levels.

In addition to determining pregnancy status, there are some indications that the blubber attached to biopsies might be used to help determine stage of pregnancy (i.e., the length of time since conception). In all mammals, maternal serum progesterone concentrations increase dramatically during the commencement of pregnancy (Bedford et al. 1972, Gemmell 1995, Ishwar 1995, Spencer and Bazer, 2002). In some of these species, progesterone concentrations continue to rise substantially throughout gestation (Bedford et al. 1972). In these animals, progesterone levels can be used not only to detect pregnancy status but also to roughly estimate stage of pregnancy. Unfortunately, cetacean serum shows no trend in progesterone concentration throughout gestation, at least not substantial enough to help indicate pregnancy stage (Cornell et al. 1987, Robeck 1996). However, if this trend would be represented in the blubber is unknown.

In our study, to further assess the potential of using projectile biopsies to determine pregnancy status in cetaceans, we extracted and quantified progesterone from blubber samples (similar to those obtained from biopsies) of three species of small delphinids: the short-beaked common dolphin (*Delphinus delphis*), northern right-whale dolphin (*Lissodelphis borealis*), and Pacific white-sided dolphin (*Lagenorhynchus obliquidens*). We examined whether blubber progesterone concentrations distinguish reproductive status in these cetaceans, and we specifically examined whether they accurately differentiate pregnant females from non-pregnant

mature ones. We profiled changes in blubber progesterone concentration throughout gestation to determine whether pregnancy stage can be estimated. Finally, we investigated how differences in blubber depth or anatomical location affect progesterone concentration. From these analyses, we attempt to assess the degree in which progesterone concentrations obtained from blubber can correctly classify pregnancy status.

Methods

General

This research is composed of two studies: one to validate our technique and the other to examine whether progesterone concentration varies by anatomical sampling site. The validation study examined three delphinid species and compared females of different reproductive states, focusing on the comparison between pregnant and non-pregnant mature females. In addition, we examined whether blubber progesterone concentration changed significantly during gestation to determine whether pregnancy stage could be estimated.

The anatomical sampling study was conducted to determine if progesterone concentration varied at different places on the body, at different depths below the surface of the skin, or both. For the first part of this study a single *L. borealis*,

determined to be recently pregnant via examination of uterine morphology, was sampled at seven locations, and blubber progesterone concentrations were compared between these locations to determine if the concentrations varied enough to lead to erroneous pregnancy designations. For the second part, progesterone was quantified at three different depths below the surface of the skin in ten pregnant *D. delphis* to determine again if sampling site variability was likely to lead to misclassification of pregnancy status.

Samples

All but one of the blubber samples were taken from dolphins incidentally caught in the California gillnet fishery and collected by observers in the California/Oregon Gillnet Observer Program, between 1991 and 2003, following the protocol delineated in Jefferson et al. (1994).

The single non-fishery sample, from a recently post-pregnant and stranded dead *L. borealis*, was collected by the Southwest Fisheries Science Center Stranding Program. When sampled, this carcass was in the initial stages of decomposition as evidence by moderate drying and wrinkling of the skin. However, there was no prevalent sloughing or cracking of the skin, and the animal appeared un-bloated. In addition, there was no strong decay odor, and the blubber was firm and only slightly blood-tinged.

All fishery samples were collected from the dorsal, mid-thoracic area; data regarding specimen length, girth, and sex were recorded. Pregnancy status, number of corpora, corpus luteum size (where applicable), and length of fetus (if present) were also noted. In summary, we acquired blubber samples from 73 *D. delphis* (18 pregnant, 19 non-pregnant and mature, and 36 immature), 29 *L. borealis* (5 pregnant, 6 non-pregnant and mature, and 18 immature), and 8 *L. obliquidens* (1 pregnant, 2 non-pregnant and mature, 4 immature, and 1 non-pregnant, mature, with distended uterus). Samples were stored between 1 and 132 months in aluminum foil at -20°C before they were extracted. The effect of storage time on measured concentration of progesterone was examined.

Sample Preparation

Cross-sectional sub-samples (150 mg; about the amount obtained by a small biopsy), spanning from epidermis to the subcutis distal to the muscle (~15 mm), were sub-sectioned from the large slabs of blubber collected in the field. Care was taken to cut away any areas of discoloration resulting from freezer storage. The thin columns of blubber were then placed into tared homogenization tubes (see below) and weighed. All slabs were sub-sampled and processed in triplicate.

To examine the variation of progesterone concentrations between different anatomical locations, we sampled the recently post-pregnant *L. borealis* specimen at multiple body sites. Blubber slabs, similar to those collected by the observer program,

were taken from seven locations along the left side of the specimen. Each slab was then sub-sampled six times, resulting in a total of 42 sub-samples. The sub-sampling and weighing procedures were the same as described above.

To determine the relationship between blubber depth and progesterone concentration, we examined the outer, middle, and inner layers of the blubber. Sub-sampling procedures were similar to those described above; however, larger vertical sub-samples were taken, each weighing approximately 500 mg. These were then further subdivided into three nearly equal horizontal subsections, each weighing between 100 and 200 mg. These were then weighed in preparation for extraction. Three sub-samples were taken from each of the ten pregnant *D. delphis*, for a total of 30 sub-samples. These sub-samples were further horizontally subdivided, as described previously, for a grand total of 90 vertical sections. From these 90 sections, we examined whether progesterone concentration varied with blubber depth, in such a way, that misclassification of pregnancy status from biopsies was likely.

In attempts to examine the impact of postmortem decay on progesterone concentration, six sub-samples from the blubber of a pregnant *L. borealis* (previously frozen stored at -20ºC) were incubated at approximately 22ºC for 52 hours (to approximate the postmortem time of a stranded carcass with minimal signs of decay). Progesterone was measured in these sub-samples and compared to concentrations we obtained directly from the frozen blubber.

Steroid Extraction

The sub-samples were homogenized in 1000 µl 100% ethanol using a automated, multi-tube homogenization instrument (FastPrep Instrument Qbiogene) and were processed for eight 45-second periods at a speed of 6.5 m/sec in specialized lysing matrix tubes available from the instrument manufacturer. The homogenates in ethanol were mixed, via a multitube vortex mixer (VWR International, VX 2500), and 500 µl were aspirated from each tube and placed into 12 x 100 mm disposable glass culture tubes. The homogenates were centrifuged (3000 rcf, 10 min), and the supernatants collected. They were evaporated under compressed air using an Evap-O-Rac (Cole-Palmer EW-01610-15) while incubating in 25°C water. Two milliliters of ethanol:acetone (4:1) were added to the residue, vortexed, and centrifuged as before. This solution was evaporated, and to the new residue, 1000 µl diethyl ether were added. The samples were again vortexed, centrifuged (3000 rcf, 15 min), and evaporated. To the resulting residue, 1000 µl acetonitrile was added and thoroughly vortexed. Then hexane (1000 µl) was added, vortexed, and centrifuged (20 min). The solvents formed two immiscible layers with hexane on top. The acetonitrile layer was collected and re-extracted with 1000 µl hexane, centrifuged (20 min), and the final acetonitrile layer was aspirated and evaporated. The final residue was centrifuged (2500 rcf, 5 min) and frozen at -20°C until analyzed.

This extraction method was modified from one delineated in Mansour *et al.* (2002). The modifications were implemented to make the procedure easier and less expensive to use by reducing the total number of steps, employing an automated

multitube homogenization instrument, and substituting compressed air for nitrogen. Twenty samples (eight pregnant, six non-pregnant and mature, and six immature) were extracted four times; twice using our final procedure and twice using the one delineated in Mansour *et. al*, (2002). The results of these extractions were examined to assess the comparability of the two procedures.

Enzyme Immunoassay

Prior to analysis, we re-dissolved all samples in 500 µl of phosphate buffered saline (pH 7.5) containing 1% bovine γ-globulin and mixed them thoroughly using the multitube vortex mixer at medium speed for 15 minutes. Progesterone levels were measured using a commercially available enzyme immunoassay kit, DSL-10-3900 (Diagnostic Systems Laboratories, Inc., Webster, TX) with a standard curve range between 0.33 and 1000 ng/ml. The reported inter-assay coefficient of variation (COV) ranged from 3.4-7.0% and intra-assay COV ranged from 4.1-5.0%. Immediately before a sample was added to the assay plate, it was mixed (30 sec) using a mini-vortex mixer. To control the measurement error contributed by all extraction and quantification steps, each sample was extracted and measured, at minimum, three times and reported as the average nanograms of progesterone per wet weight of sub-sample.

Extraction efficiency was determined for each group of extractions by spiking selected sub-samples with dilutions of cold progesterone, ranging from 0 ng to 45 ng

(45 ng is equal to 300ng/g for a 150mg sample and is within the range expected for pregnant females), in the matrix tubes before initial homogenization. We extracted and quantified the progesterone in these sub-samples according to the procedure described above. The extraction efficiency was calculated as the amount of quantified progesterone (via enzyme immunoassay analysis) of the spiked samples minus the quantified amount in the non-spiked samples, all divided by the original amount of progesterone added (spiked) before extraction. The efficiency range was 63.3 - 95.9% with a mean of 71.1%.

Data analysis and interpretation

To compare the concentrations of different reproductive classes, we analyzed the data with a one-way ANOVA followed by a post-hoc Tukey test. The concentrations were log-transformed to reduce heteroscedastic variation from measurement error. An additional one-way ANOVA/Tukey test was conducted with the log-transformed concentrations to test if significant differences in progesterone concentrations were seen between the seven anatomical sampling locations. To assess the relationship between progesterone concentration and gestation time (as estimated by fetus length), a linear correlation analysis was performed with the non-transformed concentrations. Finally, a simple linear regression analysis was employed to assess the effect of storage time on measured progesterone concentration.

To examine progesterone concentration as a function of blubber depth, we obtained average concentrations at each layer (i.e., outer, middle, and inner) in ten individuals. The significance of the differences in these average concentrations was assessed via the Krustal-Wallis test, with the data grouped by layer. This non-parametric test was employed so that females with considerably higher blubber progesterone would not dominate the results.

To assess the difference in concentration in identical samples using the different extraction techniques, a pair t-test was employed. The description of the coefficients of variation within methods and between methods were also obtained. A student t-test was used to test the significance of differences in the average concentration between samples incubated at ambient temperature and those left frozen.

All statistical comparisons in this study were considered significant at $P \leq 0.05$. Information regarding the depth of sample, anatomical location of the sample, and reproductive status were kept blind to those who were extracting and quantifying the steroids.

Results

Blubber samples from pregnant females had dramatically more progesterone than those from non-pregnant mature and immature females ($P << 0.001$ for both comparisons). Pregnant *D. delphis* had on average 16 times more blubber

Table 2. Progesterone concentrations in the blubber of pregnant, non-pregnant and immature females of four cetacean species. The progesterone concentrations are corrected for extraction efficiency (see text) and are reported as ng/g of blubber extracted. Average values are displayed with standard error.

	D. delphis	*L. borealis*[a]	*L. obliquidens*	*B. acutorostrata*[b]
Pregnant	**261 ± 29**	**312 ± 44**	**161**	**132 ± 22**
Average	**132**	**196**	**-**	**22.8**
Min	**415**	**402**	**-**	**454**
Max	**18**	**5**	**1**	**22**
N				
Non-pregnant/mature	**13.7 ± 1.8**	**15.0 ± 7.5**	**12.1 ± 8.4**	**Not available**
Average	**6.75**	**2.11**	**3.75**	
Min	**33.3**	**34.7**	**20.5**	
Max	**19**	**6**	**2**	
N				
Immature	**16.5 ± 2.7**	**14.2 ± 2.30**	**18.1 ± 9.1**	**1.95 ± 0.32**
Average	**0.92**	**0.98**	**0.11**	**1.36**
Min	**48.2**	**33.1**	**34.4**	**3.43**
Max	**36**	**18**	**4**	**6**
N				

[a] Does not include the *L. borealis* sample with distended uterus
[b] From Mansour *et. al* 2002

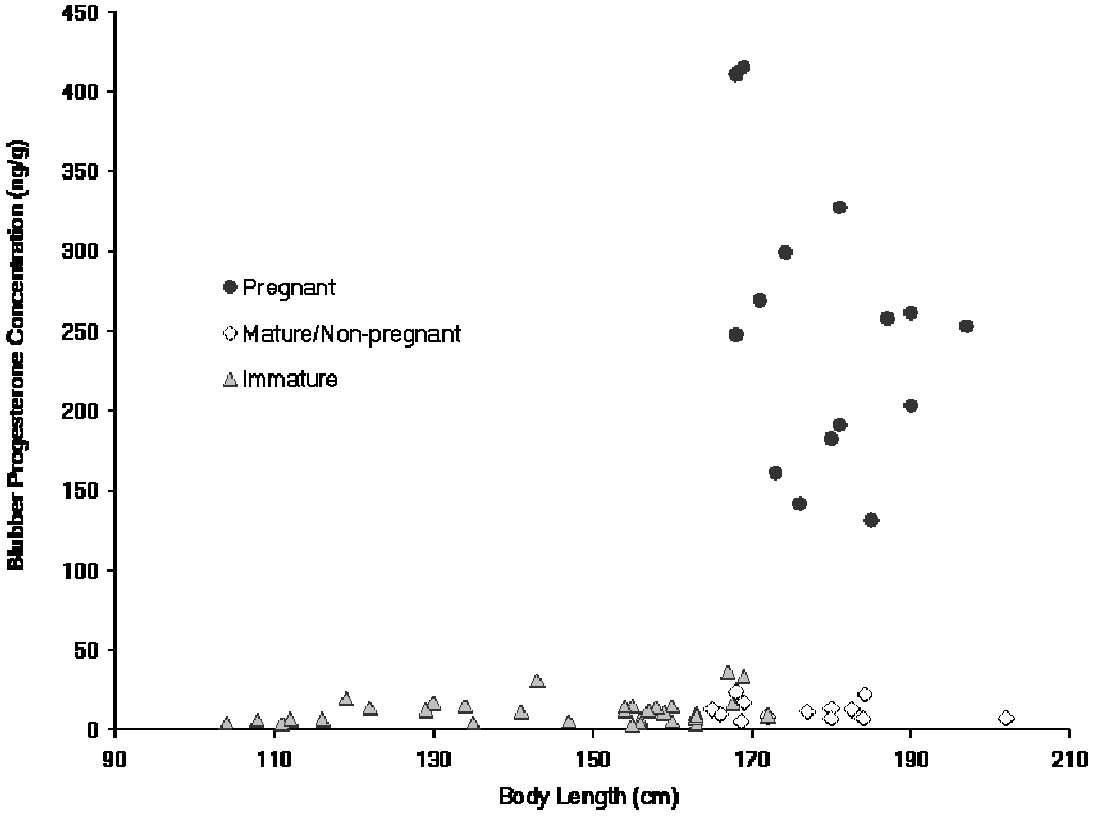

Figure 8. Blubber progesteorne concentrations as a function of total body length in pregnant, mature but not pregnant, and immature females *D. delphis*.

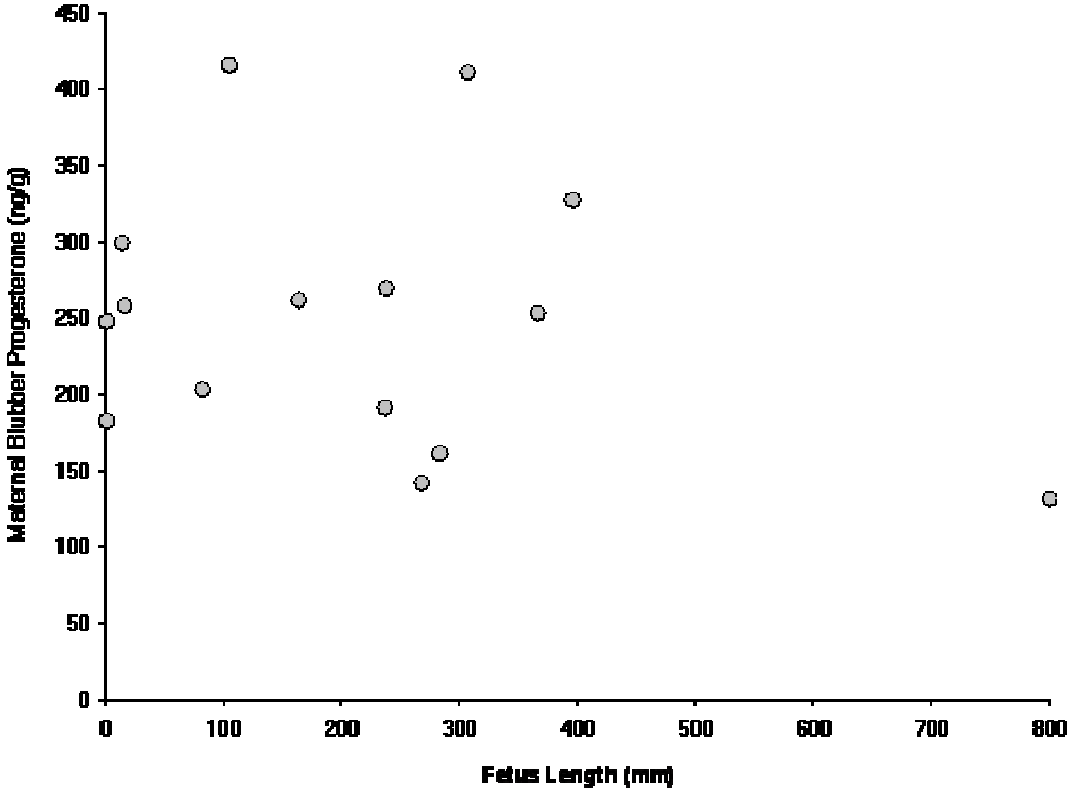

Figure 9. Blubber progesteorne concentrations as a function of total body length in pregnant, mature but not pregnant, and immature females *D. delphis*.

progesterone than non-pregnant mature dolphins (Table 2). Moreover, there was no overlap in the range of concentrations of these two reproductive states, but rather a four-fold difference existed between the lowest observed levels in pregnant animals and the highest seen in non-pregnant ones (Fig. 8). No significant difference was seen between progesterone concentrations of immature and non-pregnant mature females ($P = 0.49$). Similar results were seen for both *L. borealis* and *L. obliquidens* (Table 2).

Although a 16-fold difference existed between pregnant and non-pregnant mature animals, no trend in progesterone concentration with stage of pregnancy was observed. Blubber progesterone concentrations taken from 15 pregnant *D. delphis* showed no correlation between progesterone concentration and fetal length ($r = 0.241$, $P = 0.387$; Fig. 9). However, we note that the sample set contained only one fetus larger than 40 cm, which is approximately the halfway point of fetal development in this species. Given this sample distribution, an increase or decrease in concentration during the second half of gestation could have been missed. Too few specimens were collected from the other species to conduct a meaningful correlation analysis.

Average blubber progesterone concentrations were relatively similar at different depths below the skin (Fig. 10). Although concentrations from the middle layer were on average 1.45 and 1.39 times higher than in the outer and inner layer respectively, we did not find significant differences between the three depths ($\chi^2 = 3.16$, $P = 0.21$). More importantly, there were no substantial differences found between layers; they were not at the same magnitude as the difference observed between pregnancy states. As a result, where in the blubber layer a sub-sample was obtained would not cause a

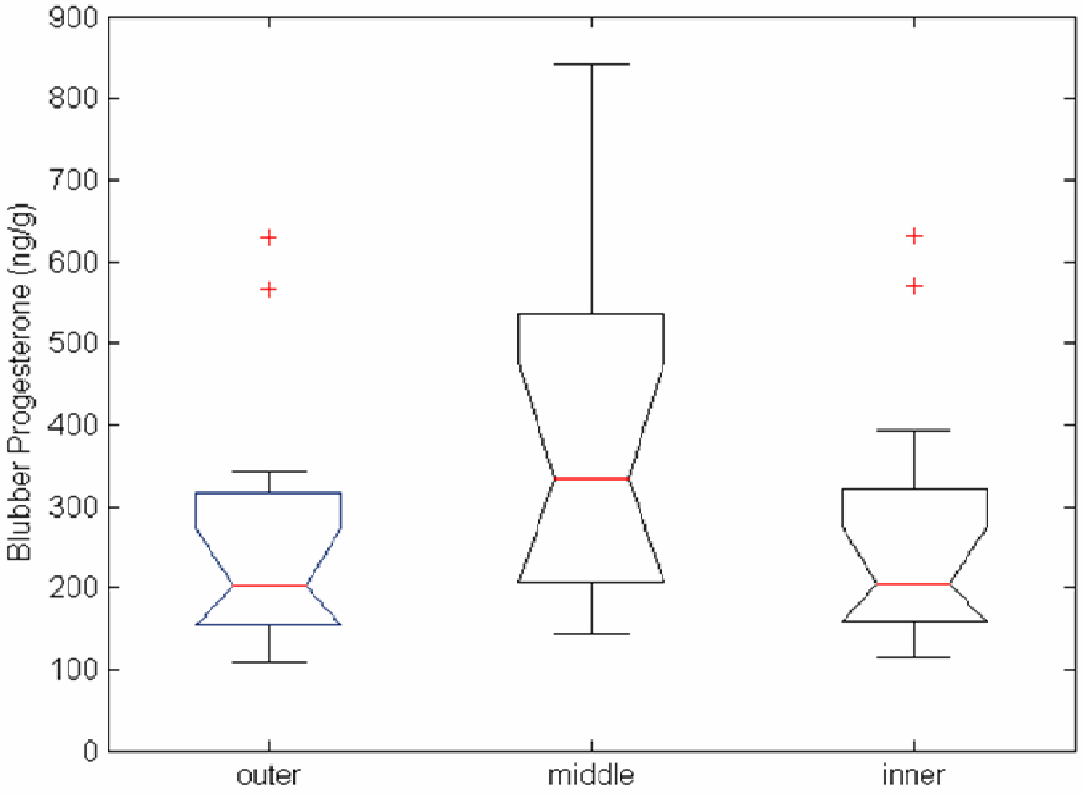

Figure 10. Blubber progesteorne concentrations for outer, middle, and inner blubber layers. The concentrations were quantified from ten pregnant D. delphis. Horizontal box lines represent the lower quartile, median, and upper quartile values from bottom to top. Whisker lines indicate range of concentrations and the pluses represent outliers (1.5 time interquartile range). Points of inflection represent upper bound to the 95% confidence interval. The lower CI bounds were not shown as they were lower than the first quartile line. No significant differences were found between any of the layers.

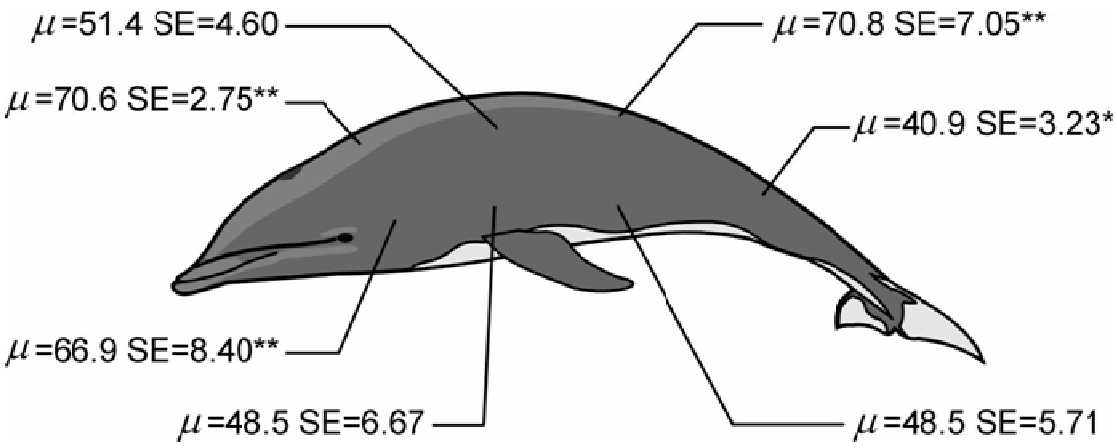

Figure 11. Blubber progesteorne concentrations at seven anatomical locations in a recently pregnant L. borealis. Six subsamples were quantified at each location. Though there are significant different between concentrations found at the peduncle sampling location (*) and three of the more anterior sampling areas (**), these differences were relatively small compared to those found between pregnant and non-pregnant animals (see text)

misclassification, because the concentrations at any layer were still within the range found for pregnant animals.

The blubber samples from the recently post-pregnant *L. borealis* yielded two important findings. First, though there were significant differences between the peduncle sample and three of the more anterior samples ($F = 4.37$, $P = 0.0026$; Fig. 11), they were not substantial. The variation of blubber progesterone concentration due to anatomical location should not compromise the accuracy of pregnancy designation. The lowest concentration (40.9 ng/g in the peduncle) was still 57.8% of the highest concentration (70.8 ng/g in the dorsal midline, posterior to the flipper). When compared to the results shown in Table 2 where the highest concentration in the non-pregnant *L. borealis* females (34.7 ng/g) is only 17.7% of the lowest concentration in the pregnant ones (195 ng/g), we found that the variation due to body location was diagnostically small. Second, we found that blubber progesterone concentrations of post-pregnant *L. borealis* were higher than the levels we saw in non-pregnant females but still much lower than those seen in pregnant females. This was the only value in our sample set where the levels were between those seen in pregnant animals and those seen in non-pregnant animals, and the only one that could have been misclassified.

We found no significant relationship between storage time at -20ºC and measured progesterone concentration in *D. delphis* blubber samples. This finding was consistent across the three reproductive groups: pregnant ($r = 0.310$, $P = 0.260$), non-pregnant mature ($r = 0.411$, $P = 0.169$), and immature ($r = 0.153$, $P = 0.375$).

Though the six samples incubated at ambient temperature for 52 hours exhibited signs of decay (discolored tissue and pronounced odor), we found no significant differences between their average progesterone concentrations and those from the frozen samples ($t = 0.617$, $P = 0.551$), suggesting that progesterone does not decrease rapidly in blubber samples kept at ambient temperature. In fact, average concentrations were slightly higher in the incubated samples (218.6 ng/g versus 199.7 ng/g).

When comparing the two extraction procedures (ours and the one described in Mansour *et al*. 2002), we found no significant differences in the concentrations using paired t-tests for each of the reproductive groups (pregnant p=0.23, non-pregnant and mature p=0.56, and immature p=0.44). We also found that the variability between extractions of identical samples using the same procedure was as high as the variability between procedures. The average paired coefficients of variation (COV) were 19.9% for the Mansour *et al.* procedure and 18.1% for the one delineated in this paper. Between procedures, the COV value was 19.6%. These results suggest that the data derived from the two procedures are comparable however; they also show that variation is high using either procedure. Therefore, replicate extractions are recommended when specific concentration is desired. Nonetheless, this amount of variation is small when compared to the differences in average concentration between pregnancy states in these delphinids and therefore is not problematic for pregnancy determination.

Discussion

Although progesterone has been previously extracted and quantified from cetacean blubber (Mansour et al. 2002), the data in this study are the first from delphinids and the first that show differences between pregnant and non-pregnant mature cetaceans, clearly a crucial distinction when detecting pregnancy. All but one of the female cetaceans (n = 110) examined in this study could be assigned to their correct reproductive status based solely on blubber progesterone concentration. In addition, this study indicates that potential sources of misclassification due to the vagaries of projectile biopsy sampling are unlikely to affect pregnancy determination. Thus, we are confident that blubber progesterone is an accurate indicator of cetacean pregnancy in wild populations. This is fortunate because there are currently no other practical alternatives except in very circumscribed situations.

The blubber progesterone levels of pregnant and immature animals found in the three species examined in this study were higher than those found in minke whales (Mansour et al. 2002). However, in both studies, pregnant animals were found to have much more blubber progesterone than immature animals; 60-fold more in minke whales and 16-fold more in the three species of delphinids studied here. Similarly, we also found that blubber progesterone concentrations in pregnant females were much higher (19 times) than those found in non-pregnant mature animals. For our subjects,

this indicates that the higher progesterone levels are, as expected, associated with pregnancy and not maturity.

The differences in blubber progesterone concentration that distinguish pregnant and non-pregnant animals can be contrasted with the differences found by examining serum and urine samples. Although elevated levels of progesterone in serum and progesterone metabolites in urine have previously been used as indicators of pregnancy in *Tursiops truncatus* (Cornell et al. 1987), *Orca orcinus* (Duffield et al. 1995), *Balaenoptera physalus* (Olafsson and Kjeld 1986), and *Phocoenoides dalli* (Temte and Spielvogel 1985), the differences between pregnant and non-pregnant concentrations of progesterone and progesterone metabolites overlap substantially (Robeck 1996, Sawyer-Steffan et al. 1983). In contrast, blubber progesterone concentrations from pregnant and non-pregnant cetaceans did not overlap in this study or the one conducted by Mansour *et al.* (2002). The only specimen (the *L. borealis* that was sampled in seven anatomical locations) that fell between the levels observed for pregnant and non-pregnant animals was a recently post-pregnant female transitioning between reproductive states (discussed below).

Although blubber progesterone concentration seemed to be an accurate indicator of pregnancy status, it did not provide information to differentiate stage of pregnancy in the specimens of *D. delphis* examined here. Instead, as in cetacean serum (Cornell et al. 1987), the level of progesterone in blubber showed no clear trend with time in gestation.

Blubber progesterone concentrations obviously must track serum concentrations, albeit lagging in some complex manner. In other cetartiodactyls, strong correlations between serum and subcutaneous adipose tissue have been observed (Hamudikuwanda et al. 1996, Hillbrand and Elsaesser 1983), although adipose concentrations have been found to lag behind those in serum by about 16-24 hours when progesterone levels are increasing in the serum and 32-50 hours when levels are decreasing (Hillbrand and Elsaesser 1983). More research is required to elucidate the specifics of the dynamic relationship of progesterone concentrations in cetacean blood and blubber, but the data obtained in this study are in agreement with the common wisdom that they are tightly associated.

Important for sampling via projectile biopsy, we observed that blubber progesterone concentration did not substantially change with anatomical location or depth of sampling. This is in contrast to other non-polar macromolecules, such as naturally occurring triglycerides, which are generally thought to be strongly stratified in both spatial distribution, concentration, and identity (Aguilar and Borrell 1990, Olsen and Grahl-Nielsen 2003). Differences in these relative concentration distributions may be due to differences in intercellular transportation of the lipophilic macromolecules. Steroids like progesterone are thought to diffuse passively from the blood into adipose tissue with minimal metabolic processing (Hillbrand and Elsaesser 1983, Mead et al. 1986). In contrast, most triglycerides must be broken into their basic metabolic units (i.e., glycerols and fatty acids) before they can be transported

through cell membranes (Mead et al. 1986), possibly leading to the regulation of triglyceride distribution in the blubber.

Although sampling location does not appear to affect pregnancy determination, there are several situations not addressed in this study that could potentially lead to misclassification. Corpora lutea that are associated with non-fertile (i.e., not leading to conception) estrous cycles also elevate serum progesterone concentrations. However, these levels are usually lower than the average concentrations seen in pregnant animals (Brook et al. 2004). Because we did not obtain samples from females in this state we were unable to document its effects on blubber progesterone concentrations. A similar situation arises with pseudo-pregnant females. In this condition, the reproductive tract behaves as though it were pregnant; the corpus luteum is maintained and produces progesterone concentrations in the serum at levels similar to those seen during pregnancy (Robeck et al. 2001). Although we saw no evidence of this condition in the samples we examined, it is observed in captive animals, and if present in wild cetaceans, it would likely produce misclassifications of status. Both conditions, non-fertile estrous cycles and pseudo-pregnancies, would lead to over-estimates of pregnancy rates in wild populations based on blubber progesterone levels. The rates of these conditions are unknown, and as such, their potential bias cannot be precisely estimated.

Another potential source of error in utilizing blubber progesterone levels to determine pregnancy, is the timing of the elevation and decline in blubber progesterone with respect to commencement and termination of pregnancy. In our

study, we observed that in embryos with total lengths < 1cm or approximately 2 to 3 weeks postconception (Šterba et al. 2000), blubber progesterone levels were between 183 and 248 ng/g, well within the total range we see for females with larger fetuses and much higher than those found in non-pregnant animals. This suggests that progesterone levels rise quickly in the blubber at the commencement of pregnancy. Clearly, at some earlier point during gestation, blubber progesterone concentrations are not effective at distinguishing pregnancy status. We suspect that the blubber progesterone levels rise to moderate levels post-estrous, as they do in the blood, and then rise further after implantation. Thus, the concentrations seen during the very earliest stages of pregnancy would likely overlap with the levels seen immediately after non-fertile estrus.

Similarly, after parturition, when progesterone levels are declining, it is likely that there is a short period when progesterone levels would be ambiguous. Females in this state, though no longer pregnant, are likely to have blubber progesterone concentrations that are still elevated. How long blubber progesterone remains elevated is unknown. However, we do know that in the beach cast, recently post-pregnant *L. borealis* had a distended uterus, indicating that it had been pregnant within the few days before its stranding. We do not know exactly how long after the termination of its pregnancy the animal was sampled. Nevertheless, this female's progesterone levels were already down to 30% of the lowest level we observed for *L. borealis* carrying a fetus. In most mammals, after normal parturition, the uterus regains pre-pregnancy state within a short period (Dolezel et al. 1991, Henell et al.

1983, Katila 1988, Ouellette and Ronald 1985). For example, in cattle, uterine morphology returns to non-gravid shape within ten days post-parturition (Dolezel et al. 1991). Given this short physiological window in which the uterus is distended and the dramatically lower blubber progesterone levels found in the post-pregnant *L. borealis*, it is likely that the decrease in progesterone occurs rapidly after parturition. Knowing the precise relationship between apparent pregnancy (i.e., elevated levels of blubber progesterone) and true pregnancy (i.e., the presence of an embryo/fetus) would allow us to more accurately estimate pregnancy rates in the wild.

Another plausible explanation for the lower levels of progesterone found in this animal is that the processes involved in decay reduced the levels of progesterone as a proportion of tissue mass. In attempts to address this issue, we incubated blubber sub-samples at temperatures roughly equal to those that a carcass might be exposed to before it could be sampled. We found no significant differences between their average progesterone concentrations and those from the frozen samples. In fact, average concentrations were slightly higher in the incubated samples (218.6 ng/g versus 199.7 ng/g). This suggests that progesterone does not decrease rapidly in blubber under 'ambient' conditions. This finding is consistent with other studies that show progesterone can be stable in various biological samples at temperatures above 20ºC (Eissa *et al.* 1995, Galama *et al.* 2004, Groschl *et al.* 2001, Wiseman *et al.* 1983).

Although there are several potential sources of error inherent in determining pregnancy status via blubber progesterone concentrations, to our knowledge there are no other practical means to obtain non-lethal estimations of pregnancy rates in wild

cetacean populations. Given the importance of determining these rates for population dynamic models and the lack of other means to do this, we believe that this approach, when coupled with projectile procedures, will be a useful tool for researchers.

The material found in Chapter Two is an adapted version of the text that was published in Marine Mammal Science 22:1-16. I was the primary researcher and author. The co-authors M. L. Trego and C. I. Marks helped generate replicate raw data and A. Dizon supervised the research that formed the foundation of this chapter.

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IV. CHAPTER THREE

Blubber testosterone: A marker of male reproductive status in short-beaked common dolphins

Abstract

We developed and implemented a novel approach to detect reproductive seasonality and estimate the proportion of sexually mature male dolphins biopsied in the water off California. By quantifying testosterone in the blubber of skin biopsies androgen level was assessed and then evaluated as an indicator of seasonality and reproductive maturity. Blubber testosterone (BT) was measured in 116 male short-beaked common dolphins, *Delphinus delphis*, collected from fishery bycatch or beach strandings. When these concentrations were compared between maturity states (determined independently) we found average ($\mu \pm$ SEM) BT levels of mature *D. delphis* (14.3 ± 3.0ng/g) were significantly higher those of pubertal (2.5 ± 0.5ng/g; $p = 0.006$) and immature animals (2.2 ± 0.3ng/g; $p < 0.0001$). We also found that BT concentrations in mature males were much higher in summer months than those measured the rest of the year, indicating seasonality in mating. The data collected from the fishery and stranding samples were then employed as a reference set to estimate the proportion of mature males found in 299 biopsied *D. delphis* (of unknown maturity state) sampled off California; we modeled the probability of maturity as a function of BT concentration and ordinal date. From two resulting models, we estimated that between 0.24 and 0.38 of the biopsied males were mature.

Introduction

An approach that assesses testosterone levels in projectile biopsies could provide pertinent data on seasonal breeding preferences and the proportion of sexually mature males within free-ranging cetacean populations. The increase of testosterone during postnatal development is a key factor that defines puberty in male mammals (Preslock 1980). Consequently, testosterone concentration is commonly used as the endocrine indicator of male reproductive maturity in many mammals, including cetaceans. The relationship between serum testosterone concentrations and maturity state has been previously documented for several cetacean species including *Phocoenoides dalli* (Temte 1991), *Delphinapterus leucas* (Robeck *et al.* 2005), and *Tursiops truncatus* (Kirby 1990).

Testosterone concentrations have also been used to reveal seasonal breeding patterns. A common mammalian characteristic of seasonal reproduction is a dramatic increase in testosterone production prior to peak breeding (Kaplan and Mead 1993, Lincoln 1998, Williams *et al.* 1998, Buck and Barnes 2003, Muteka *et al.* 2006). Previous studies have effectively evaluated seasonal breeding preferences using serum testosterone concentration in different species by documenting the presence or absence of this pattern in *Tursiops truncatus* (Schroeder and Keller 1989), *Delphinapterus leucas* (Robeck *et al.* 2005), and *Stenella longirostris* (Wells 1984).

These previous studies have predominately used serum and, very recently, fecal material, to quantify testosterone in non-captive animals (Robeck *et al.* 2005, Rolland *et al.* 2005). However, serum is exceedingly difficult to obtain from free-ranging

cetaceans except under highly circumscribed conditions and therefore is impractical to be used in population level assessments. Fecal samples have also been used to assess male maturity state (Rolland *et al.* 2005) but opportunities to collect these samples are limited, often needing special detection systems (Rolland *et al.* 2006), and do to relatively small scat size, fecal material can be difficult to collect for many dolphin species.

Fortunately, blubber is a useful alternative to fecal material and serum. This lipophilic tissue accumulates high concentrations of steroid hormones (Deslypere *et al.* 1985, Kellar *et al.* 2006) and is attached to most skin biopsies collected using projectile biopsy techniques. Moreover, these biopsies are routinely obtained for studies that examine many aspects of cetacean biology including genetic relationships, diet, and contaminant loading (Todd *et al.* 1997, Hooker *et al.* 2001, Hobbs *et al.* 2003, Escorza-Trevino *et al.* 2005). Although we are not aware of any previous studies that have quantified testosterone in blubber, we do know that progesterone, a reproductive steroid hormone with similar solubility and chemistry, has been successfully quantified in blubber and used as a marker for cetacean pregnancy (Mansour *et al.* 2002, Kellar *et al.* 2006). In fact, concentrations of progesterone in blubber exceed three times those found in blood (Kellar *et al.* 2006). These high concentrations make quantification of steroids easier and likely more reliable.

Quantifying blubber testosterone (BT) in skin biopsies to determine reproductive seasonality and sexual maturity could provide demographers and life

historians access to additional data that cannot be acquired using traditional approaches. Historically, this information has been determined by examining the gonadal tissue of dead specimens. Consequently, demographic and breeding information is derived from individuals collected from strandings, harvests or incidental bycatch (Calzada *et al.* 1996, Heise 1997, Kasuya *et al.* 1997). This opportunistic collection of samples has the drawback that harvesting activities or the conditions that create strandings dictate the timing, location, and composition of the sample sets (Read 1990, Zeh *et al.* 1995, Hohn *et al.* 1996). An approach that utilizes easy-to-obtain biopsy samples collected more randomly from live animals would reduce some of these problems.

In our study, to assess the potential of using projectile biopies to determine male maturity status and seasonality, we extracted and quantified testosterone from small blubber samples (similar to those obtained from biopsies) of short-beaked common dolphins (*D. delphis*) incidentally the California gillnet fisheries (Barlow and Cameron 2003). Each male was classified as immature, pubertal, or mature based on testis histology and mass in combination with standard specimen length. We then compared testosterone concentrations for each maturity state to evaluate whether BT level to discriminate these states. From these data, using a logistic regression structure, we modeled the probability of maturity as a function of BT concentration alone and then as a function of BT concentration plus ordinal date. We estimated model parameters using a Bayesian approach and assessed the relative fit of each model based on Decision Information Criterion (DIC). From the resulting models in

conjunction with biopsies collected from free-ranging *D. delphis* inhabiting the waters off California, we described the seasonal variation in testosterone concentrations and estimated the proportion of sampled males that were mature.

Methods

Samples:

Reference Collection – Fishery/Stranding Samples

The blubber samples (n = 116) used to develop and validate our approach were obtained from male dolphins for which we had associated gonadal tissue for independent assessment of maturity. The majority of these (n = 106) were incidentally caught in the California gillnet fishery and collected by observers in the California/Oregon Gillnet Observer Program between 1991 and 2005. The remaining specimens (n=10) were collected by the Southwest Fisheries Science Center Stranding Program. Each blubber sample was collected from the dorsal, mid-thoracic area following the protocol described in Jefferson et al. (1994) and stored in aluminum foil at -20°C before being processed. The right testis (left when right was not available) of each individual was collected for morphometric measurements, mass determination and histological examination.

Experimental Data Set - Biopsy Samples

Skin biopsies (n= 299) were obtained via projectile sampling of free-ranging *D. delphis* in the waters off California (Fig. 12). These samples were stored at -20° in 2ml cryovials until processed. The sex of each biopsied animal was determined by the

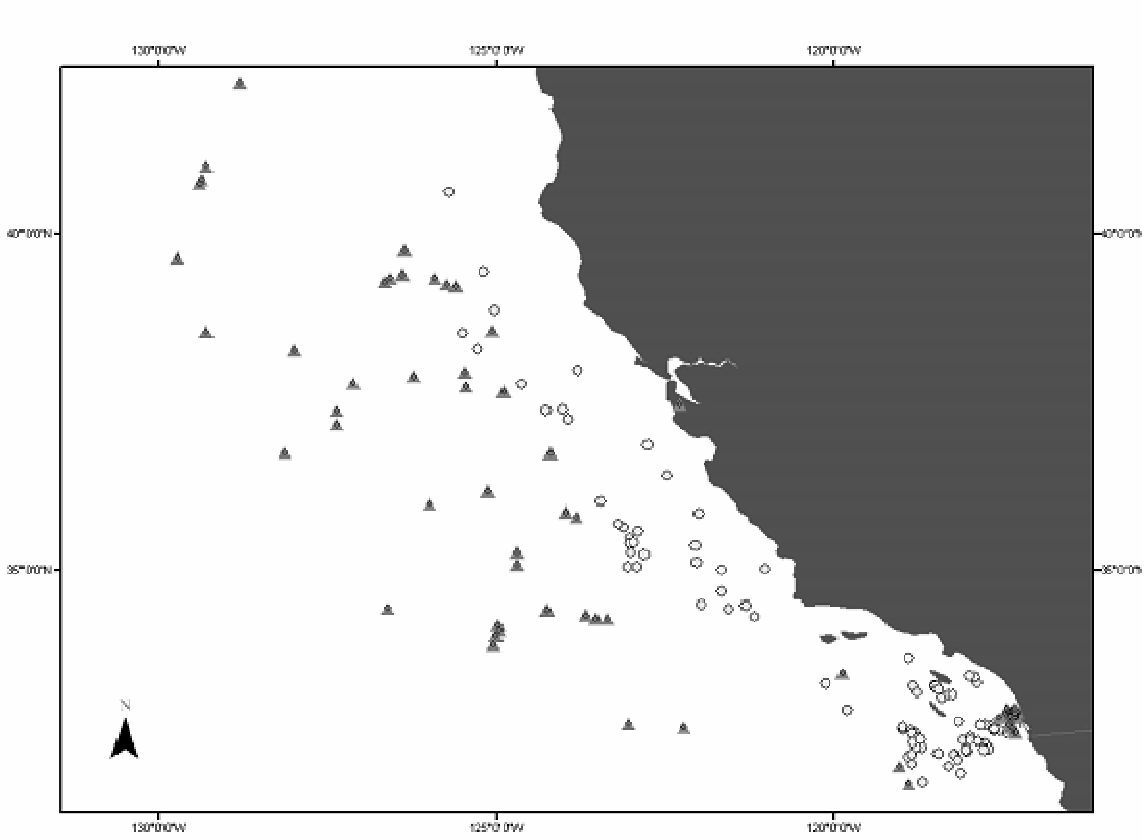

Figure 12. Geographic distribution of the blubber samples from fishery-killed (circles) and biopsied (triangles) male *D. delphis* used in this study.

presence of sex specific genes found in the epidermis layer of the skin. Two assays were used: standard electrophoresis (Fain and LeMay 1995) on all of the biopsies and quantitative PCR (Morin *et al.* 2005) on a subset of 30 randomly selected samples to assess our rate of error. Only 261 skin samples contained more than 50.0mg of blubber, the minimum needed for our assay.

Sample processing

Sexual maturity state of each animals sampled for the reference collection was determined independently of BT concentration from histological preparations of tissue excised at the testis' mid-length following methods delineated in Akin *et al.* (1993). Thin sections, stained with haematoxylin and eosin, were examined at magnifications of up to 600 x for the presence and abundance of spermatozoa and size of seminiferous tubules (Fig. 13). We used the criteria delineated in Collet and St. Girons (1984) to classify each individual as immature, pubertal, or mature. The maturity classifications were graphed to show the relationship between specimen length and testis size as a qualitative check for potential outliers/misclassifications (i.e., the majority of immature animals are shorter and have smaller testes than mature animals).

To investigate the BT concentration variation between anatomical locations, we sampled three fishery bycaught males at nine different body sites. The males were identified as mature following the same criteria listed above and were all collected between October 18 and December 11, 2000 (chosen in part to minimize potential

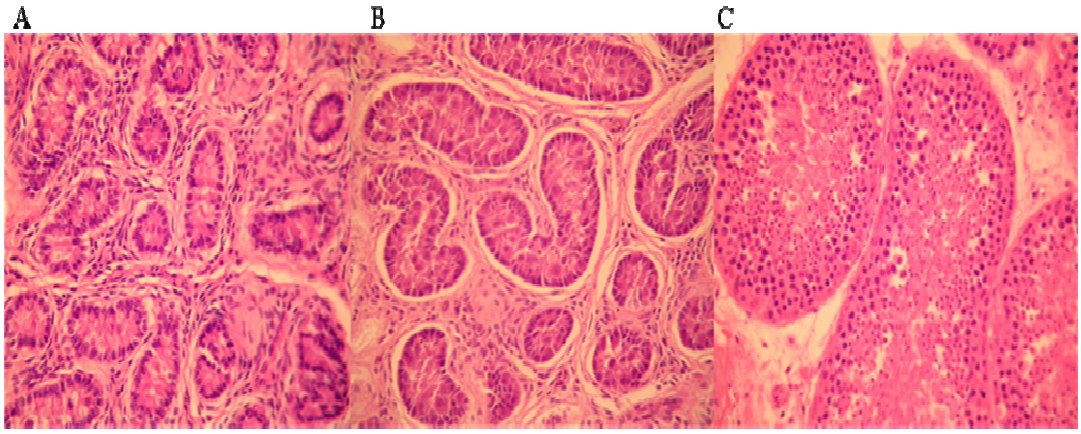

Figure 13. Compound light microscopy images showing histological characteristics of testes development in short-beaked common dolphins. Three stages shown: A) immature (abundant interstitial tissue, narrow tubules, and few spermatogonia); B) pubertal (less interstitial tissue, larger seminiferous tubule diameter, more spermatozoa, and some spermatocytes); and C) mature (much larger tubules, many spermatocytes, and presence of spertazoa). All images at 400x magnification.

effects of storage time and seasonal differences in BT production). The blubber was sub-sampled following the same procedure delineated in Kellar *et al.* (2006).

We also examined the relationship between testosterone concentration and blubber depth relative to the epidermis. For this comparison we sub-divided full depth samples (epidermis to muscle) from mature males (n=7) and into equal thickness sub-samples representing three layers (inner, middle, and outer), each approximately 150mg. The specific depth of each was relative to the thickness of the individual's overall blubber layer. These were then processed the same as all other samples.

Procedures for steroid extraction were identical to those described by Kellar *et al.* (2006). The blubber slabs collected from the fishery specimens were sub-sectioned into thin columns (~150mg), approximately the amount obtained using a standard biopsy dart. The blubber tissue was homogenized in 1000µl 100% ethanol using an automated, multi-tube homogenization instrument (FastPrep Instrument, Q-Biogene, Irvine, CA). It was processed for eight 45-second periods at 6.5 m/sec in lysing tubes provided by the instrument manufacturer. Steroids were extracted and isolated using the multi-step organic solvent approach delineated in Kellar *et al.* (2006). The resulting residue containing the testosterone was frozen at -20°C until analyzed. Prior to final analysis, samples were redissolved in 250µl of phosphate buffered saline (pH 7.5) containing 1% bovine γ-globulin and then mixed using a multi-tube vortexer (VWR Scientific Products; Morrisville, NC, U.S.A.), at medium speed for at least 15 minutes.

Testosterone concentrations were determined using a commercially available enzyme immunoassay kit, DSL-10-3900 (Diagnostic Systems Laboratories, Inc., Webster, TX) The reported inter-assay coefficient of variation (COV) ranged from 3.4-7.0%, and intra-assay COV ranged from 4.1-5.0% with a standard curve range between 0.1 and 25 ng/ml. The five highest documented cross-reactive steroids for the assay were as follows: Testosterone at 100%, 5α-Dihydrotestosterone at 6.6%, 5-Androstane-3β,17β-diol at 2.2%, 11-Oxotestosterone at 1.8%, and Androstenedione 0.9%.

Linearity and Accuracy Validation

Serial dilutions of pooled blubber extracts from three mature male dolphins were used to test for parallelism with the known standard controls. Slopes at the central linear portion (~0.5 B/B_0) of the log transformed binding curve were compared. Six replicates of the serial dilution curve were generated from two assays and compared against the corresponding standard curve and a T-test (replicate curves paired against intra-assay standard) was used to test for significant differences in the slopes.

The effects of potential inhibition of the blubber extracts on concentration measurements were also examined. We compared concentrations of the 10ng/ml assay standards that were spiked (1:1) with (1) the 0ng standard and (2) pooled blubber extracts from three immature males. Significant differences the six replicated of each using a non-paired T-test.

Extraction efficiency was determined for each group of extractions by spiking selected sub-samples with eight dilutions of cold testosterone ranging from 0 ng to 5 ng in the matrix tubes before initial homogenization. We extracted and quantified the testosterone in these sub-samples according to the procedure described above. The resulting extraction efficiency rate from a group was divided from each testosterone measurement of the non-spiked sub-samples within that group to correct for estimated losses of testosterone during the extraction procedure. The average efficiency rate across all extractions was 72.1% (Range: 60.0 - 98.9%).

Data analyses

Data analyses were conducted in three parts. The first assessed BT measurement variability by comparing concentrations of different a) anatomical locations, b) depths relative to epidermis, and c) sampling seasons. The second determined the correlations between BT concentration and maturity state from the reference collection. The third estimated the proportion of mature males in the experimental data set using a Bayesian statistical model derived from the reference collection.

All testosterone concentrations were log transformed prior to analysis to reduce heteroscedasticity (i.e., with increasing testosterone concentration we find increasing measurement variance). Four single-factor ANOVAs were used to compare BT concentrations between (1) maturity states (immature, pubertal, and mature), (2)

seasons of sampling [summer (June, July, August), autumn (September, October, November), and winter (December, January, February)], (3) sampling depths below the surface of the skin (outer, middle, inner) and (4) anatomical locations of samples (nine different sampling sites). When significant differences were found these analyses were followed by Tukey/Kramer post-hoc tests. Linear regression analysis was used to determine if there was a significant effect of storage time (-20°C, wrapped in tin foil) on BT concentration over the collecting period (range: 2.5-189 months) using samples from mature males collected during the apparent non-breeding season[4]. We also examined the relationship between the testis mass and BT concentration using Pearson's correlation analysis. All preceding statistical comparisons were conducted using Matlab 7.0 (The Mathworks Inc. Natick, MA), with an alpha value of 0.05.

Additionally, we modeled maturity state (M) as a function of BT concentration (t) using the following standard logistic structure:

$$M_i \sim Bernoulli(p_i)$$

$$p_i = \frac{1}{1 + e^{-(\alpha_0 + \alpha_1 t_i)}}$$

[4]These samples were chosen because we had sufficient number within this group (n = 43) and they likely had higher initial testosterone concentrations than corresponding immature males thus increased our likelihood of detecting any trend with time (i.e., changes at higher concentrations are easier to detect than those at lower concentrations).

where M_i is 0 for non-mature (immature and pubertal) or 1 for mature, t_i is BT concentration for individual i, and α_0 and α_1 are the estimated parameters of the model. This approach is appropriate given the Bernoulli distribution of the data (i.e., an animal is either sexually mature or not at the time of sampling) [5]

.

To allow for variation in BT concentrations due to possible seasonal fluctuations we constructed a second model that incorporated an interaction term between BT and date. Because ordinal date is cyclical, this interaction term was modeled as a generalized sine function in the following structure:

$$p_i = \frac{1}{1 + e^{-(\alpha_0 + \alpha_1 t_i + (\alpha_2 + \alpha_3 t_i) abs(\sin(d_i / 2 - \alpha_5))^{\alpha_4})}}$$

where d_i is the ordinal date as expressed in radians (see below) and α_2, α_3, α_4, and α_5 are the estimated parameters of the interaction term. Ordinal date (j) was converted to radians (d) using the following approximation:

$$d = j\left(\frac{2\pi}{365_{days}}\right)$$

[5] We considered using a three-step or cardinal regression model to allow for an estimate for the proportion of pubertal animals within the population but decided against it. Our intent was to estimate the proportion of sexually mature animals that were sampled and therefore the focus was on these mature males. By adding a third class of maturity (pubertal) we felt that the associated added complexity to the model could take away from that focus and make interpretation of the results more muddled and perhaps overreaching. Also, relatively little is known about the maturation process in these dolphins and staging of different maturity states is subject to interpretation, consequently we felt that the more conservative, two stage model, most appropriate.

Other structures could have been used to model the interaction of season on the relationship between testosterone concentration and maturity state. We chose this function from first principals given the cyclical nature of the sine function and the fact that it is relatively flexible and straightforward to interpret. However, this function is locally symmetric about the maximum and therefore assumes the rise in average testosterone concentrations throughout the population is equal and opposite to the rate of decline. Therefore, for any one iteration, the slope of the rise is equal to the negative of the slope of the decline; however, the composite of the model (i.e., the set of median values of estimated testosterone concentration across all iterations at each day throughout the year) is not restricted (and not likely) to be symmetric. Because the estimate of the proportion of mature males results from a distribution created across all iterations the local symmetry of the sine function does not affect this estimation as much as if it were a point estimate was made from a single curve using best fit parameters.

Parameters were estimated via Bayesian based model constructed by the authors using WinBugs 1.4.1 (Spiegelhalter *et al.* 2003). Vague (non-informative) priors were specified for each of the parameters: α_0, α_1, α_2, and α_4 distributed as normal (mean=0.0 and variance=10^6), α_3 distributed as uniform between 0 and 100, and α_5 also distributed as uniform between -1.0 and 1.0. A Bayesian approach was used to incorporate model uncertainty into the subsequent estimates of the proportion of mature males of the biopsied animals. This is particularly important because sampling

effort of reference specimens was not uniform with respect to ordinal date leaving months with few or no samples representing specific maturity groups; however, this Bayesian approach incorporates much the subsequent uncertainty resulting from clumped reference sampling into the estimates of proportion mature of the unknown biopsy samples. An iterative log likelihood approach was also possible but we felt that the resulting Bayesian models would be more flexible for future applications.

Using the parameters estimated from our reference sample, we generated posterior probability distributions from both models to infer the proportion of mature male dolphins we biopsied in the waters off California.

Results

Linearity and Accuracy Validation

Slopes of the competitive dose curves from the serial dilutions of pooled blubber extracts and the known standard controls were not significantly different (t = -0.511 p=0.96, Fig. 14), indicating that the assay was primarily measuring the same antigen in the control and the blubber extracts (i.e. displaying parallelism). We also found no significant differences between the assays standards (10ng/ml) spiked with the blubber extract and those spiked with the 0ng/ml negative control (t = 0.561, p=0.62) though average concentration in the extract spiked were slightly higher (μ=5.09ng/ml SE=0.30) than those spiked by the negative control (μ=4.77ng/ml SE=0.48).

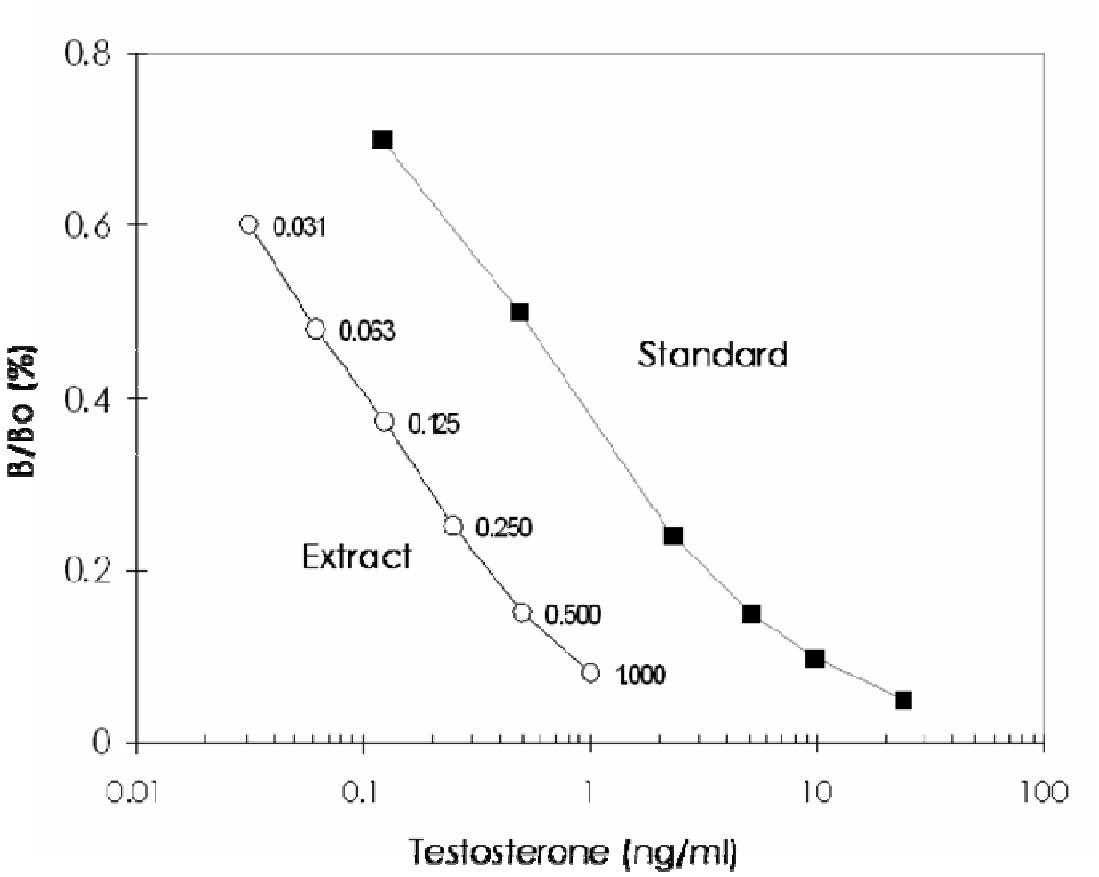

Figure 14. Competition curves showing parallel displacement between known standard concentrations and serial dilutions of pooled blubber extracts of unknown concentration. Dilution coefficients of the extracts are labeled to the right of each corresponding measurement.

Table 3. Testosterone concentrations in the blubber of immature, pubertal, and mature males Delphinus delphis. The concentrations are corrected for extraction efficiency (see text) and are reported as (ng/g of blubber extracted. Average values are displayed with standard error.

	Mature	Pubertal	Immature
Spring Median Mean ± SE Range n	15.7 15.7 - 1	2.5 2.5 ± 1.3 1.2 - 3.8 2	1.7 1.7 - 1
Summer Median Mean ± SE Range n	59.4 53.9 +/- 2.0 16.9 - 83.0 8	6.7 6.7 - 1	2.5 2.7 ± 0.6 1.2 – 4.8 6
Fall Median Mean ± SE Range n	7.0 9.0 +/- 2.0 2.1 - 53.7 26	2.4 2.5 ± 0.6 1.5 - 3.9 4	1.6 2.0 ± 0.3 0.6 - 5.6 25
Winter Median Mean ± SE Range n	4.8 6.6 +/- 1.1 1.8-21.1 22	1.8 1.9 ± 0.2 1.6 - 2.5 7	2.7 2.6 ± 0.4 0.77 – 5.2 13
All seasons Median Mean ± SE Range n	6.9 14.4 +/- 2.6 1.8 - 83.0 57	2.0 2.5 ± 0.4 1.2 -6.7 14	1.9 2.3 ± 0.2 0.6-5.6 45

Reference Collection-Fishery/Stranding Samples

Of the 116 fishery-killed males examined, 43 were classified as immature, 14 pubertal, and 57 mature (two had tissue too degraded to classify). A qualitative inspection of these classifications with respect to testis mass and total length does not show any clear outliers/misclassifications (Fig. 15) though maturity groups do overlap in both criteria. Mature males had significantly higher BT concentrations than pubertal ($P = 0.006$) and immature animals ($P < 0.0001$). Average concentrations (Table 3) in mature animals (14.4 ng/g) were more than four times higher than in pubertal (2.5 ng/g) and six times higher than in immature males (2.2 ng/g). BT levels in pubertal and immature males were not significantly different ($P = 0.98$).

Testosterone concentrations appeared to vary by season only in mature males. Summer concentrations in these animals were much higher ($P < 0.0001$) than those from fall and winter (Table 3, Fig. 15). Conversely, immature and pubertal concentrations did not vary significantly by season. This pattern is likely due to physiological changes associated with seasonal breeding, and it results in a period during the summer in which there is no overlap in BT between mature and non-mature (immature and pubertal) males.

Right testis weights ranged from 1.6 to 107g in immature, from 45.3 to 390g in pubertal and 191 to 1418g in mature males. Not surprisingly, a significant positive

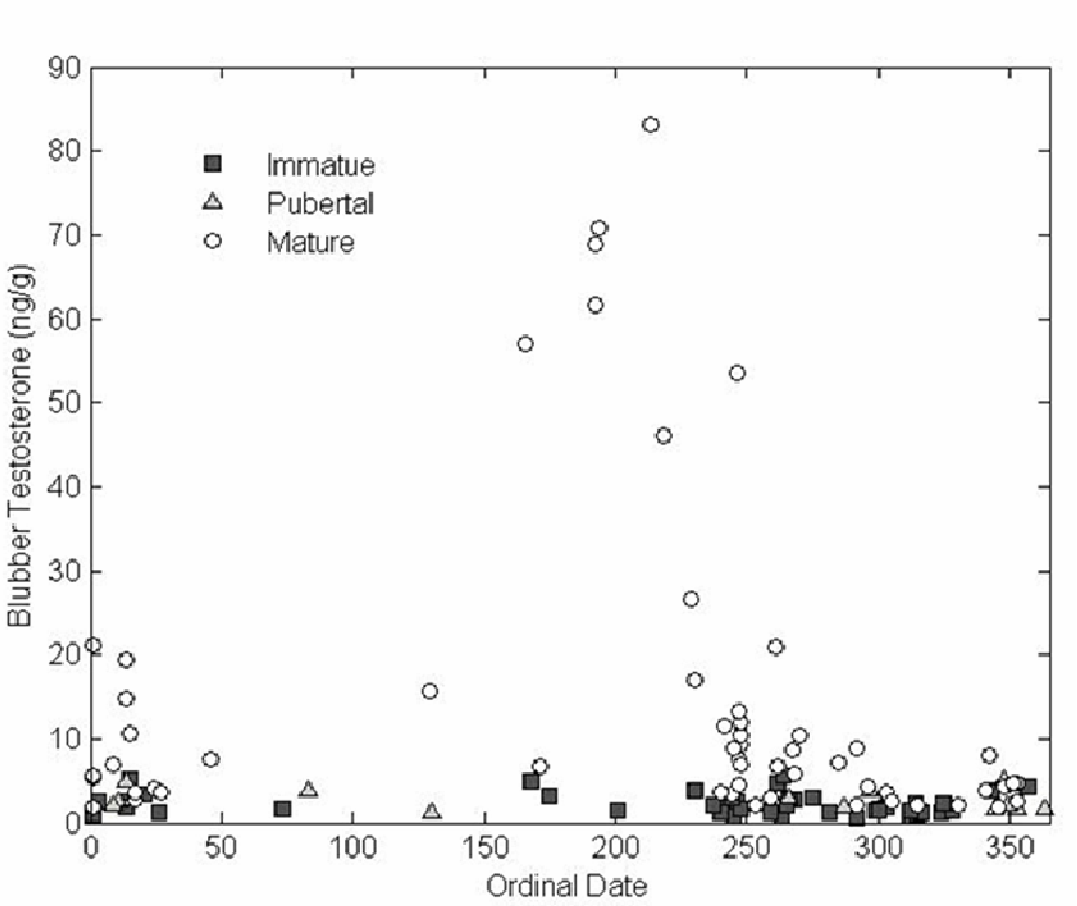

Figure 15. Blubber testosterone concentration as a function of ordinal date for immature, pubertal, and sexually mature male short-beaked common dolphins.

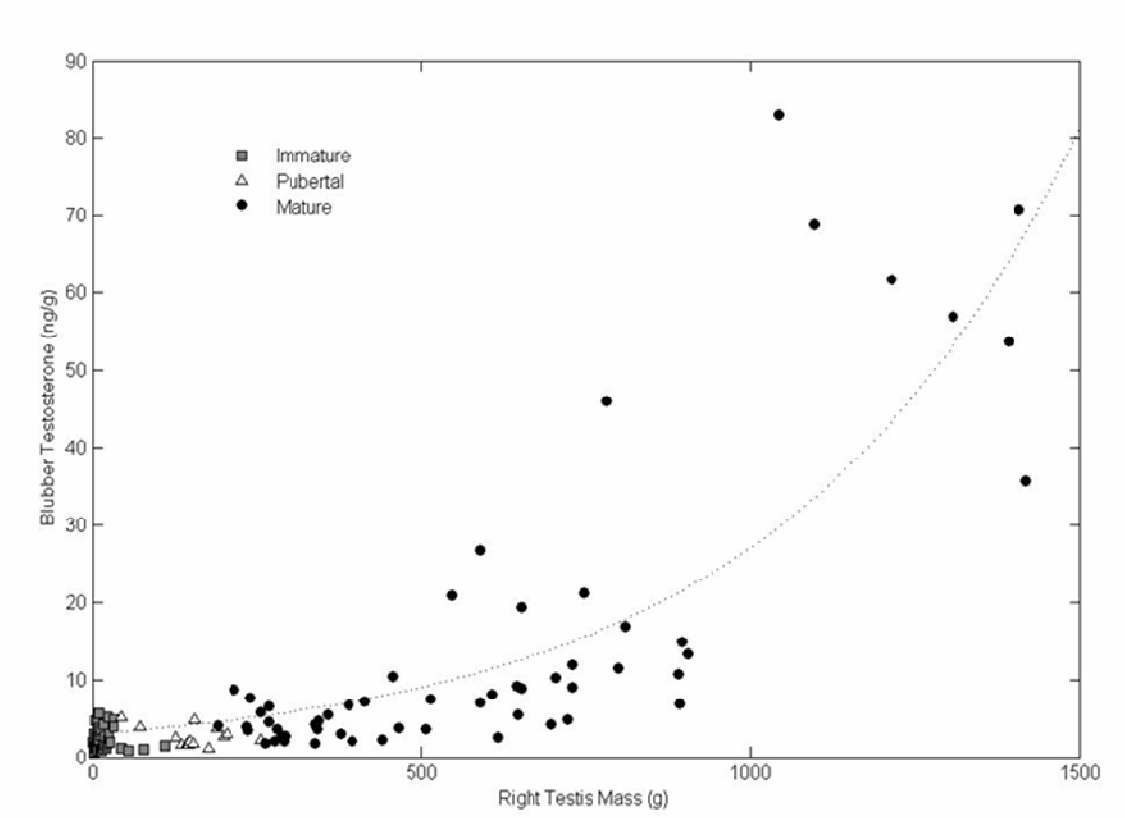

Figure 16. Blubber testosterone concentration as a function of right testis mass for immature, pubertal, and sexually mature male short-beaked common dolphins.

correlation ($\rho = 0.77$, $P < 0.0001$) was found between testis mass and blubber testosterone concentration across maturity states (Fig. 16). In fact, BT appears to increase exponentially with increasing testis weight according to the following relationship:

$$BT = 3.03e^{0.0022\text{testis mass (g)}}$$

Bootstrap 95% CI are 1.64 – 4.06 and 0.0019-0.0028 respectively as determined by least-squares linear regression analysis.

We found no significant differences in BT concentration at different sampling depths relative to the epidermis (Fig. 17); median concentrations ranged from 4.48ng/g in the outer layer to 5.7 ng/g in the middle layer ($F = 2.09$, $P = 0.146$).

Significant differences in BT concentration were found between the dorsal fin sample and seven of the eight more ventral samples ($F = 5.39$, $P = 0.043$; Fig. 18); the exception being the posterior peduncle ($P = 0.31$). On average concentrations from the dorsal fin were 28.6% lower than the region where we found the highest BT levels (i.e. the anterior peduncle).

We found no significant relationship between storage time at −20°C and measured BT concentration in *D. delphis* blubber samples. This finding was consistent across the three reproductive groups: mature ($r = 0.22$, $P = 0.14$), pubertal ($r = 0.16$, $P = 0.59$), and immature ($r = 0.21$, $P = 0.21$).

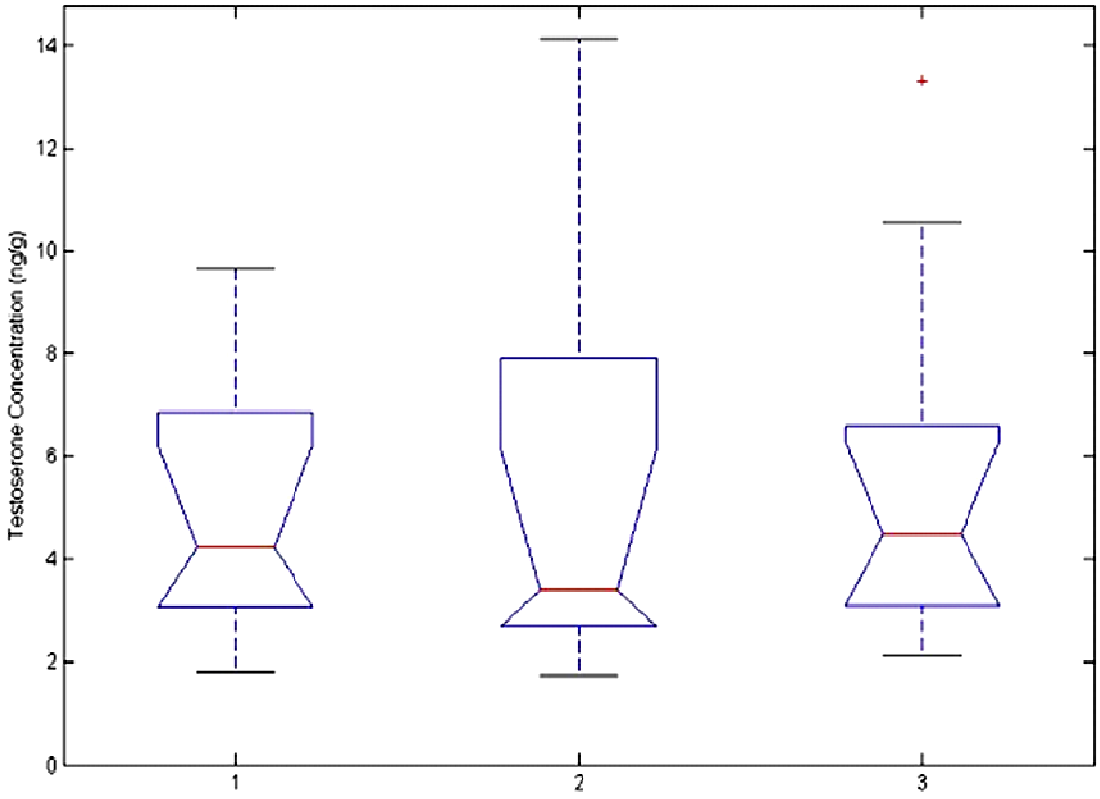

Figure 17. Blubber testosterone concentration for inner (1), middle (2), and outer (3) blubber layers. The concentrations were quantified from ten mature D. delphis. Horizontal box lines represent the lower quartile, median, and upper quartile values. Whisker lines indicate range of concentrations and the pluses represent outliers (1.5 time interquartile range). Points of inflection represent upper bound to the 95% confidence interval. The lower CI bounds were not shown as they were lower than the first quartile line. No significant differences were found between any of the layers.

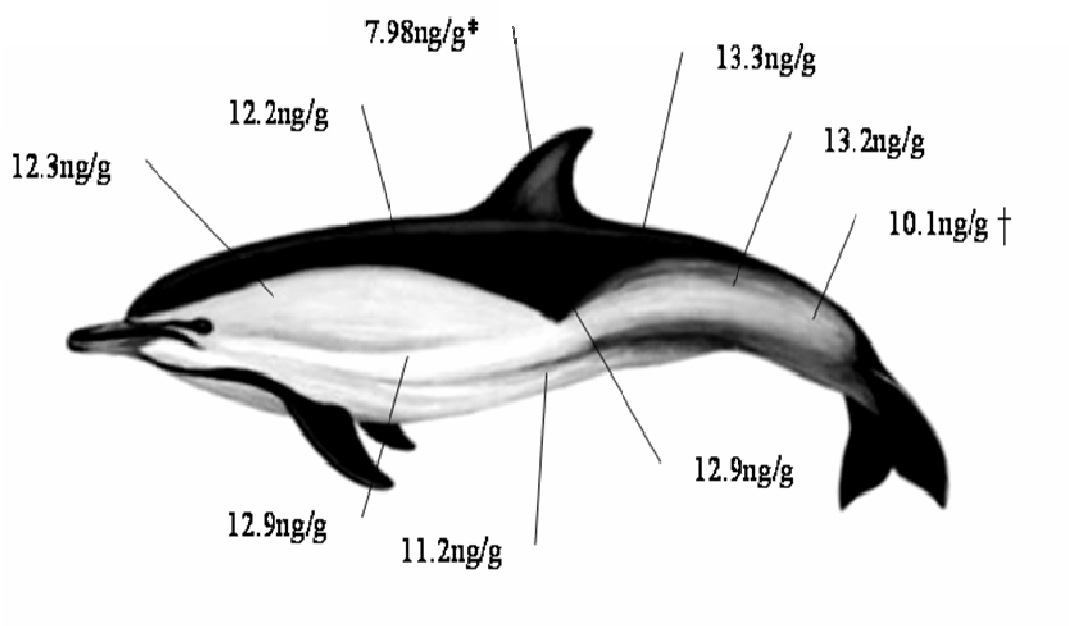

Figure 18. Mean BT concentrations as nine anatomical locations in 3 mature male *D. delphis*. There were significantly lower concentrations found in the dorsal fin (*) compared with the rest of the sampling locations except the posterior peduncle (†)

The estimated logistic model (Fig. 19) for probability of maturity relative to BT concentration was:

$$p_i = \frac{1}{1 + e^{-(-3.7+2.9t_i)}}$$

A summary of the distributions for each parameter is provided in Table 4. The testosterone concentration in which the p(maturity) = 0.5 was 4.1ng/g (95%BI = 3.5-5.0 ng/g).

The inferred model (Fig. 20) incorporating the interaction between ordinal date and BT concentration was:

$$p_i = \frac{1}{1 + e^{-(-3.9+3.1t_i+(-25.3+7.2t_i)abs(\sin(d_i/2.0+0.15))^{11.5})}}$$

A summary of the distributions for each parameter is provided in Table 4.

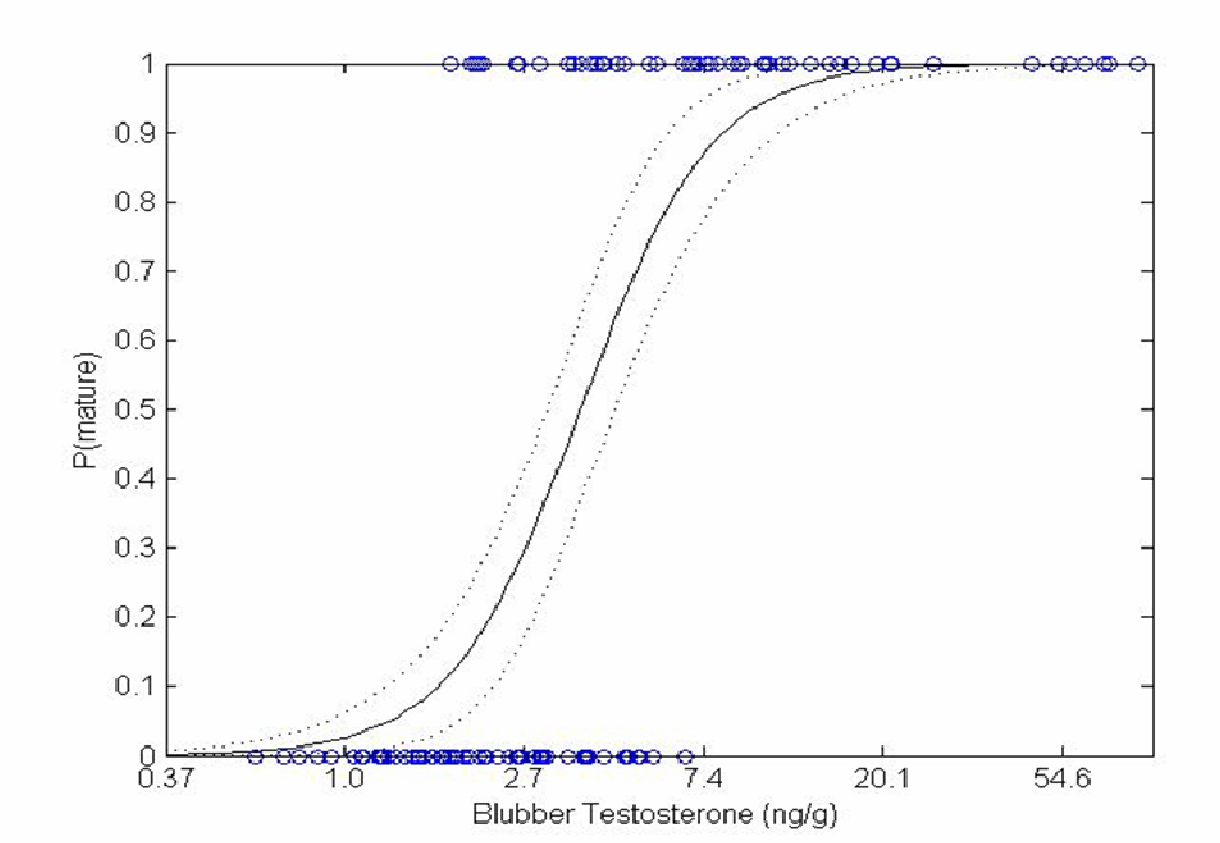

Figure 19. Logistic model for p(mature) relative to blubber testosterone concentrations. Dashed lines represent 95% confidence from 10,000 bootstrap simulations. The abscissa of the figure is natural log scaled. Model parameters are given in text.

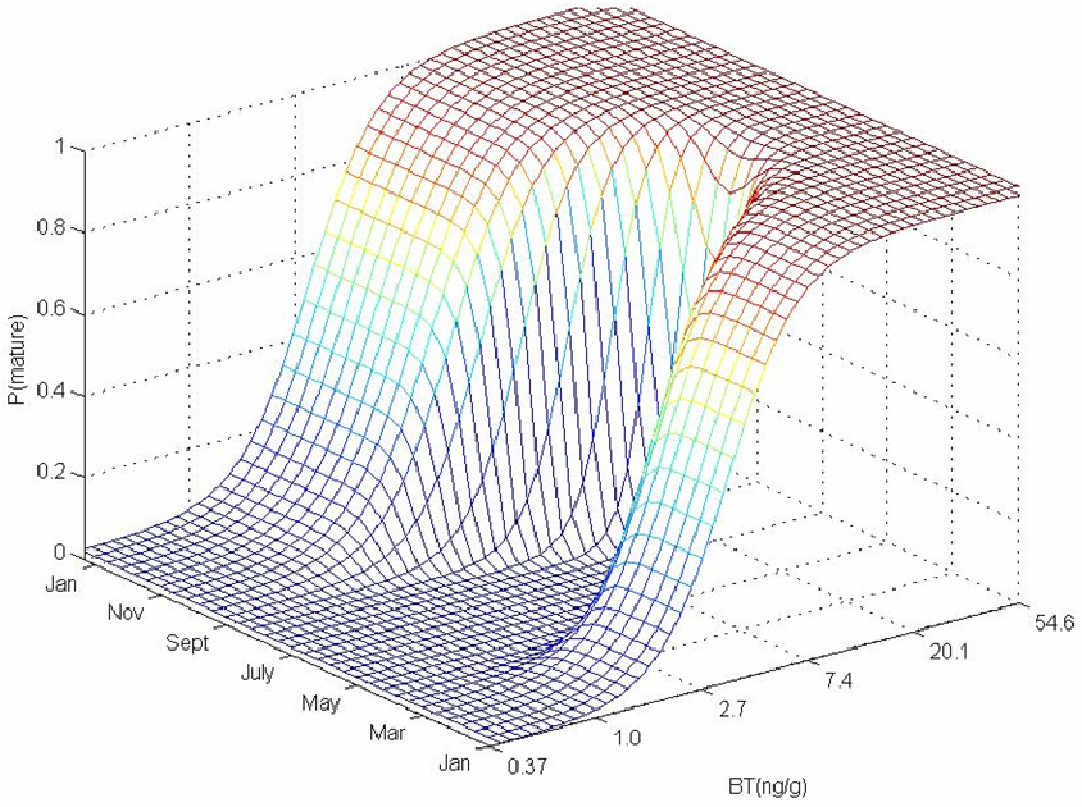

Figure 20. Logistic model for p(mature) relative to blubber testosterone concentrations and date. Model parameters given in text.

Table 4. **Bayesian inference estimates of parameters of two logistic models fitted to log transformed blubber concentrations of 116 male *D. delphis* fill in the California gillnet fishery. Median values were reported with values delineating 95% of the marginal posterior distributions, in parentheses.**

Model	α_0	α_1	α_2	α_3	α_4	α_5	**DIC**
Without Date	-3.7 (-5.3, -2.5)	2.9 (1.9, 4.0)	-	-	-	-	94.1
With Date	-3.9 (-5.5, -2.5)	3.1 (2.1, 4.4)	-25.3 (-60.2, -2.9)	7.2 (-0.9, 19.8)	11.6 (6.7, 92.5)	0.15 (-0.14, 0.46)	87.1

Experimental Data Set – Biopsy Samples

Of the 261 biopsies with sufficient blubber for analysis, 138 were from males and 123 from females: a result not significantly different from a 1:1 ratio ($\chi^2 = 0.86$, $P = 0.82$).

The biopsied male dolphins exhibited a range and seasonal pattern in BT concentrations similar to those in the fishery-killed animals (Fig. 21). The mean concentration for the biopsies was 5.4ng/g with a range between 0.01 and 63.9ng/g. The highest 10% of the concentrations were from samples collected during the period from June to mid September (ordinal dates 150-260). In June, July, and early August, the log concentration frequency distribution is decidedly bimodal with no value occurring between 15 and 20ng/g. The lower concentration mode is centered at 1.8ng/g and likely composed of immature and pubertal animals. The higher peak is centered at 31.1ng/g and presumably associated with reproductively mature males.

The two logistic models we employed, one with and one without the ordinal date interaction term, estimated 0.29 (95% BI: 0.24 – 0.35) and 0.32 (0.27 - 0.38) of the biopsied male dolphins were mature, respectively. This is significantly lower than the proportion of mature males collected from the fishery.

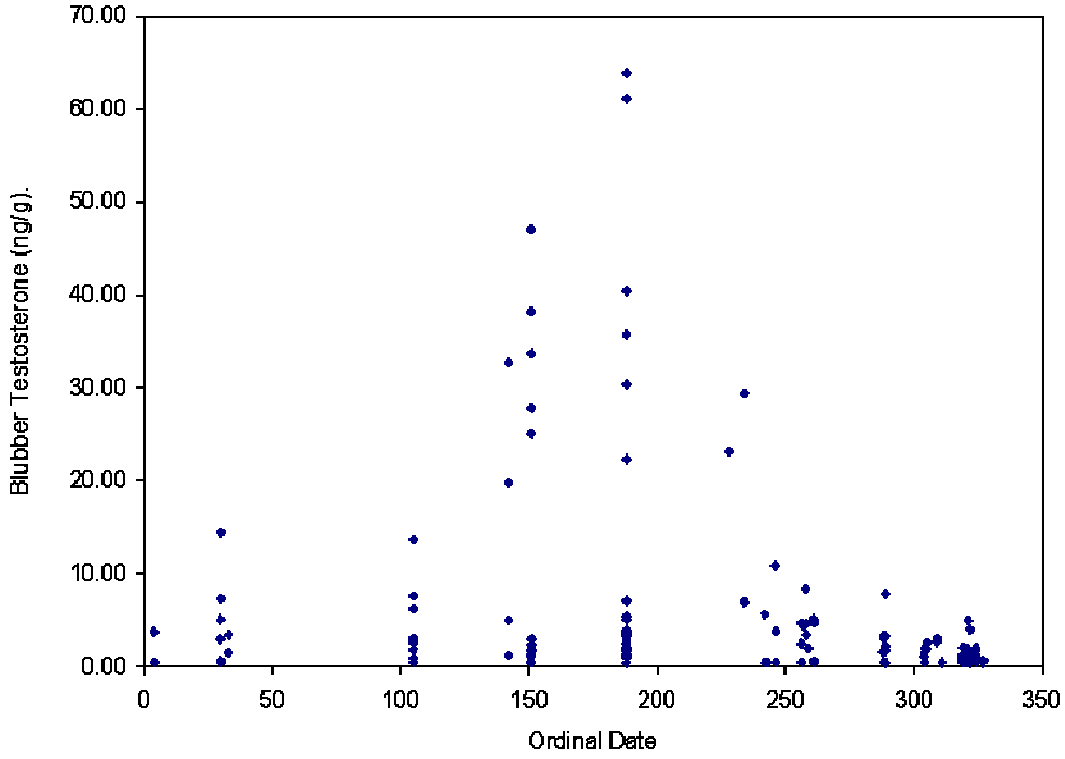

Figure 21. BT concentrations as a function of ordinal data in biopsied D. delphis in the Northeastern Pacific.

Discussion

This is the first study to report testosterone concentration in the blubber and the first to assess its utility as an indicator of reproductive seasonality and sexual maturity. We found that mean testosterone levels of mature male short-beaked common dolphins were higher than those of non-mature males. However, substantial overlap was observed in the non-breeding season, suggesting that blubber testosterone, as an indicator of sexual maturity, is not consistently effective for the diagnosis of individuals (i.e., only works for individual diagnosis if sampled during the breeding season). Instead, BT can be used to estimate the proportion of males within a population that are sexually mature as long as appropriate reference data (i.e., testosterone concentrations from animals of known maturity status) are used for comparison. We also found strong seasonal fluctuations in average testosterone concentrations with maximum levels observed from June through August, suggesting that this sample displays reproductive seasonality with highest testosterone levels in summer and early fall. We also note that by sampling during mating season, we can greatly minimize (perhaps eliminate) classification error and thus obtain more accurate estimates of the proportion of mature animals sampled.

BT concentrations in the short-beaked dolphins investigated here were similar to those previously reported for serum in other cetacean species. For example, Kirby (1990) examined 15 adult, 7 pubertal and 16 immature captive *Tursiops truncatus*

over several years and found average testosterone serum concentrations to be 14.12, 1.76, and 0.26 ng/ml, respectively. However, we expected that the lipid-rich blubber would accumulate higher concentrations per weight of the lipophilic testosterone. For instance, progesterone concentrations in the blubber are 3-6 times higher per mass of extracted sample (assuming the density of water is a close approximate for that of serum) than those found in the blood (Hillbrand and Elsaesser 1983, Hamudikuwanda *et al.* 1996, Kellar *et al.* 2006). Perhaps the lower relative testosterone concentrations reflect catabolic processing occurring in the blubber. Aromatase (CYP19), an enzyme found in lipid-rich adipose tissues that converts testosterone to estradiol (Zhao *et al.* 1995), has been shown to be active in cetaceans (Wilson *et al.* 2005) and could be acting locally to reduce residence time of blubber testosterone, resulting in lower relative concentrations. To our knowledge, no comparable enzyme that breaks down progesterone has been documented in cetacean blubber.

The data from both our biopsies and reference collection show a pronounced elevation of BT concentrations during the summer and early fall indicating that these short-beaked common dolphins are seasonally reproductive. We observe samples with higher BT concentrations from May (in biopsies) though September (in biopsies and references samples). Larger average testis size found during these months complement this finding (Fig. 22). However, estimating the specific timing of heighten reproductive activity cannot be precisely estimated because 1) the dynamics of testosterone in the blubber are unknown and 2) the relationship between BT and

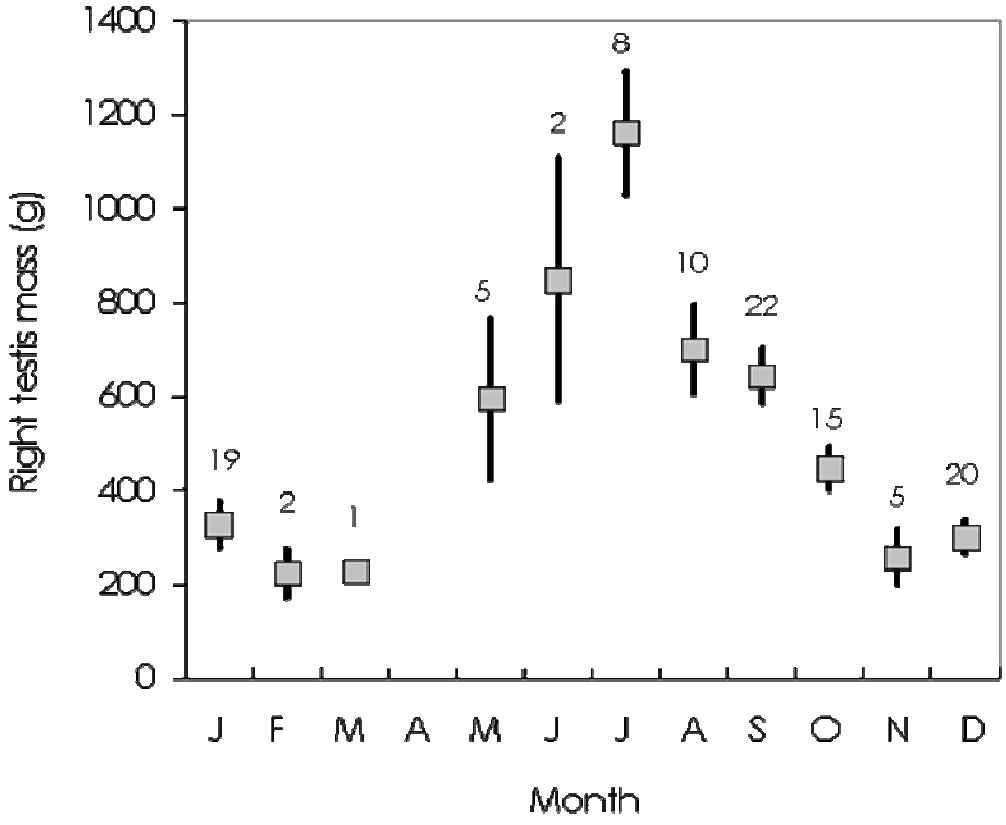

Figure 22. Average right testis mass of stranded and fishery bycaught "non-immature" D. delphis (i.e., those with right testis mass < 100g). Square markers indicate mean mass and error bars represent standard error. The sample size is presented adjacent to corresponding marker. The figure includes additional specimens not represented in the reference collection (those for which no blubber was collected).

testis function has not been precisely delineated. For instance we know that in some captive cetaceans, there is evidence of a lag between testosterone elevation and maximum sperm production or conception (Schroeder and Keller 1989, Robeck *et al.* 2005). If the *D. delphis* we investigated, exhibit a similar lag, then the actual breeding season commencement and termination would occur later in the year, possibly weeks later than BT levels would indicate.

Although the exact timing of the breeding season cannot be precisely determined, the seasonal BT fluctuations indicate that these *D. delphis* are seasonal breeders with one breeding peak. This finding is contrary to a previous study that indicates multiple peaks for *D. delphis* in the Southern California region (Evans 1975). Using fetal and calf lengths, Evans found two calving seasons: one in late fall and the other in early summer. It is not clear why our finding is different but we do know that the animals that Evans indicted as calving in late fall (from winter conceptions) were all from a single school and perhaps this anomalous group is not representative of the majority of animals in the region. Alternatively, our sampling effort (an aggregate of strandings, fishery bycatch, and biopsies) may not have fully captured the characteristics of the entire population and therefore we did not obtain representative blubber samples from males reproductively active during the winter. Other studies that have examined annual breeding patterns in temperate *D. delphis* populations in the Northern hemisphere also indicate one reproductive peak, and their timing is similar to the animals we examined, with highest calving frequency

occurring in late spring into early summer and peak conception in late summer (Perryman and Lynn 1993, Ferrero and Walker 1995, Murphy *et al.* 2005, Westgate and Read 2007).

The two models generated to estimate the probability of maturity given BT concentration (one with the ordinal data interaction term and one without) behaved similarly except for non-mature individuals sampled during the summer breeding months. For these animals, the model without ordinal date assigns a higher probability of maturity; this is because this model does not incorporate the seasonal fluctuations in BT levels. In other words, when we find low concentrations in summer samples we are more confident that those samples are from non-mature animals than winter and fall samples with similar concentrations. The model that includes ordinal data captures this relationship between season, testosterone concentration, and maturity state. However, because we have many fewer samples during summer compared with fall and winter, our model selection criteria, DIC, does not strongly favor either model. With additional summer samples, we expect that the model, which includes ordinal date, will be favored.

The estimates of the proportion of mature males that were biopsied, generated from the two models we created (without ordinal date, 0.32 and with ordinal date, 0.29), are comparable to values reported for other *D. delphis* populations. However, it should be noted that the previous studies show that the proportion of mature males

sampled is highly variable; ranging from 0.22±0.029SE in the eastern North Atlantic (Murphy *et al.* 2005) to 0.61±0.039SE in the western North Atlantic (Westgate and Read 2007). The only data from the Pacific comes from the central North Pacific where Ferrero and Walker (1995) found 0.30±0.055SE of males sampled were mature. The majority of animals represented in these previous studies were collected as bycatch in gillnet fisheries. It is unlikely that the true proportion of mature males differs greatly in the populations sampled. More likely is that differences in non-intentional demographic selectivity inherent to the fisheries are responsible for the high variability in the estimates. The vastly different age structures reported in each of these studies adds evidence to this assessment. We suspect that biopsy collection is also selective relative to demographic status; for instance, larger animals are probably sampled disproportionally more than smaller animals, meaning that adults are over-represented in biopsy sample sets. Additional work is needed to assess the presence/degree of this type of selectivity in projectile biopsy sampling in order to provide estimates of proportion mature of a sampled population.

The anatomical location of a sample can also affect the accuracy of our approach. Samples taken from the dorsal fin appear to contain lower concentrations of testosterone than those collected from other sites. This is likely due to the relatively low levels of lipid per mass of sample. Mature animals sampled in the dorsal fin would be more likely misclassified as being non-mature and this could lead to an underestimate of the proportion of mature animals sampled. Because we found

relatively high variation of testosterone concentration in the dorsal fin tissue, we recommend eliminating such samples from the analysis instead of trying to correct for the differences in expected concentration. That means the anatomical location from which the samples came should be noted whenever possible, especially when the study's goal is to estimate the proportion of mature males, and samples are collected outside the breeding season when the average concentration difference between maturity states is smaller. In the future, we plan to develop a method to normalize BT concentrations as a function of total lipid content; this may reduce some of the problems associated with differences in sampling location and increase the utility of this approach.

The manuscript from which Chapter Three was derived has been accepted for publication in Marine Mammal Science. I was the primary researcher and author. The co-authors listed in this publican either helped generate raw data or supervised the research from which this chapter was formed. This chapter was written in collaboration with M. L. Trego, C. I. Marks, S. J. Chivers, K. Danil, and F. I., Archer.

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V. CHAPTER FOUR

Pregnancy patterns of pantropical spotted dolphins (*Stenella attenuata*) in the eastern tropical Pacific determined from hormonal analysis of biopsies and correlations of the patterns with the purse-seine tuna fishery

Abstract

The spotted dolphin population in the eastern tropical Pacific (ETP) remains depleted, and recent estimates of abundance show no indications of recovery. One hypothesis for lack of recovery is that continued chase and encirclement by the tuna fishery negatively affects reproduction. Insufficient life history sampling in this region over the last decade makes traditional estimation of population reproductive rates impossible. Here, we examine the current reproductive patterns of these dolphins using, for the first time, a molecular method to assess pregnancy state from blubber progesterone concentrations in biopsy samples. Blubber progesterone was quantified in 212 biopsies from female offshore spotted dolphins sampled between 1998 and 2003 in the northeastern tropical Pacific. These concentrations were found to be sharply bimodal with no value observed between 49 ng/g and 87 ng/g, a finding consistent with the concentration gap between known pregnant and non-pregnant dolphins. Given that high blubber progesterone ($\geq$ 87 ng/g) indicates pregnancy, we found that 11.8% of the biopsied females were pregnant. This is substantially lower than an estimate of the proportion pregnant found in the fishery kill over the same region (22.3%) between 1973 and 1992. To try to ascertain the potential cause of this discrepancy, the relationship between pregnancy and fishery exposure was analyzed, and we found that pregnant females were exposed to significantly less fishery activity than non-pregnant ones ($P < 0.046$), suggesting that the fishery has an inhibitive

effect on pregnancy. However, there are several caveats to this finding, and how this relationship might explain the discrepancy between these data sets is unclear. We also examined and modeled spatial patterns of reproduction and found that pregnancy was more aggregated than random ($P = 0.020$), with the highest proportion pregnant in the mouth of the Gulf of California, an area with relatively low reported fishery activity. Because this is a first attempt at applying this technique to a wild population, we are careful in the interpretation of the results. However, it appears that it is a promising tool for assessing reproductive rates in populations of free-ranging cetaceans.

Introduction

In harvesting yellowfin tuna, ETP purse seine fishing vessels have exploited the common association between tuna and spotted dolphins (as well as dolphins of other species), as dolphin schools are chased, herded, and encircled in order to catch the tuna swimming underneath (Perrin 1969). From the 1950s to the early 1990s, this practice generated high dolphin mortality and a substantial decline in abundance, especially in the northeastern stock of offshore pantropical spotted dolphins (Smith 1983, DeMaster *et al.* 1992, Dizon *et al.* 1994, Wade 1994), and consequently led to their listing as "highly depleted" under the Marine Mammal Protection Act. Although changes in seining methods in the early 1990s drastically reduced direct fishery mortality (Joseph 1994, Gosliner 1999), this stock has shown no signs of recovery (Wade *et al.* 2002, Gerrodette and Forcada 2005, Wade *et al.* 2007). One explanation

for the lack of recovery is that even though reported fishery mortality has dropped off precipitously, the continued chase and encirclement of these dolphins has unobserved detrimental effects on their health, including reducing reproduction (Archer *et al.* 2003).

Historically, concern regarding their depleted status had prompted numerous studies into the biology of these animals, often focusing on reproductive rates (many reviewed by Perrin and Hohn (1995)). Early studies employed observers to collect biological samples, including reproductive tracts, of the incidentally killed dolphins (Perrin *et al.* 1976, Perrin *et al.* 1977, Hohn *et al.* 1985, Myrick *et al.* 1986). The tracts were examined for fetal presence and other indicators of pregnancy, and the resulting data were used to estimate reproductive rates for the entire population (Perrin *et al.* 1977, Myrick *et al.* 1986, Chivers and Myrick 1993, Chivers and Demaster 1994). Thousands of samples were collected in this way from NE offshore spotted dolphins, creating one of the largest collections of reproductive data of any cetacean population ever studied and providing the overwhelming majority of the life-history information we have for these dolphins today (Myrick *et al.* 1986). However, since 1993, with the decline in dolphin mortality and the cessation of the systematic collection of specimens, too few reproductive tracts have been collected to accurately estimate pregnancy rates using fishery-killed dolphins. In addition, there are concerns about age selectivity of the kill, as neonates and post-weaned juvenile animals are under-represented (Barlow and Hohn 1984, Archer and Chivers 2002).

Recent studies into the relationship between pregnancy status and blubber progesterone (BP) content provide an avenue that could allow sufficient sampling to help re-assess pregnancy rates of these dolphins (Mansour *et al.* 2002, Kellar *et al.* 2006). The results from these studies document large non-overlapping differences in BP concentration between known pregnant and non-pregnant (mature and immature) female cetaceans and show that compared to the variation between pregnancy states, that BP is relatively homogeneous throughout an individual's blubber layer (Kellar *et al.* 2006). When coupled with projectile biopsing, in which hundreds of blubber samples can be collected during a routine abundance cruise, BP can be used as a pregnancy diagnostic appropriate for population-level estimates.

In this study, BP levels of biopsies (both projectile and cores from tagging operations) of NE spotted dolphins were quantified and pregnancy status was determined. We compared the proportion of pregnant females in our sample to those from the incidental fishery kills. We also examined geographic variation in reproduction and correlations between pregnancy status and indices of fishery exposure.

Methods

Sample collection

Historic Fishery Kill

The historical data on incidental fishery kills for this study were collected by scientific observers from the National Marine Fisheries Service and the Inter-American Tropical Tuna Commission between 1974 and 1993. The reproductive tracts (n = 1146) were examined for the presence of a fetus, and both ovaries were collected for subsequent examination and characterization of associated corpora. For each specimen, the time and location of collection were noted along with additional biological data including total specimen length and developmental coloration phase. As with all sample sets covered in this study, we estimated the proportion pregnant as the fraction of pregnant females out of all females sampled, regardless of maturity state. In most cetacean literature, "pregnancy rate" is based on the fraction of pregnant females of all *mature* females. We use the "proportion pregnant" here to distinguish our index from this parameter. The reason for this difference is that we could not definitively assign maturity state for biopsied animals. We did not biopsy obvious small calves or large animals accompanied by small calves, but we did target all other animals, which accordingly included large juveniles. This also applied to the sampling during tagging (see below).

Reference Specimens

Though too few specimens have been collected during the International Dolphin Conservation Program Act (IDCPA) necropsy program (1999-2001) for population-level reproduction analysis, those that were collected were used in this study as reference samples to help ground-truth the relationship between pregnancy state and

blubber progesterone concentration, which had not been previously determined for pantropical spotted dolphins. For these reference specimens (n = 9), blubber was collected from the lateral mid-thoracic region (ventral to the dorsal fin) along with both ovaries (used to determine maturity state (DeMaster 1978; 1984)), and observations of fetal presence. Blubber was trimmed into 100mg full-thickness pieces (similar in size to a typical biopsy), then processed and measured identically to all other samples.

Biopsies

Two types of skin biopsy samples were used in this study. The first type was obtained via crossbow (projectile) darting with a 6mm OD stainless steel collection tip similar to that described in Palsbøll *et al.* (1991). These biopsies had varying amounts of blubber, ranging from zero to 213mg with an average of 98.0mg. They were most commonly obtained from the posterior flank and tailstock regions posterior to the dorsal fin. The geographic sampling area was large (although representing only the eastern third of the NE spotted dolphin range), bounded by the Central American mainland, 111°W, 5°N, and 25°N (Fig. 23). The samples were collected during four research cruises from 1998 – 2003 (n = 417).

The second type of biopsy was a byproduct of attaching plastic identification tags (Roto-tags, Dalton Supplies, Nettlebed, England) (Scott *et al.* 1990) to encircled dolphins as part of a 2001 study (Chase Encirclement Stress Studies or CHESS) (Forney *et al.* 2002) tracking movement patterns and investigating the effects of chase

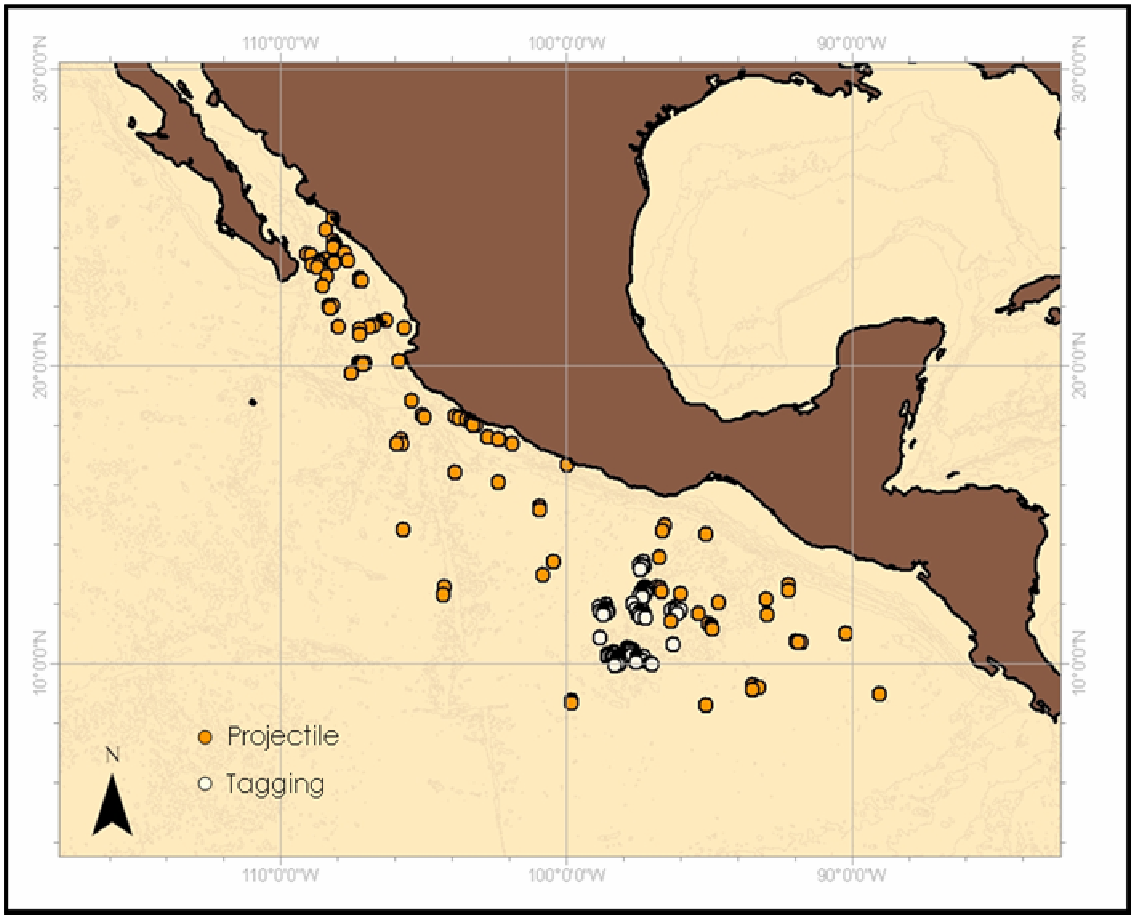

Figure 23 Geographic distribution of the female *S. attenuata* blubber samples acquired from projectile biopsing (dark circles) and roto-tagging (light circles) that were analyzed to determine progesteorne concentration.

and encirclement. With the application of a tag to the tailing edge of the dorsal fin, a small skin core (3-4mm in diameter and 2-3cm in length) was produced (Scott *et al.* 1990). The blubber from these cores ranged in mass from 0.052mg to 0.188mg (n = 215). The geographic area covered during the 2001 study was much smaller than our projectile biopsy sampling area; it was centered around 12°N and 97°W (Fig. 23).

Hormone Extraction

A FastPrep automated homogenization instrument (Q-Biogene Inc. Irvine, CA) was used to pulverize blubber samples in 1000ul of 100% ethanol following a previously described procedure (Kellar *et al.* 2006). Unlike in Kellar *et al.* (2006), after complete homogenization the entire contents of the homogenization tubes were poured (instead of aspirating only 500ul for further processing) into 12 x 100 mm disposable glass tubes. The homogenization tubes were washed with an additional 500ul of ethanol. The wash was then collected and added to the 12 x 100mm collection tubes. A mixture of ethanol and acetone was added to this solution such that the final volume was 4 ml at 4:1. The rest of the hormone extraction procedures were identical to those described in Kellar *et al.* (2006). The final residue was stored at -20°C until quantified.

Hormone Quantification

A commercially available enzyme immunoassay was used to measure the progesterone in each extraction following a previously described protocol for blubber samples (Kellar *et al.* 2006). The coefficient of variation (COV) at approximately the 50% binding concentration for inter-assay and intra-assay comparisons was 3.2 and 4.2, respectively.

Six blubber samples of non-pregnant females were spiked with known quantities of cold progesterone (0.0-45.0ng) to estimate extraction efficiency for each group of extractions (groups ranged from 16 to 30 samples including these controls). The controls were extracted and quantified identically to the rest off the samples. Efficiency was calculated as the amount of measured progesterone minus the amount determined in the non-spiked sample, all divided by the amount of progesterone added (spiked) to the sample prior to extraction. The average efficiency was 82.7% (SE=7.8%).

Sexing

A standard PCR assay (Fain and LeMay 1995) was used to determine the sex of the biopsied animals. For 35 of the tagged animals, we had additional data from field determinations of gender (gross examination of external morphology). This information was kept blind until after molecular assaying was complete and therefore provided a check of assay accuracy.

Data Analysis

All statistical analyses were performed using Matlab 7.0 (The Mathworks Inc. Natick, MA). Progesterone measurements from the biopsies were compared against those from fishery-killed females of known pregnancy and maturity state. For these comparisons biopsy data were stratified into two groups (likely pregnant and non-pregnant) based on the concentration frequency distribution; the distribution was inspected for local minima between 50 and 100 ng/g, a range between known non-pregnant and pregnant female dolphins (data aggregated for four species: pantropical spotted [this study], short-beaked common, northern right whale, and Pacific white-sided dolphins [Kellar *et al.* 2006]). Two Student T-tests were used to compare the concentrations from biopsies to those from the fishery-killed dolphins, for each pregnancy state. In addition, blubber progesterone measurements from projectile biopsies and tagging biopsies were separately compared in the same way.

Chi-squared contingency analyses were used to test the significance of comparisons of proportion pregnant. The estimates from biopsies were compared with those from the 1973-1992 fishery kill. In addition, the biopsy sampling area was stratified into three equal regions according to gaps in the biopsy sample distribution, and for each region the preceding comparisons were re-analyzed.

Statistical significance of spatial variation in proportion pregnant as determined from the biopsies was analyzed using permutation tests of two metrics: Ripley's K (Ripley 1977, Besag and Clifford 1989) and average nearest neighbor distance (Diggle *et al.* 1976, Sinclair 1985, Besag and Clifford 1989). These were calculated for the observed samples and then each sample set was randomized with respect to pregnancy state while keeping the proportion pregnant the same as the observed. The resulting permutation tests were constructed on 20,000 replicates, and the significance of the spatial variation in proportion pregnant was tested against what would be expected at random given the sampling locations.

Fishery Effort

A weighted relative index of fishing exposure (Archer in prep) was estimated for each biopsied female using Inter-American Tropical Tuna Commission (IATTC) data indicating the time, date, and location of dolphin sets (i.e., purse-seine operations in which offshore pantropical spotted dolphins were encircled to capture tuna) in the days and months prior to the sampling event in which the biopsy was obtained. The index generally gives greater weight to sets the closer in time and space they were to the sampling event. The weighting system is based on a species-specific movement model (Archer in prep) that incorporates tag-recapture data at different temporal and spatial scales. Three indices were calculated for every biopsy, each reflecting a different temporal/spatial window or ambit (the duration prior to the sampling event

in which weighted sets were added together). We used 70, 140, and 180-day ambits in attempts to include a large enough duration to be physiologically meaningful (i.e., a duration in which we expect to be able to quantify any change in proportion pregnant within the population) without making the duration so large that the power to differentiate individual fishery exposure was unduly compromised. Fishery exposure levels were then compared in pregnant and non-pregnant biopsied females using a difference-of-means permutation test (Manly 1991) (comparable to Student's t-test but does not require that parametric assumptions are met) because the distribution of the fishery exposure index for our sample set was decidedly multimodal.

Spatial Mapping

Using ArcMap 9.1 (ESRI, Redlands, CA), ordinary Kriging prediction maps were created as approximate illustrations of the spatial patterns in proportion pregnant and the associated fishery index estimations. All default values were used in the parameter and model selection with the exception that the number of neighboring points which were used for interpolation was raised from 5 to 30 in a effort to capture broad regional patterns instead of local, likely ephemeral, heterogeneities.

Results

Progesterone concentration was measured in nine female spotted dolphins of known pregnancy state (5 immature, 2 non-pregnant mature, and 2 pregnant).

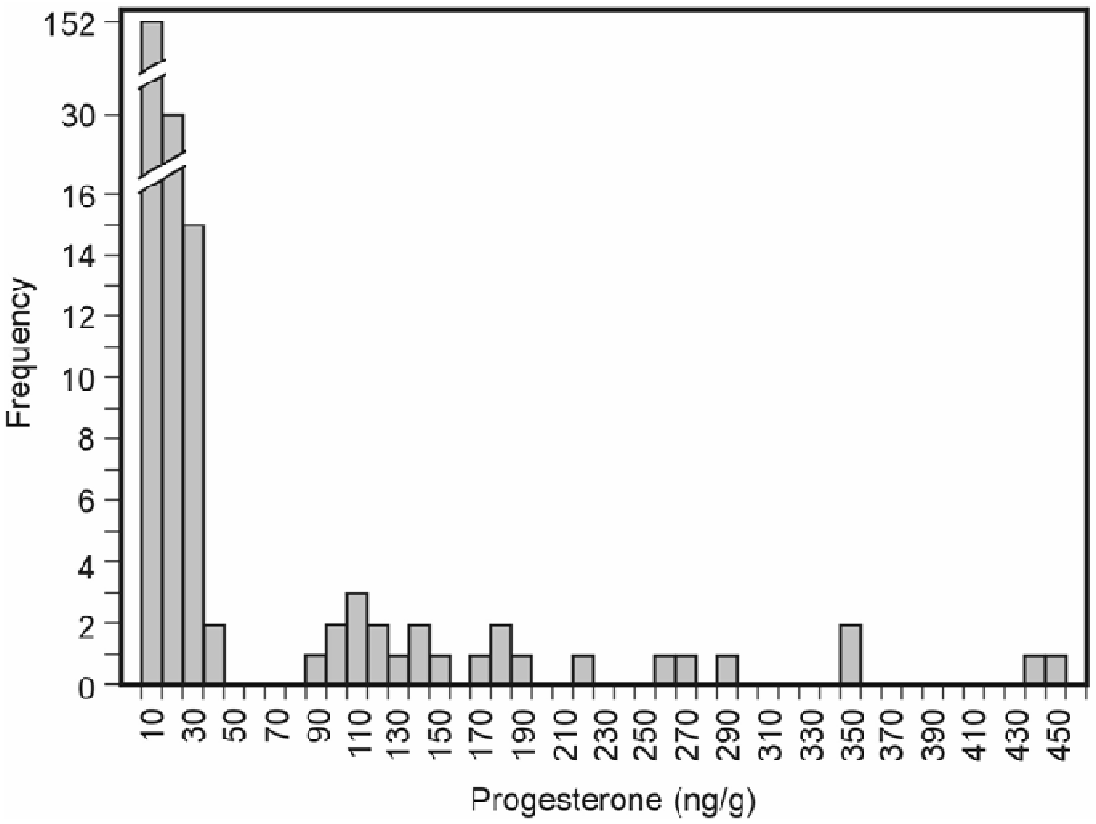

Figure 24. Frequency distribution of blubber progesterone concentrations (ng/gram of blubber extracted) in biopsied female *S. attenuata*.

Table 5. Student t-test results from comparisons of blubber progesterone concentrations of *S. attenuata* biopsies with concentrations found in blubber samples from five cetacean species of known reproductive state. Concentrations from known animals were acquired from necropsy samples processed in this study (s. attenuata) and previous studies (reference at bottom of table). P values are given significant differences ($\alpha = 0.05$) are indicated with asterisks.

S. attenuata (Biopsy) **vs.**	**Non-Pregnant (immature & mature)**	**Pregnant**
S. attenuata (Necropsy)	T=0.24 P=0.92	T=1.51 P=0.41
D. delphis	T=2.94 P=0.006*	T=1.57 P=0.15
L. borealis	T=3.69 P=0.001*	T=2.2 P=0.09
L. obliquidens	T=1.50 P=0.21	Not Available
B. acutorostrata	T=6.82 P=0.001*	T=2.03 P=0.06

Concentrations in the pregnant animals (398.7 ± 129SE) were on average 63 and 79 times greater than those in non-pregnant mature (5.84 ± 0.36SE) and immature (8.29 ± 4.0SE) females respectively. No overlap in progesterone concentrations was observed between pregnancy states, irrespective of maturity, with the highest measurement of a non-pregnant dolphin at 12.1 ng/g and the lowest measurement in a pregnant one at 269 ng/g.

A total of 632 biopsy samples were collected from *S. attenuata* in the study area between 1999 and 2003, and a little over half (320) were identified as females using the Fay and LaMay sexing assay. Of these, 212 samples (129 projectile biopsy; 83 tagging biopsy) contained sufficient blubber for hormone extraction and analysis. The progesterone concentration frequency distribution was bimodal, with no value observed between 48.1 and 89.3ng/g; a finding consistent with the break between known pregnant and non-pregnant delphinids (Fig. 24). We therefore classified those females below 48.1 ng/g as non-pregnant and those above 89.3ng/g as pregnant. The gap is smaller than the one we found for the references sample, but this is to be expected given the large difference in sample size in the two data sets. There were no significant differences between dolphins that were biopsied and those that were sampled from recent fishery bycatch, irrespective of designated reproductive state (pregnant: t-stat = -1.53, p = 0.37; non-pregnant: t-stat = 0.24, p = 0.82) (Table 5). Significant differences were found when the biopsies were compared in a similar fashion with previously reported values for other cetacean species (Table 5);

Table 5. Progesterone concentrations in the blubber of pregnant and non-pregnant females of five cetacean species. The progesterone concentrations are corrected for extraction efficiency (see text) and are reported as ng/g of blubber extracted. Average values are displayed with standard error.

	S. attenuata (Biopsy)	*S. attenuata* (Necropsy)	*D. delphis*[b]	*L. borealis*[b]	*L. obliquidens*[b]	*B. acutorostrata*[a]
Pregnant	**201 + 24.8**	**398 + 128**	**261 − 29**	**312 + 44**	**161**	**132 + 22**
Average	**89.3**	**269**	**132**	**196**	**-**	**22.8**
Min	**518**	**527**	**415**	**402**	**-**	**454**
Max	**21**	**2**	**18**	**5**	**1**	**22**
n						
Non-pregnant (immature & mature)	**6.77 ± 0.63**	**6.49 ± 0.97**	**15.5 ± 2.9**	**14.5 ± 2.0**	**16.1 ± 6.2**	**1.95 ± 0.32**
	0.23	**4.10**	**0.92**	**0.98**	**0.11**	**1.36**
Average	**49.9**	**12.11**	**48.2**	**34.7**	**34.4**	**3.43**
Min	**188**	**7**	**55**	**24**	**6**	**6**
Max						
n						

[a] From Mansour *et. al* 2002
[b] From Kellar *et. al* 2006

Table 7. Blubber progesterone concentrations (ng/g) found in projectile and tagging biopsies. No significant differences were found between the types of biopsies irrespective of reproductive state.

Tissue Type	Non-Pregnant (immature & mature)	Pregnant
Biopsies (All)	**6.77 ± 0.62 (n=188)**	**201 ± 24.8 (n=24)**
Projectile	**6.36 ⊥ 0.80 (n=113)**	**228 ⊥ 31.6 (n=16)**
Tagging	**7.39 ± 0.99 (n=75)**	**162 ± 37.5 (n=8)**

however, the differences were only among non-pregnant females, and they were small compared to the average disparity between pregnant and non-pregnant animals in all species reported (Table 6). We also found that concentrations of blubber progesterone were not significantly different between projectile biopsies and tagging biopsies regardless of reproductive state (Table 7). The t-statistics were 1.16 (P=0.269) and -0.81 (P=0.422) for pregnant and non-pregnant females respectively.

Spatial hypothesis testing

Though spatial testing was not one of the primary goals of this study, we present the results here because they affect the subsequent analyses comparing the proportion pregnant in the historic fishery and biopsy data sets.

Assuming high blubber progesterone concentration is diagnostic of pregnancy, the nearest-neighbor permutation test indicated that, within the biopsy samples, pregnant individuals were more clumped spatially than one would expect at random (P = 0.020), with a mean nearest neighbor distance of 8.54-nmi (Fig. 25). This finding was concordant with the result of the Ripley's K permutation test, which found that pregnant animals were more aggregated than 97.5% of the permuted simulation runs (n = 5,000 for each distance) at a scale from 6.0 to 180-nmi (Fig. 26). Proportion pregnant was greatest, by a substantial margin, in the northern portion of the sampling range (Fig. 27).

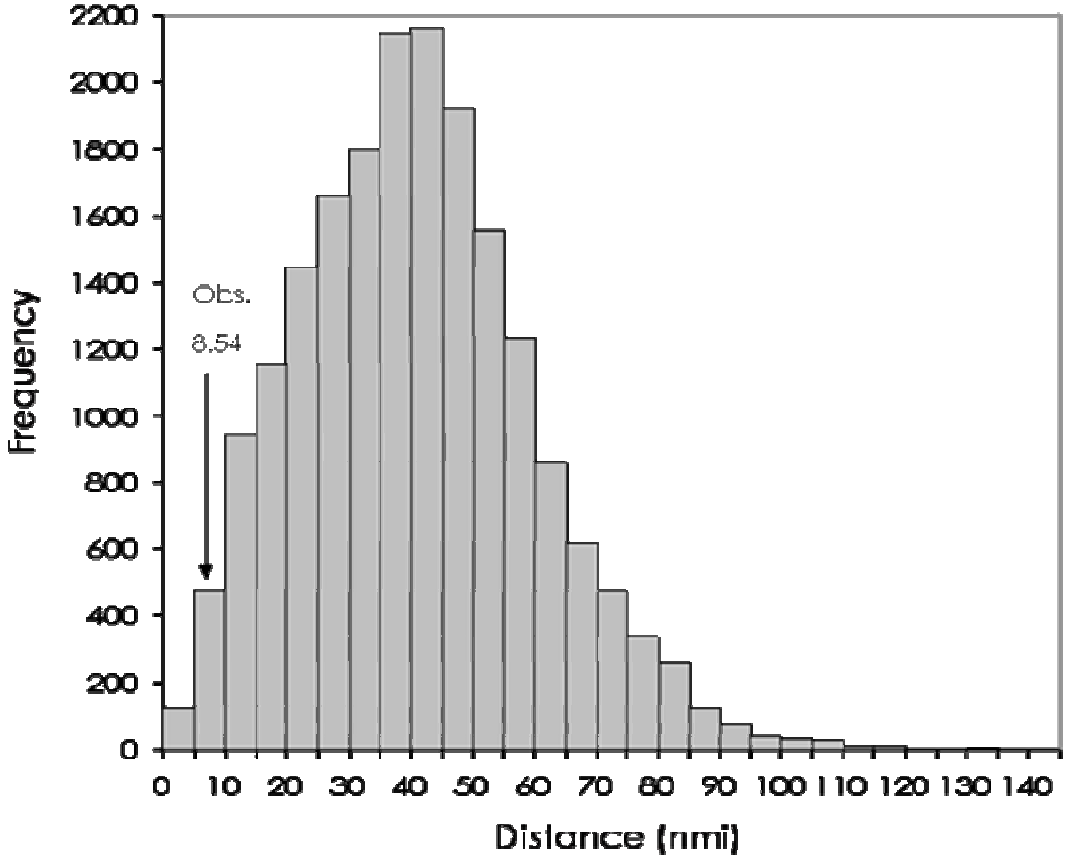

Figure 25. Results of the randomized nearest neighbor distance analysis. The distribution represents the frequencies of mean nearest neighbor distances of pregnant animals, given our sampling locations, if pregnancy was randomly distributed with respect to area. It was derived from 25,000 simulated runs in which pregnancy state was randomized. The arrow indicates were on the distribution the observed mean nearest neighbor distance (8.54-nmi) was found. This value was lower for 98.9% of the simulated runs ($p = 0.023$).

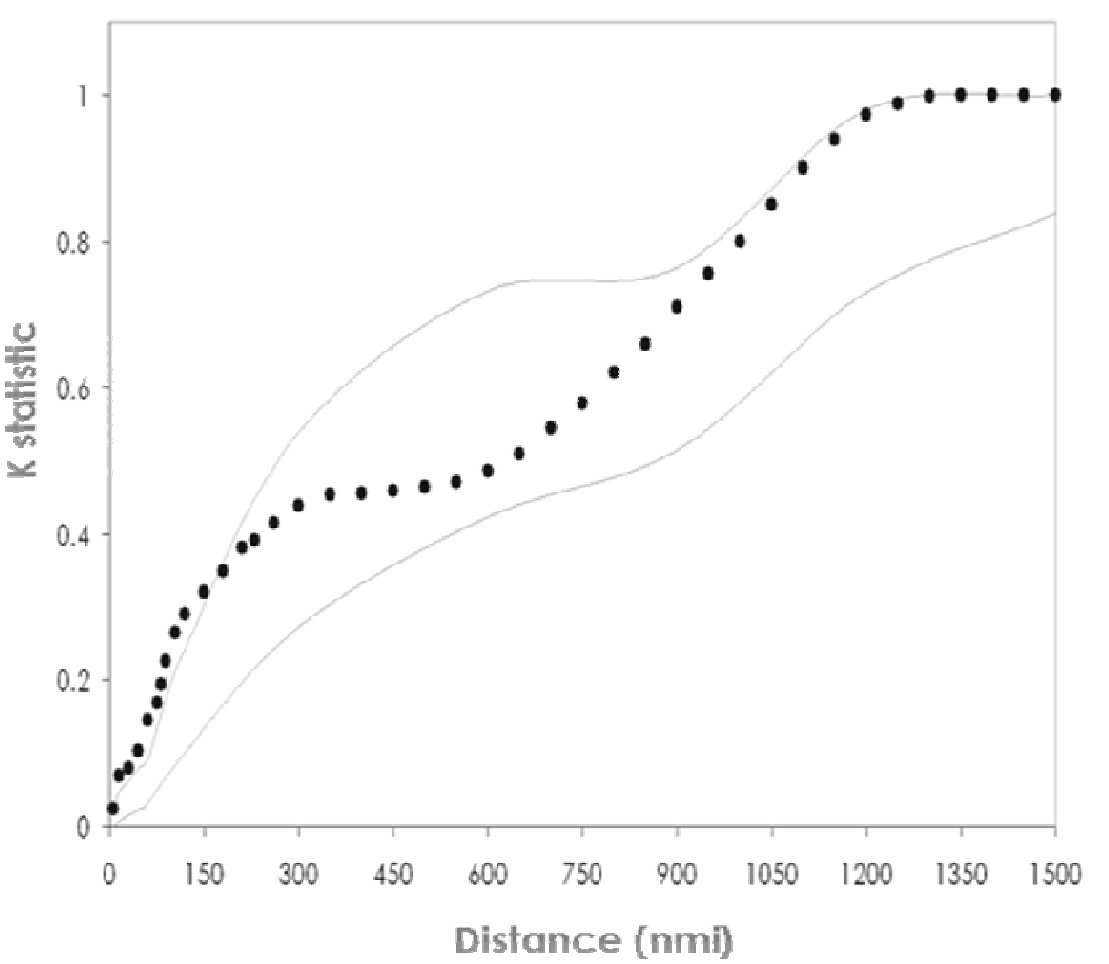

Figure 26. Results of spatial pattern analysis of the distribution of 24 biopsied pregnancy *S. attenuata* relative to all females sampled (n =212) using Ripley's K statistic (Ripley's 1977). The function K(d) was calculated for each 20-nmi interval(0-300-nmi) and 50-nmi interval (>300-nmi). The derived sample statistic $K(d)/n_{pregnant}$ was plotted against distance (dotted line). The solid light lines represent the 95% simulation envelope for total spatial randomness. The envelope was derived by permuting (5000 simulations/distance interval) reproductive state for the entire set of sampling sites while fixing the total number of simulated pregnant animals at 24 (equal to the observed number)..

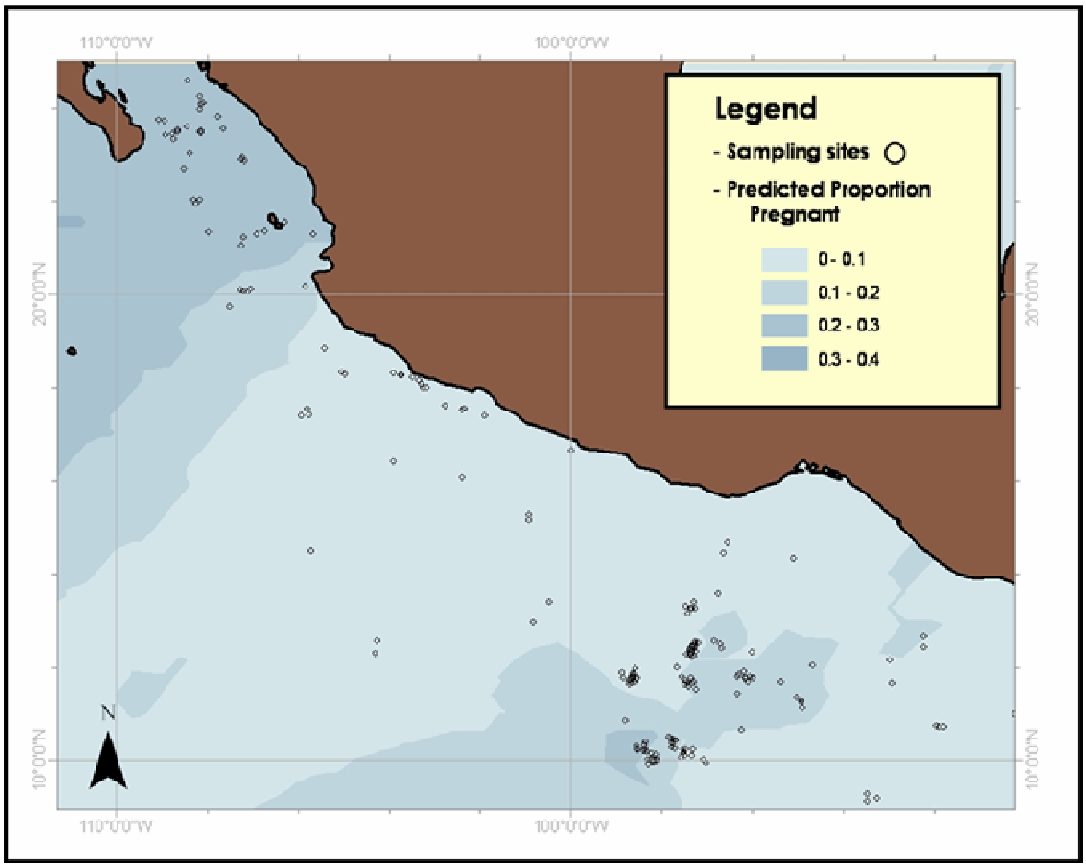

Figure 27. Prediction map from an ordinary Kriging interpolation of the proportion pregnant for our biopsies of female *S. attenuata*. The open circles indicate the sampling locations and darker areas indicate higher levels of proportion pregnant (interpolated)

After discovering this potential spatial pattern in pregnancy, the historic fishery-kill data were divided into three sub-regions (north, central, and south - Fig. 28); delineated by breaks in the biopsy sampling distribution. This allowed us to test whether the historic fishery kill[6] had similar spatial patterns with respect to pregnancy and to assess whether any region had a significantly higher proportion pregnant in the fishery-kill data (Fig. 29). The subsequent 2x3 contingency analysis indicated that proportion pregnant in the historic fishery-kill varied significantly among the three regions, ($\chi^2 = 11.81$, $P = 0.004$). Individual 2x2 contingency tests showed that the northern region was significantly higher in proportion pregnant than either of the two more southern regions (central: $\chi^2 = 5.18$, $P = 0.02$; southern: $\chi^2 = 12.00$, $P < 0.001$), but we found no significant differences between the southern two ($\chi^2 = 0.80$, $P = 0.88$).

Comparison of proportion pregnant

We found the proportion of pregnant females of all females biopsied (likely mature plus non-neonate juveniles) was 0.118 (SE = 0.022). This result is significantly lower ($\chi^2 = 11.04$, $P < 0.001$) than the overall proportion of pregnant females sampled from the historic fishery bycatch (0.216 ± 0.011SE) within the same sampling area. However, after stratifying both data sets into the three geographic sub-regions, we found that in only two of the three areas that the biopsies were

[6] Because there was no *a priori* stratification of the *biopsy data,* we did not compare the proportion pregnant between these regions with this sample set.

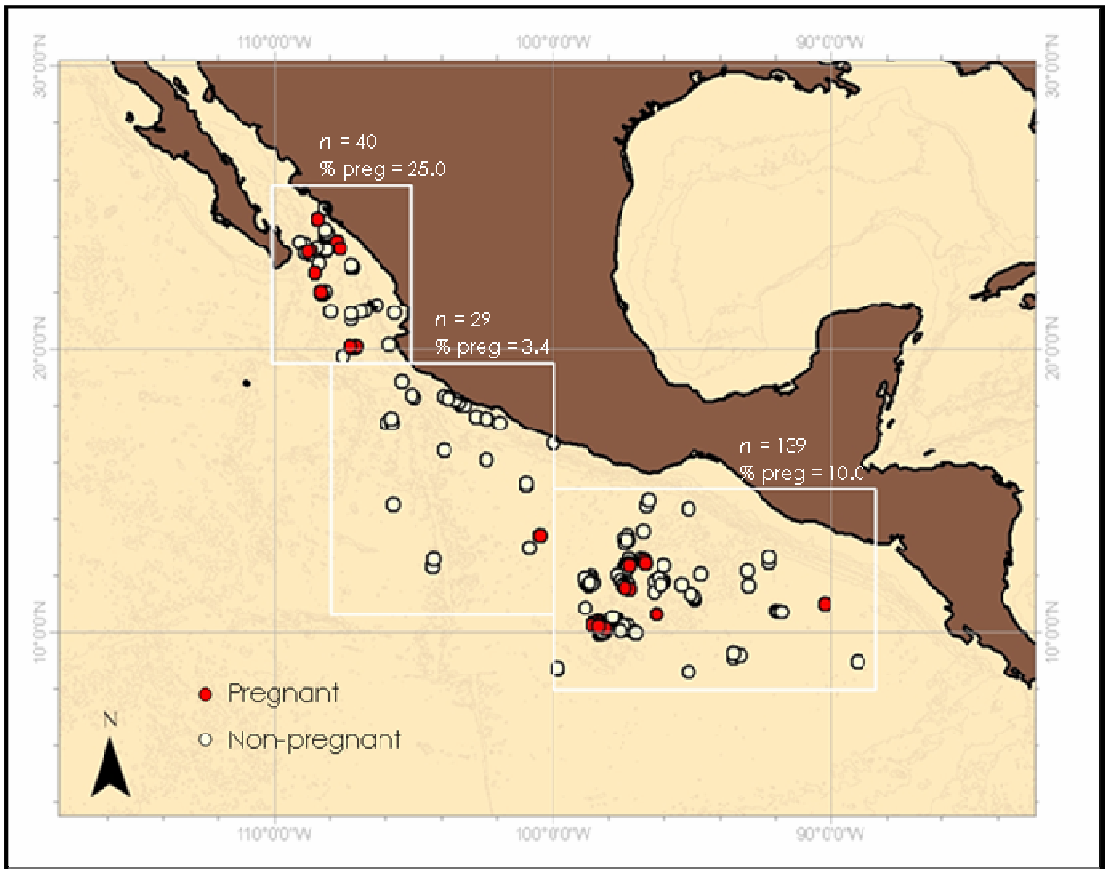

Figure 28. Geographic distribution of the female *S. attenuata* biopsies designated as pregnant (dark circles) and non-pregnant (light circles) as determined from blubber progesterone concentrations. Using these data, three regional geographic strata were delineated)light boxes) at breaks in the sampling distribution. These strata were used for comparisons within the historic kill and between the biopsies and the historic fishery kill.

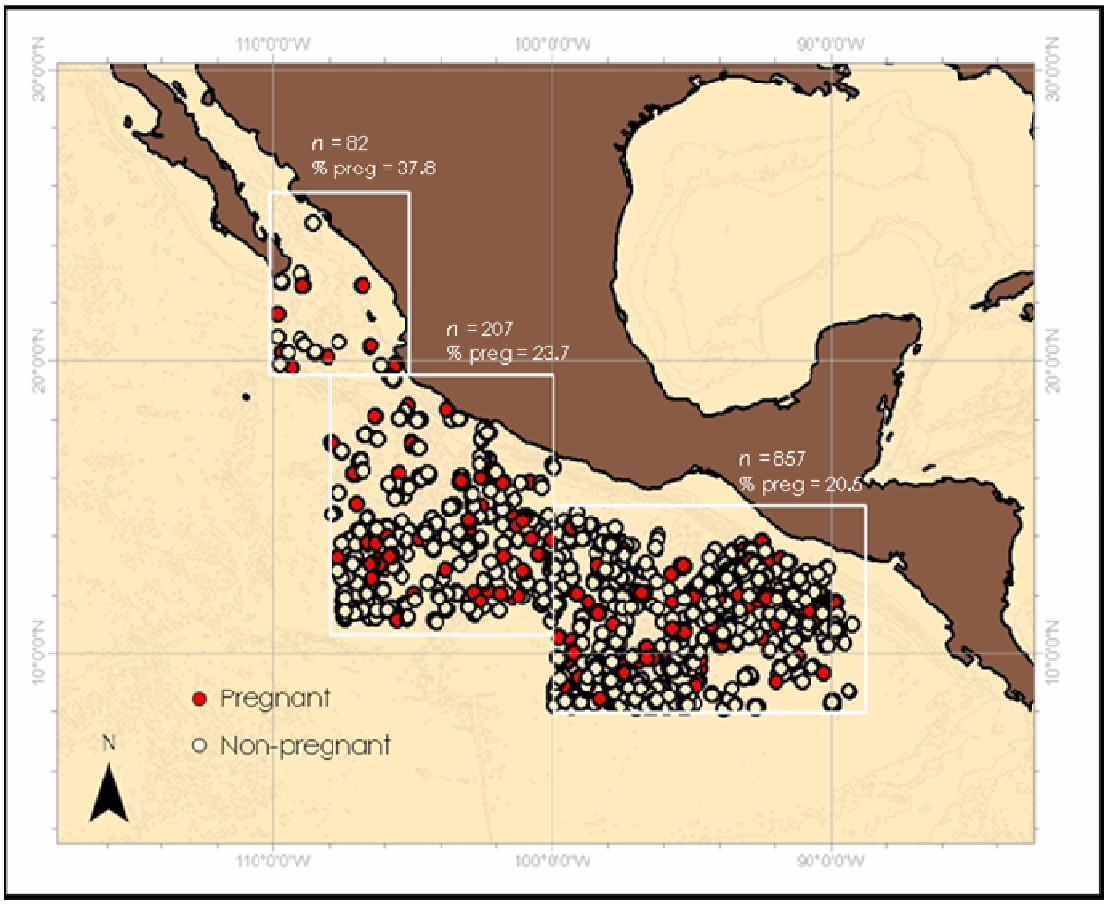

Figure 29. Spatial distribution of pregnant and non-pregnant female northeastern spotted dolphins in the historic fishery-kill from 1974-1992, constrained to the same area and time-of-year covered by our biopsy sampling from 1997 to 2003. The light colored boxes indicate the strata of comparison used to 1) investigate spatial differences in proportion pregnant within the historic kill and 2) assesses regional differences in proportion pregnant between the kill and the biopsies.

represented by significantly lower proportion pregnant (central $\chi^2 = 5.08$, $P < 0.023$; southern $\chi^2 = 7.82$, $P < 0.004$); the exception being the northernmost sampling sub-region, at the mouth of the Gulf of California, which was not significantly different from the historic fishery-kill ($\chi^2 = 1.44$, $P < 0.22$). However, the last can be perhaps ascribed to inadequate statistical power given the smaller sample sizes for the northern strata.

Fishery Exposure

In our biopsy sample, pregnant animals had substantially lower fishery exposure indices than non-pregnant ones (Table 8). Permutation tests indicated that these differences were significant for all three ambits, the 70-day ($\Delta X_{stat} = -95.6$, P = 0.0456), the 140-day ($\Delta X_{stat} = -76.7$, P = 0.0052) and the 180-day ($\Delta X_{stat} = -69.2$, P = 0.0062) (Fig, 30), suggesting that there is an inverse relationship between fishery exposure and proportion pregnant. Moreover, there is also an apparent spatial pattern to the fishery exposure index and the density of sets, with lowest levels seen in the north (where we find the highest proportion pregnant) and the highest levels in the south around 10 degrees (Fig. 31). It was judged that there were too few points (specifically from pregnant animals) to spatially stratify the data and to make similar comparisons within each geographic area that would be meaningful.

Table 8. **Median (SE_{median}) fishery-exposure indices, for the three ambits captured, of pregnant and non-pregnant biopsied female *S. attenuata*. The indices are significantly lower in pregnant animals indicating a negative relationship between fishery-exposure and pregnancy. P-values were calculated from Δ-mean permutation tests in which pregnancy state was permuted (while keeping the proportion pregnant constant) to generate the null distribution in the difference in index means.**

Ambit	Median fishery-exposure index ± SE_{median}		*P*-value
	Pregnant	Non-pregnant	
70-day	84.8 ± 14.0	180.4 ± 7.0	0.0456
140-day	138 ± 18.1	214.7 ± 7.4	0.0052
180-day	181.4 ± 20.8	250.6 ± 7.6	0.0060

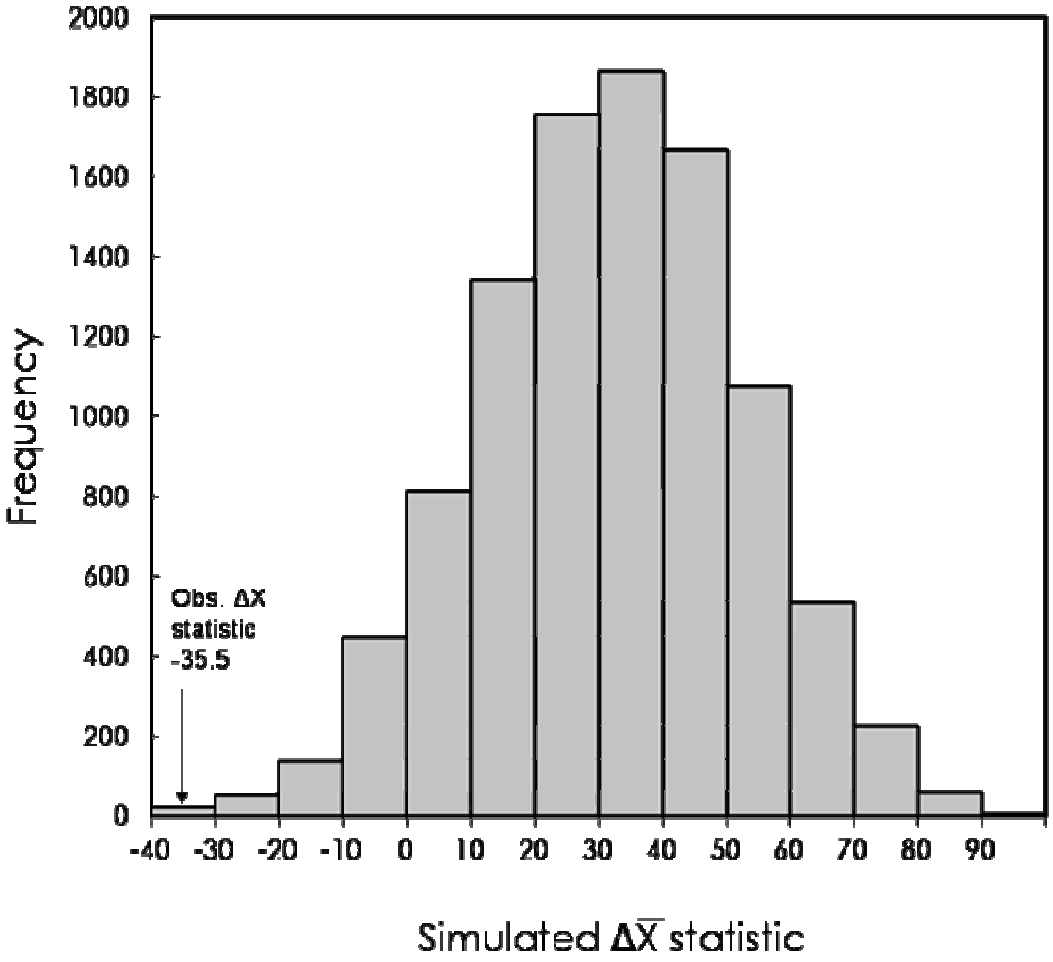

Figure 30. Frequency distribution of simulated (permuted) difference of means statistic for Archer's fishery exposure index (140-nmi ambit) of biopsied pregnant and non-pregnant females *S. attenuata*. The observed statistic (indicated with arrow) is lower than 99.5% of the permuted values representing the null distribution.

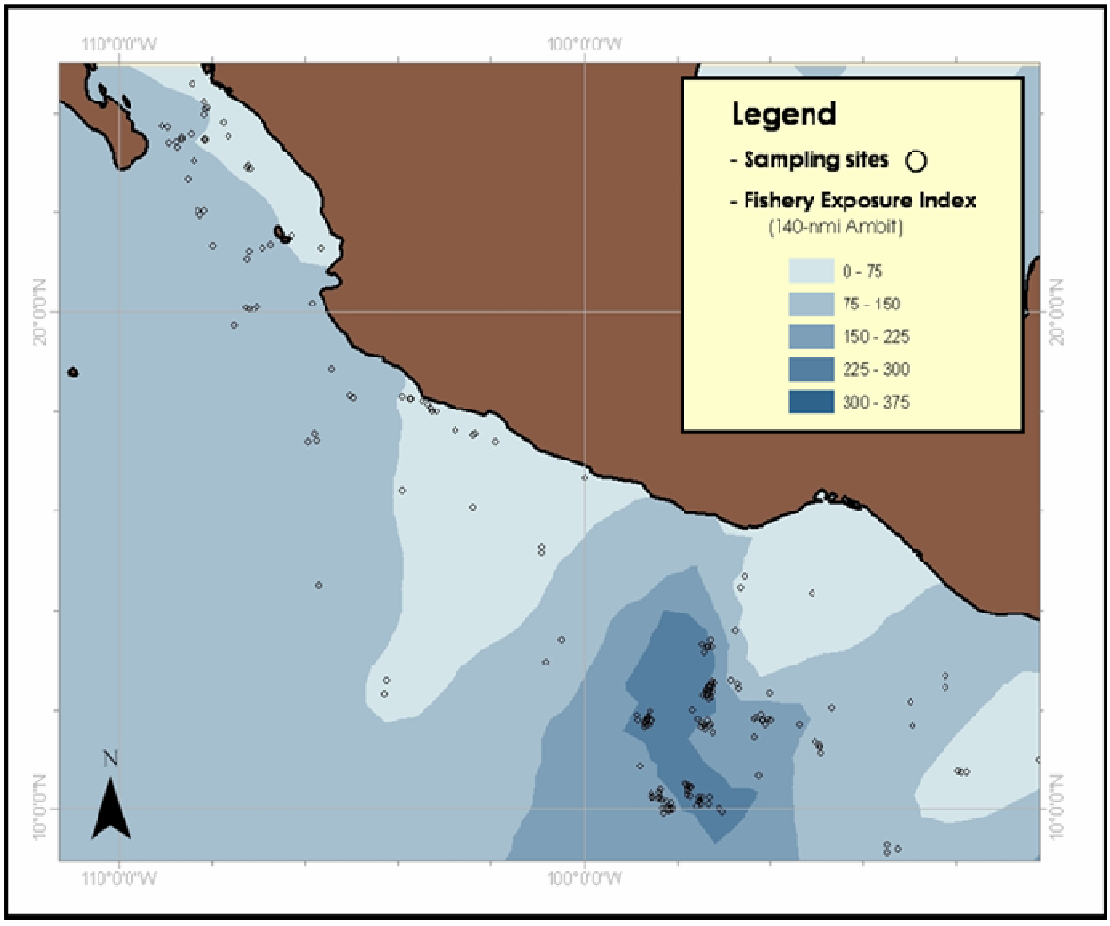

Figure 31. Prediction map from an ordinary Kriging interpolation of Archer's fishery exposure index (140-nmi ambit) for our biopsies of female *S. attenuata*. The open circles indicate the sampling locations and darker areas indicate higher levels of exposure.

Discussion

Performance of blubber progesterone as a marker of pregnancy

The most challenging obstacle for this study was that the reference set of blubber from spotted dolphins of known pregnancy state to test the accuracy of blubber progesterone as a diagnostic of pregnancy in this species was rather small (n = 9). However, several lines of evidence indicate the veracity of this potential diagnostic:

1) Blubber progesterone has been shown to be an accurate indicator of pregnancy state in all species of cetaceans examined previously (four delphinids [Kellar *et al.* 2006] and one mysticete [Mansour *et al.* 2002]), each demonstrating large non-overlapping differences in blubber progesterone concentrations between known pregnant and non-pregnant females. These concentrations were remarkably similar across all delphinids measured (Table 1)

2) The blubber progesterone concentrations from the reference set of nine recent fishery-killed spotted females of known pregnancy state (our reference set) were similar to these other delphinids with each value falling within the range of concentrations relative to pregnancy state.

3) The frequency distribution of blubber progesterone concentrations in the spotted dolphin biopsies was bimodal, with no value between 48.1 and 89.3 ng/g. This hiatus is similar across all species examined (in this study and previous studies) and in specimens of known reproductive state, it reflects the separation between non-pregnant and pregnant females[7]. These lines of evidence strongly indicate that blubber progesterone is an accurate indicator of pregnancy state in *S. attenuata*.

Fishery Exposure

It has been hypothesized that fishery-induced stress from frequent repeated chase and encirclement of dolphin schools might lead to reduced reproductive success in the highly affected populations (Curry 1999, Cramer and Perryman 2002, Archer *et al.* 2004b). Our finding that pregnant animals had less previous fishery exposure than non-pregnant animals is consistent with this hypothesis. Individual dolphins are chased, captured, and released on average of eight times a year (2-50 times annually depending on school size) (Perkins and Edwards 1999). The high annual rate of chase and encirclement of dolphins during tuna fishing may negatively affect reproductive physiology, altering the rate of pregnancy throughout this population.

[7] Given sufficient sampling, it is likely that one would find intermediate values indicating a transition between pregnancy states or perhaps as a signal of ovulation. However, because these events are short in duration relative to the amount of time a female spends in pregnancy, lactation, or as an immature animal over her lifetime, they would be rare events and therefore difficult to catch via biopsy sampling.

There is additional research consistent with these findings. Crammer *et al.* (in review), showed that the proportion of calves within spotted dolphin populations was negatively correlated with fishing effort. In other Cetartiodactyla, numerous stressors (including some that appear much more benign than chase and encirclement) have been shown to affect all major aspects of reproductive endocrine activity, leading to irregular estrus, fewer ovulations, fewer successful fertilizations, implantation failure, spontaneous abortion, and elevated infant mortality (Moberg 1976, Hennessy and Williamson 1983, Moberg 1991, Wilson *et al.* 1998a, Wilson *et al.* 1998b).

How this interference may affect the proportion pregnant is difficult to predict and depends on which stage of pregnancy or reproduction in general is most vulnerable to frequent chase and encirclement. We discuss two potential scenarios here:

(a) If the most vulnerable aspects of reproduction are ovulation, fertilization and/or early pregnancy then one would expect to find a lower proportion pregnant in areas with higher fishing effort. This scenario fits with conventional stress-response theory in which ovulation and implantation are thought to be most susceptible to chronic stressors (Moberg 1976, Coubrough 1985). Any effect on ovulation rate would be difficult to quantify, but there is evidence that early fetal

mortality of dolphin populations impacted by the fishery is unusually high (Perrin *et al.* 2003).

(b) If late pregnancy and neonate survival are more vulnerable then we might expect a positive correlation between proportion pregnant and chase and encirclement frequency because mothers with calves are physiologically less likely to become pregnant (lactation has inhibitive effects on ovulation)(McNeilly 2001). If a calf is killed well before weaning or as a fetus before birth, then it is likely that its mother would become pregnant again earlier than had the calf or fetus not died. If this situation occurred at a sufficient rate, it would lead to a measurably higher proportion pregnant within the population (but a lower birth rate). Several studies have indicated that dolphin calves, especially neonates, are disproportionally more vulnerable to chase and recapture than dolphins of other demographic classes (Archer *et al.* 2001, Archer *et al.* 2004a, Edwards 2006, Noren and Edwards 2007).

Given the fact that we found pregnant animals had significantly lower fishery exposure levels, the results of this study are more concordant with scenario 'a'; they suggest that fishery exposure reduces the likelihood of a female being pregnant (i.e., either becoming pregnant or maintaining a pregnancy). However, this conclusion is tempered by several important caveats. First, there was apparent spatial pattern to the fishery exposure index with the density of sets lowest in the north (where we find the

highest proportion pregnant [Fig. 5]) and the highest levels in the south around 10 degrees (Fig. 9) so it is impossible to establish a definitive causal relationship. There are several alternative explanations that could explain this result that might also have a similar spatial pattern, for example any oceanographic/habitat conditions that increase the likelihood of becoming and staying pregnant might be poor for tuna fishing. Second, the sampling in this study was not random; the biopsies were taken opportunistically, leading to clumped sampling in both time and space. This may result in some pseudo-replication characteristics within the sample set. Currently there are too few samples, especially of pregnant animals (n=24) to adequately address this concern. Third, the exposure index calculation does not incorporate information about population range, nor does it adjust for proximity to land masses. Both could lead to edge effects that gave animals sampled near the boundaries of their range disproportionately lower exposure values compared to animals in the center of the range. This is especially important because nearly half of the pregnant dolphins we sampled came from within a few degrees of the northern boundary of their range. Further honing of the exposure index measurement and additional sampling in the core of the population would help address this caveat.

Comparing proportion pregnant between historic fishery-kill and biopsy data

The biopsy samples were composed of a much smaller proportion pregnant than what was found in the historic fishery-kill over the same sampling area. There is no clear reason for these large differences. However, plausible explanations include

(1) differential sampling selectivity with respect to reproductive or developmental state, (2) inter-annual variation in spatial distribution of reproductive animals, (3) changes in habitat leading to reduced pregnancy rate and, (4) ongoing fishery effects on reproduction.

1) Differential sampling selectivity/bias

Age-frequency distributions of spotted dolphins collected in the fishery kill indicate that the kill is biased; juveniles (5-15 year olds) are substantially underrepresented (Archer and Chivers 2002). This has potentially large ramifications for the historic-fishery/biopsy comparison. Remember the metric, proportion pregnant, reported in this study, is the number of pregnant females as a fraction of all females sampled. By disproportionally sampling non-juvenile females, we expect that the proportion pregnant within the fishery-kill is biased higher than what we would find in the actual population. If biopsy sampling is less biased or differently biased with respect to age, this could easily account for the differences seen in proportion pregnant in this study. However, we do not know how or whether biopsy sampling is selective relative to age. We do know that calves under a year old and mothers with calves under a year old were not targeted for biopsy sampling. With this restriction, one would expect that the biopsy data would be, like the fishery kill, biased high compared to the fraction of pregnant females within the

actual population because female calves cannot be pregnant and mothers with calves are disproportionally less likely to be pregnant compared to other adult females (lactation generally suppresses ovulation). However, it is unknown whether this bias is as large as that resulting from the fishery-kill; consequently, it is uncertain whether the differences in selectively of the two sampling methods (i.e., fishery bycatch and biopsy) are responsible for the large differences in proportion pregnant.

2) Inter-annual variation in spatial distribution

From the fishery-kill data, we find that annual variation in reproductive rates in any one region of the ETP can be substantial (Barlow 1985). If our biopsy sampling captured a year or more of low regional pregnancy rates that were not reflected in the pregnancy rates of the entire population this could lead or contribute to the disparity in proportion pregnant between the biopsies and the historic fishery kill.

It is not known why we see such large differences in the proportion pregnant in the fishery kill from year to year in certain areas, but perhaps if the movements of females of different reproductive groups are even partially driven by differential nutritional requirements, then year-to-year fluctuations in oceanographic conditions could help explain these sometimes dramatic regional changes. More sampling in this region over several additional years

and an examination of the correlation between pregnancy state and different oceanographic conditions and prey distributions should help us investigate whether normal regional (here we mean across the entire sampling area) annual variation in reproduction is responsible for the low proportion pregnant in the biopsies obtained for this study.

3) Changes in habitat leading to reduced pregnancy

General changes in the habitat of the ETP from the time of the historic fishery kill (1974 – 1993) until our recent biopsy sampling (1998 – 2003) could also affect the rate at which pregnancies were conceived and sustained. For instance, changes in the oceanography of the region might alter the preferred prey distribution or abundance leading to suboptimal nutritional conditions for reproduction. Although numerous studies have documented a strong oceanographic regime shift (from warm to cool) in the North Pacific starting in 1976 that had effects (though somewhat dampened) on the conditions in ETP (Fiedler 2002), this does not appear to be correlated with the relatively large differences in proportion pregnant between our biopsy sample set and the historic fishery kill. We found no significant differences in proportion pregnant within the historic fishery kill before or after the shift in our study region, and the majority of the fishery-kill data used in our analysis were obtained after the change in conditions. No other decadal climate shift in the tropical Pacific has been documented since the 1976-77 event. Although

from this cursory examination of published oceanographic trends it does not appear that changes in the general habitat of the ETP can account for the differences in proportion pregnant between our two sample sets, a much more focused investigation of the relationships between pregnancy state and various oceanographic conditions and prey composition and abundance indices is needed before habitat change can be ruled out as a contributing cause of the differences in proportion pregnant.

4) Fishery effects on reproduction over time

The most fundamental change in fishing practices between the two time periods is that dolphin mortality was high when the historic fishery-kill data were collected and orders of magnitude lower when the biopsies were collected. If kill was selective with respect to pregnancy state, then the pregnancy rate of the population would likely differ between the two periods. As noted above there is evidence that post-weaned, pre-sexually mature females were underrepresented in the kill (Archer and Chivers 2002); i.e., the adults and calves suffered disproportionately higher mortality. If true, the population, at the time of high fishery mortality, would have had a higher proportion of post weaned immature females within the population and therefore a lower proportion pregnant per female (all maturity states). However, any reduction in rate would not be represented in the kill itself;

instead, it would have a high proportion pregnant because disproportionately fewer immature females would be collected and result, in essence, in the scenario described in the section "*1) Differential sampling selectivity/bias*".

Beyond the sampling biases with respect to pregnancy status, it is not clear how, the fishery kill might have affected the proportion pregnant within the kill or population. Reduction in sub-lethal effects on reproduction of the fishery (ex., chronic stress from repeated chase and recapture) does not appear to be responsible for the disparity in pregnancy rate between the two data sets because fishery activity (number of dolphin sets in the region per year) was similar when both the historic kill was sampled and the biopsies collected. Nor would a density-dependant change in reproductive rates appear responsible. When our first fishery-kill sample was collected in 1974, the northeastern spotted dolphins had been set on for over a decade and their population had already been reduced to 20% of the estimated pre-exploitation level (approximately the abundance today).

There does not appear to be a straightforward way to test which potential factor or factors described above may be contributing to the disparity in proportion pregnant between the biopsy samples and those from the historic fishery kill, though additional sampling with greater temporal and spatial coverage may help, especially if high regional variability was a significant factor during biopsy sampling. In addition, we are attempting to assess the length distribution of spotted dolphins that make

themselves available for biopsy and compare it to lengths in the population as a whole as determined from aerial photographs to investigate the potential selectivity of biopsy sampling. Because of the numerous potential confounding factors delineated above and the limited avenues to generate independent data, this will likely remain a difficult question to answer fully.

Spatial Variability in Proportion Pregnant

The spatial variability analyses indicated that reproductive state is not randomly distributed with respect to space. Both the fishery and biopsy data indicate that pregnancy rates were highest in the mouth of the Gulf of California. The explanation may be as simple as the different areas contain demographically distinct populations with different reproductive rates. There are several additional plausible explanations for this type of selective spatial clumping:

1) Pregnant females move to areas of higher forage density:

Many different mammals segregate by demographic or reproductive condition by selecting habitats that can meet their specific nutritional requirements (Seagle and McNaughton 1992, Labisky and Fritzen 1998, Bowyer 2004, Millspaugh *et al.* 2004, Ciuti *et al.* 2006). Pregnant females, especially during mid to late gestation, experience large increases in energy requirements (Millar 1977, Jonsson *et al.* 1995, Reynolds and Kunz 2000) and changes in dietary needs that can prompt movement to more productive or

more nutritionally appropriate habitats (Bonenfant *et al.* 2002). In our case, we find the highest proportion pregnant in the mouth of the Gulf of California an area with relatively high productivity (Collins *et al.* 1997, Bustos-Serrano and Castro-Valdez 2006). Therefore "the differential nutritional requirements concept" appears to be an explanation consistent with our observations. However, we know that directly after pregnancy the nutritional requirements are on average even higher while lactating (Hadjipieris and Holmes 1966, Millar 1975; 1977, Bowen *et al.* 2001); in some mammals the difference is as much as three times as great (Millar 1977). So although nutrition may be a driver for reproductive partitioning or differential location selectivity, we would not necessarily expect only pregnant animals to be disproportionately occupying the areas of highest nutritional output or highest prey concentration; lactating females would be subject to the same drive. In addition, this explanation presupposes that there is some drawback to these areas such that when a post-weaned female is not either pregnant or lactating she would more likely than chance move to an area of more limited nutritional resources; otherwise one would expect that all animals, regardless of reproductive condition, would seek out areas of high productivity.

2) Chase and Encirclement Altered Reproductive Physiology Regionally

Because fishing effort is not evenly distributed in space with respect to dolphin abundance, we expect that dolphins in certain areas experience

different levels of chase and encirclement; this is evident in our fishery exposure analysis. If chase and encirclement is generating altered physiological activity at a level that negatively affects reproduction as suggested by the exposure analysis, it might alter the relative spatial distribution of pregnancy.

Again the specific effects of high fishery exposure on pregnancy rate could be complicated, depending on which aspects of reproduction are most vulnerable; for instance, we might not necessarily expect that high fishery exposure would lead to lower pregnancy rates, especially if the late pregnancy or early postnatal periods are most affected. As noted above, this is because mortality at these stages may allow mothers to become pregnant again faster than had they successfully raised and weaned their offspring.

3) Mature females segregate spatially

Because we cannot differentiate maturity state in biopsied females, it also may be that mature females are disproportionately segregating from immature/post-weaned animals on a regional basis. If this is true, the factors delineated above may still contribute to the spatial variability we find in pregnancy state, but they would be acting on somewhat different physiological, behavioral, and/or social attributes associated with the different maturity groups instead of the different pregnancy states. Previous studies

have observed selective maturity partitioning in several free-ranging dolphin and porpoise species. They show reproductively immature animals schooling differently and in different areas compared with mature dolphins (Kasuya 1972; 1976, Kasuya and Ogi 1987). However, as noted above, the fishery-kill data do not suggest strong segregation by demographic or reproductive state.

It may be that more than one of these potential factors play some role in the geographic variation of pregnancy. However, irrespective of the potential causes, we reiterated that there does not appear to be strong segregation (though it is significant) by either pregnancy or maturity state. For instance, in the historic fishery-kill, as we find all demographic classes are represented in all areas examined. Instead, the signal suggests a *disproportionate* preference of certain areas by different reproductive/demographic groups.

Conclusions

This study is the first to use a non-lethal approach in attempts to estimate the proportion of animals pregnant sampled from a wild dolphin population. As such, we try to be cautious in the interpretation of our results and delineate areas that need further investigation. However, we do find that BP concentrations in this species appear to be similar to other delphinids and diagnostic of pregnancy state. We showed that it is possible to collect sufficient samples to investigate spatial patterns of

pregnancy and make meaningful estimates of reproduction. We found a negative correlation between exposure to fishing effort and proportion pregnant. We also found spatial patterns in the proportion pregnant. However, what factors are responsible for the specific patterns we found are unknown. In addition, the potential selective nature of biopsy sampling with respect to pregnancy state may be a limitation that will be difficult to assess and consequently would limit our ability to make unbiased estimates of population pregnancy rates. Nonetheless, to our knowledge there are no other non-lethal ways to assess pregnancy levels in wild populations. Given the important nature of this information for population assessments, especially for these spotted dolphin that are frequently and continually affected by anthropogenic activities, we think that this approach has potential utility in the study of dolphin life history and warrants further application.

Chapter Four will be submitted, in part or in full, as a manuscript for publication. I was the primary researcher and author. The co-authors listed in this publican either helped generate raw data or supervised and directed the research from which this chapter was formed. This chapter was written in collaboration with M. L. Trego and F. I., Archer.

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