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Blubber Testosterone: A Potential Marker of Male Reproductive Status in Short-Beaked Common Dolphins

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

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ABSTRACT

A novel molecular technique was used to measure blubber testosterone (BT) in 114 male short-beaked common dolphins, *Delphinus delphis*, collected from incidental fishery bycatch and strandings. When these concentrations were compared across maturity states, the mean (± SEM) BT levels of mature *D. delphis* (14.3 ± 3.0 ng/g) were significantly higher than those of pubertal (2.5 ± 0.5 ng/g, $P = 0.006$) and immature animals (2.2 ± 0.3 ng/g, $P < 0.0001$). BT concentrations in mature males were significantly higher in summer months (53.9 ± 2.0 ng/g) than during the rest of the year (7.9 ± 0.69 ng/g, $P < 0.0001$), indicating reproductive seasonality. An analysis of BT in different anatomical locations showed that hormone concentrations were not homogenous throughout the body; the levels in the dorsal fin were significantly lower than in most other areas ($F = 5.39$, $P = 0.043$). Conversely, we found no significant differences in BT concentration with respect to subepidermal depth ($F = 2.09$, $P = 0.146$). Finally, testosterone levels in biopsies from 138 free-swimming male *D. delphis*, of unknown maturity state, sampled off California were found to be of concentrations similar to those from the fishery bycatch and stranding samples and revealed an analogous trend with respect to ordinal date.

Key words: blubber, adipose tissue, biopsy, testosterone, androgen, sexual maturity, seasonality, testes, short-beaked common dolphin, *Delphinus delphis*.

Little is known about androgen levels in cetaceans, and yet they are important components of male endocrinology that can provide pertinent data on reproduction, development, and seasonal reproductive trends. The increase of androgens during postnatal development is a key factor defining puberty in male mammals (Preslock 1980). Consequently, concentrations of androgens, particularly testosterone, are

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commonly used as endocrine indicators 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), *Tursiops truncatus* (Kirby 1990), *Globicephala melas* (Desportes *et al.* 1994), and *Balaenoptera physalus* (Kjeld *et al.* 1992).

Testosterone concentrations have also been used to reveal seasonal breeding patterns. A common characteristic of mammalian seasonal reproduction is the 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 cetacean species by documenting the presence or absence of this pattern in *T. truncatus* (Schroeder and Keller 1989), *D. leucas* (Robeck *et al.* 2005), *Stenella longirostris* (Wells 1984), *G. macrorhynchus* (Kita *et al.* 1999), and *B. acutorostrata* (Mogoe *et al.* 2000, Kjeld *et al.* 2004).

These previous studies have predominately used serum and, more recently, fecal material to quantify testosterone levels in noncaptive 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 is therefore impractical in most population-level assessments. Similarly, although fecal samples have been used with success to assess male maturity state in free-ranging cetaceans (Rolland *et al.* 2005), opportunities to collect these samples can be limited and often require special detection systems (Rolland *et al.* 2006) to increase collection efficiency.

Sampling blubber provides another method to assess androgen levels. This lipid-rich 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 (Palsbøll *et al.* 1991). 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 study that has quantified testosterone in blubber, 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).

Quantifying the concentration of blubber testosterone (BT) in skin biopsies could provide demographers and life historians access to data on reproductive seasonality and sexual maturity that might not be easily acquired from free-swimming animals. 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 (*e.g.*, 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 relatively easy-to-obtain biopsy samples collected more randomly from live animals could mitigate some of these problems.

In our study, we extracted and quantified testosterone from small blubber samples (similar to those obtained from biopsies) of short-beaked common dolphins (*D. delphis*) that stranded or had been incidentally caught in the California gill-net

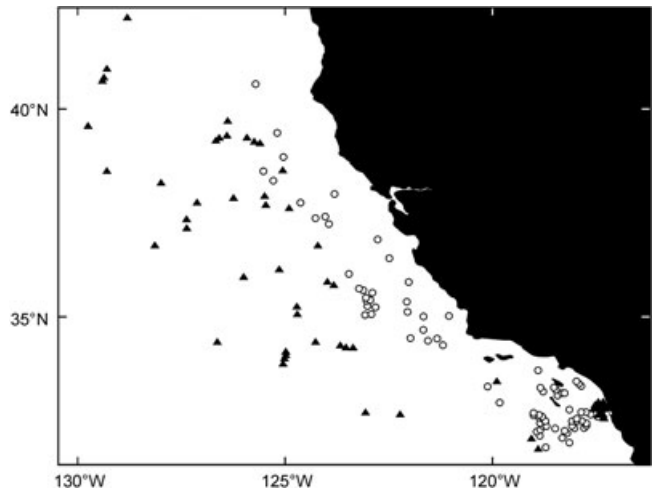

Figure 1. Geographic distribution of the blubber samples from fishery-killed (circles) and biopsied (triangles) male *D. delphis* used in this study.

fishery (Carretta *et al.* 2004). BT concentration was compared across maturity states to evaluate whether it could discriminate between these states. We also examined the variation of BT with respect to anatomical location, subepidermal depth, and ordinal date. And finally, we quantified testosterone levels in biopsied free-ranging male *D. delphis*, of unknown maturity state, and compared these levels to our reference group.

Methods

Samples

Reference collection: fishery/stranding samples—The blubber samples ($n = 114$) used to develop and validate our approach were obtained from male dolphins for which we also had testis tissue available to provide an independent determination of the state of sexual maturity. The majority of these ($n = 106$) were incidentally caught in the California gill-net fishery and collected by observers in the California/Oregon Gillnet Observer Program between 1991 and 2005 (Fig. 1). The remaining specimens ($n = 8$) were collected by the Southwest Fisheries Science Center Stranding Program. Full-thickness blubber samples ($\sim$100 cm^2) were collected from the dorsal and mid-thoracic area 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 mass determination and histological examination (Jefferson *et al.* 1994).

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. 1) from 1999 to 2006.[1] These samples were stored at –20°C in 2-mL cryovials until

[1]These samples were originally collected for genetics studies for which the epidermis was used, but most of the blubber from each was available for endocrine analysis.

processed. The sex of each biopsied animal was determined by the presence of sex-specific genes found in the epidermis of the skin. Toward these ends, two assays were used: (1) standard PCR electrophoresis (Fain and LeMay 1995) on all of the biopsies and (2) quantitative PCR (Morin *et al.* 2005) on a subset of 30 randomly selected samples to check assay reliability. Only 261 samples had >50.0 mg of blubber, the minimum needed for our assay.

Sample Processing

Sexual maturity state of each animal sampled for the reference collection was determined independently of BT measurements using histological preparations of tissue excised at the mid-length of the testis following methods delineated in Akin *et al*. (1993). Thin sections, stained with hematoxylin and eosin, were examined at magnifications of up to 400× for the presence and abundance of spermatozoa and the size of seminiferous tubules. We used the criteria delineated in Collet and Saint Girons (1984) to classify each individual as immature, pubertal, or mature. The maturity classifications were graphed to investigate the relationship between specimen length and testis size as a qualitative check for potential outliers or misclassifications (*i.e.*, the majority of immature animals are shorter and have smaller testes than mature animals).

Extraction and Quantification

The procedures for steroid extraction were identical to those described by Kellar *et al.* (2006). The blubber samples collected from the fishery and beach-stranded specimens were subsectioned into thin columns (~150 mg), approximately the amount obtained using a standard biopsy dart with the epidermis removed. The tissue was homogenized in 1,000 μL of 100% ethanol using an automated, multitube homogenization instrument (FastPrep Instrument, Q-Biogene, Irvine, CA, USA). The tissue was processed for eight 45-s periods at 6.5 m/s in lysing tubes provided by the instrument manufacturer. Steroids were extracted and isolated using the multistep 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, the samples were redissolved in 250 μL of phosphate-buffered saline (pH 7.5) containing 1% bovine γ-globulin and then mixed using a multitube vortexer (VWR Scientific Products, Morrisville, NC, USA), at medium speed for at least 15 min.

Testosterone concentrations were determined using a commercially available enzyme immunoassay (EIA) kit, DSL-10–3900 (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). The manufacturer reported interassay coefficient of variation (COV) ranged from 3.4% to 7.0%, and intra-assay COV ranged from 4.1% to 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 at 0.9%. It should be noted that the assay signal is a composite of these immunoreactive androgens; therefore, BT concentrations reported here represent this aggregate of androgens (in nanogram per gram of blubber, wet weight) in which testosterone is very likely the most prevalent. All samples were processed and quantified in triplicate.

Sampling Effects

To investigate whether BT concentration varies across anatomical sites, we sampled three fishery bycaught males at nine different body sites. The males were identified as sexually mature following the Collet and Saint Girons (1984) criteria and were all collected between 18 October and 11 December 2000 (chosen in part to minimize potential effects of storage time and seasonal differences in BT production). The anatomical sampling sites were chosen arbitrarily to represent areas of frequent biopsy sampling plus adjacent sites to better characterize the variation of blubber concentration throughout the animal. The blubber was subsampled 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 subdivided full-depth samples (epidermis to muscle) from mature males ($n = 7$) into equal-thickness subsamples representing three layers (inner, middle, and outer), each approximately 150 mg. The specific depth of each subsample was dependent upon the thickness of the individual's overall blubber layer.

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 ($\sim$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; 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 10 ng/mL assay standards that were spiked (1:1) with (1) the 0 ng standard and (2) pooled blubber extracts from three immature males. The two groups, each containing six replicates, were compared using a nonpaired *t*-test.

Extraction Efficiency and Controls

Extraction efficiency was determined for each set of extractions by spiking selected subsamples with six dilutions of cold testosterone ranging from 0 to 5 ng in the matrix tubes before initial homogenization. We extracted and quantified the testosterone in these subsamples according to the procedure described above. The resulting extraction efficiency rate was estimated as the percentage recovered in the final quantification after correcting for the intrinsic amount measured in the nonspiked samples. The average efficiency rate across all extractions was 72.1% (range 60.0%–98.9%).

To compliment the assay controls provided in the EIA, we periodically utilized positive (no tissue, with varying dilutions of testosterone) and negative (no tissue, no testosterone) extraction controls to qualitatively monitor the consistency of our measurements and test for detectable contamination. Although these were not part of every extraction set, the variation within the positive extraction controls measurements were consistent with the inter- and intra-assay COVs, and the negative extraction control measurements never contained a signal within the detectable limits of the assay. In addition, the subsamples used to estimate extraction efficiency

for each extraction set were taken from the same two individuals throughout the measurement procedure. Consequently, the interextraction variation was monitored and normalized using the extraction efficiency procedure delineated above.

Data Analyses

All testosterone concentrations were log-transformed prior to analysis to reduce heteroskedasticity (*i.e.*, increasing testosterone concentration yielded increasing measurement variance). Four single-factor ANOVAs were used to compare BT concentrations between (1) maturity state (immature, pubertal, and mature), (2) season of sampling (summer [June, July, and August], autumn [September, October, and November], and winter [December, January, and February]), (3) sampling depth below the surface of the epidermis (outer, middle, and inner), and (4) anatomical site of sample (9 different sampling sites). When significant differences were found, these analyses were followed by Tukey/Kramer *post hoc* tests. A linear regression analysis was employed to determine whether 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 mo) using samples from all reference males in each maturity class. We also examined the relationship between testis mass and BT concentration. For this a Pearson's correlation analysis was used instead of a linear regression test because upon visual inspection of these data a clear nonlinear relationship was found. All preceding statistical comparisons were conducted in Matlab 7.0 (The Mathworks Inc., Natick, MA, USA), using an alpha value of 0.05.

RESULTS

Parallelism and Accuracy Validation

The slopes of the competitive dose curves from serial dilutions of pooled blubber extracts and the known standard controls were not significantly different ($t = -0.501$, $P = 0.96$; Fig. 2), indicating that the assay was primarily measuring the same antigen in the control and blubber extracts. We also found no significant differences between the assay standards (10 ng/mL) spiked with the blubber extract and those spiked with the 0 ng/mL negative control ($t = 0.561$, $P = 0.62$), although the average concentrations in the extract spiked standards were slightly higher ($\mu = 5.09$ ng/mL, SE = 0.30) than those spiked by the negative control ($\mu = 4.77$ ng/mL, SE = 0.48).

Reference Collection: Fishery/Stranding Samples

It should be noted that for the reference animals, the sample distribution relative to ordinal date was uneven. Because the gill-net fishery from which we obtained the majority of our samples was not uniformly active year round, we have many fewer samples representing certain periods (Table 1). A disproportionate number of samples were acquired during fall ($n = 53$) and winter ($n = 42$), with fewer in the summer ($n = 15$), and almost none in the spring ($n = 4$).

Of the 114 fishery-killed males examined, 43 were classified as immature, 14 pubertal, and 57 mature, based upon histological examination of testis tissue. Mature males had significantly higher mean BT concentrations than pubertal ($q = 4.52$,

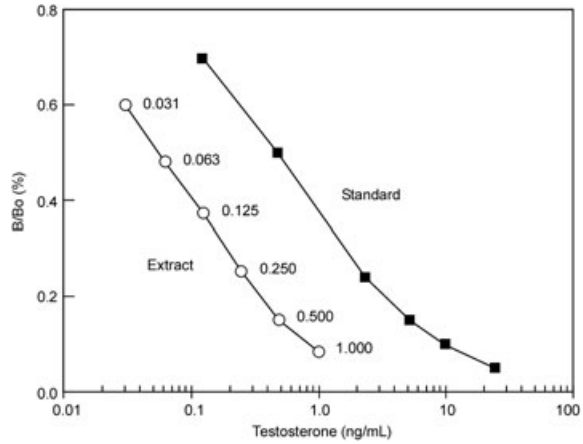

Figure 2. Competition curves showing parallel displacement between known standard concentrations and serial dilutions of pooled blubber extracts of unknown concentration. Dilution coefficients of the extracts are labeled to the right of each corresponding measurement.

Table 1. Testosterone concentrations in the blubber of known immature, pubertal, and mature male *Delphinus delphis* (*i.e.*, reference sample).

	Mature	Pubertal	Immature
Spring			
Median	15.7	2.5	1.7
Mean ± SEM	15.7	2.5 ± 1.3	1.7
Range	–	1.2–3.8	–
n	1	2	1
Summer			
Median	59.4	6.7	2.5
Mean ± SEM	53.9 ± 2.0	6.7	2.7 ± 0.6
Range	16.9–83.0	–	1.2–4.8
n	8	1	6
Fall			
Median	7.0	2.4	1.6
Mean ± SEM	9.0 ± 2.0	2.5 ± 0.6	2.0 ± 0.3
Range	2.1–53.7	1.5–3.9	0.6–5.6
n	26	4	23
Winter			
Median	4.8	1.8	2.7
Mean ± SEM	6.6 ± 1.1	1.9 ± 0.2	2.6 ± 0.4
Range	1.8–21.1	1.6–2.5	0.77–5.2
n	22	7	13
All seasons			
Median	6.9	2.0	1.9
Mean ± SEM	14.4 ± 2.6	2.5 ± 0.4	2.3 ± 0.2
Range	1.8–83.0	1.2–6.7	0.6–5.6
n	57	14	43

The concentrations are corrected for extraction efficiency (see text) and are reported as nanogram per gram of blubber extracted. The average values are displayed with standard error of the mean (SEM).

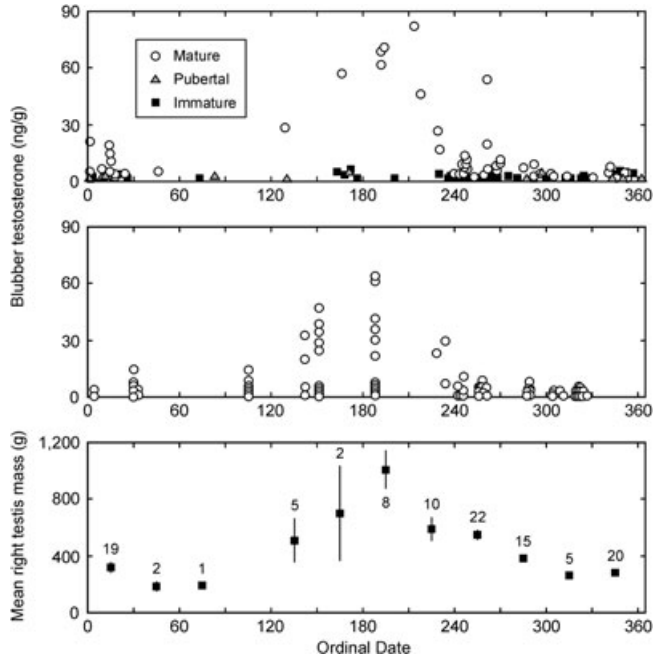

Figure 3. Annual patterns of blubber testosterone (BT) concentration and testis mass in male short-beaked common dolphins. (A) BT as a function of ordinal date for immature, pubertal, and sexually mature males that constitute the reference collection. (B) BT as a function of ordinal date in biopsied free-swimming males. (C) Average right testis mass of stranded and fishery bycatch "nonimmature" males (*i.e.*, those with right testis mass >100 g). Small circle markers indicate mean mass, and bars represent standard error of the mean. Sample size is indicated above or below each marker. Panel C includes additional specimens ($n = 109$) not represented in the reference collection (those for which no blubber was collected).

$P = 0.006$) and immature animals ($q = 9.06$, $P < 0.0001$). The mean concentrations (Table 1) in mature animals (14.4 ng/g) were more than five times higher than in pubertal (2.5 ng/g) males and almost seven times higher than in immature (2.2 ng/g) males. BT levels in pubertal and immature males were not significantly different ($q = 0.722$, $P = 0.98$).

Testosterone concentrations varied by season only in mature males. Summer concentrations in these animals were much higher ($q = 12.86$, $P < 0.0001$) than those from fall and winter (Table 1, Fig. 3A). This pattern, which is likely due to physiological changes associated with seasonal breeding, results in a period during the summer in which there is no overlap in BT concentration between mature and nonmature (immature and pubertal) males.

Right testis mass ranged from 1.6 to 107 g in immature, from 45.3 to 390 g in pubertal, and from 191 to 1,418 g in mature males. A significant positive correlation ($\rho = 0.77$, $P < 0.0001$) was found between testis mass and BT concentration across maturity states (Fig. 4). BT concentration increased exponentially with increasing

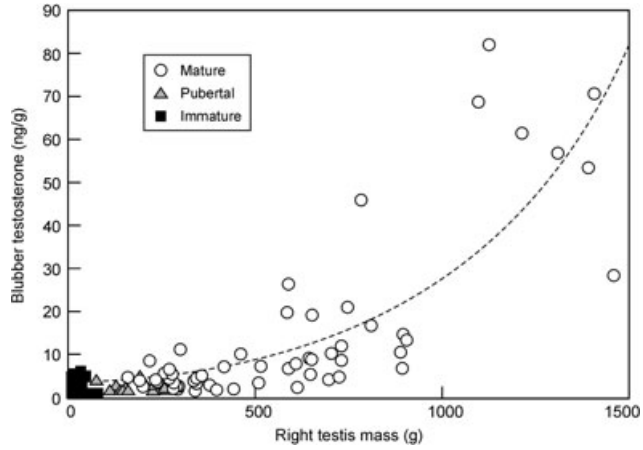

Figure 4. Blubber testosterone concentrations as a function of right testis mass for immature, pubertal, and mature male short-beaked common dolphins. A significant positive correlation ($\rho = 0.77$, $P < 0.0001$) was found between testis mass and blubber testosterone concentration across maturity states.

testis mass according to the following relationship:

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

Bootstrap 95% confidence intervals (CI) were 1.64–4.06 and 0.0019–0.0028, respectively, as determined by least-squares regression analysis.

We found no significant differences in BT concentration at different sampling depths relative to the epidermis (Fig. 5); the median concentrations ranged from 4.48 ng/g in the outer layer to 5.7 ng/g in the middle layer ($F = 2.09$, $P = 0.146$).

When examining anatomical sites, the only statistically significant differences in BT concentration were found between the dorsal fin sample and 7 of the 8 more ventral samples ($F = 5.39$, $P = 0.043$; Fig. 6); the exception being the posterior peduncle ($P = 0.31$). On average, the concentrations from the dorsal fin were 28.6% lower than those in the region in which 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 $r = 0.21$, $P = 0.21$.

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 ($\chi^2 = 0.86$, $P = 0.82$). However, it should be noted that resampling was possible, and no genetic assays were conducted to confirm individual status.

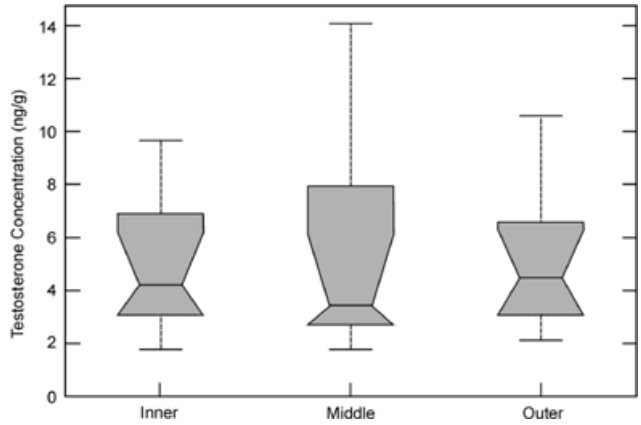

Figure 5. Blubber testosterone concentrations for inner, middle, and outer blubber layers. The concentrations were quantified from seven mature *D. delphis*. Horizontal box lines represent the lower quartile, median, and upper quartile values. Whisker lines indicate range of concentrations, and the plusses represent outliers (1.5 times interquartile range). Points of inflection represent upper bound to the 95% confidence interval (CI). 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.

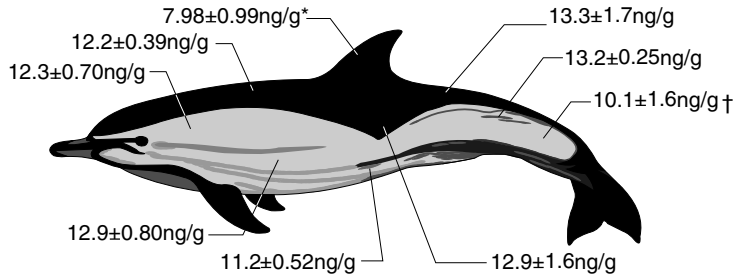

Figure 6. Mean (±SEM) BT concentrations at nine anatomical sites in three mature male *D. delphis*. There were significantly lower concentrations found in the dorsal fin (*) compared with the rest of the sampling locations except the caudal tail stock (†).

The biopsied male dolphins exhibited a range of BT concentrations that were similar to those in the fishery-killed animals (Fig. 3B). The mean concentration for the biopsies was 5.4 ng/g, with a range between 0.01 and 63.9 ng/g.

In addition, both groups exhibited similar seasonal patterns in these values. The highest 10% of the BT concentrations were from samples collected during the period from June to mid September (ordinal dates 150–260). In June, July, and early August, the concentration frequency distribution is decidedly bimodal, with no value occurring between 15 and 20 ng/g. The lower-concentration mode is centered at 1.8 ng/g and likely composed of immature and pubertal animals. The higher peak is centered at 31.1 ng/g and presumably associated with reproductively mature males.

Discussion

Given the uneven sampling distribution of the reference samples relative to ordinal date, there are periods for which the sample size is quite low (*e.g.*, spring $n = 4$). This uneven sampling limits the strength of some results, especially those characterizing the timing of testosterone production. The following is our best interpretation of the results with this proviso.

To our knowledge, this is the first study to quantify testosterone in cetacean blubber and the first to describe its variation with respect to maturity state, season, and anatomical location.

Similar to studies that have examined testosterone in serum samples, we confirmed that testosterone levels in the blubber of mature males were significantly higher than those in nonmature (immature and pubertal) males. We found an almost six-fold difference in BT concentrations in mature males *vs.* nonmature males when BT values were averaged throughout the year. The greatest differences occurred in the summer when mature males had more than 20 times higher BT concentrations than nonmature males. This result is similar to testosterone differences reported by Kirby (1990), who found a 10:1 (mature:nonmature) ratio of testosterone concentration in the serum of *T. truncatus*. However, in this study, there were no significant differences between pubertal and immature males, and there was substantial overlap between all maturity states. These results suggest that although there is likely an increase in testosterone concentration with sexual maturation, it is small compared with the seasonal testosterone fluctuations associated with sperm production in mature males.

The data from both the biopsies and reference collection indicate that the short-beaked common dolphins sampled in this study are seasonally reproductive, showing a pronounced elevation of BT concentrations during the summer and early fall. We observed higher BT concentrations starting in May (in biopsies) though September (in biopsies and references samples). Larger average testis size found in the reference dolphins during these months complement this finding (Fig. 3C). However, the specific timing of heightened reproductive activity cannot be precisely estimated because (1) the dynamics of testosterone in the blubber are unknown, and (2) the relationship between BT and testis function has not been precisely delineated. For instance, it has been shown that in some captive cetaceans, there is a lag between testosterone elevation and maximum sperm production or conception (Schroeder and Keller 1989, Robeck *et al.* 2005). If the *D. delphis* that we investigated exhibit a similar lag, then the breeding season commencement and termination would occur later in the year than what 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 indicated 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 (or multiple populations or stocks within the region), 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 timed similarly to that which we observed, with highest calving frequency occurring in late spring to early summer and peak conception in late summer (Perryman and Lynn 1993, Ferrero and Walker 1995, Murphy *et al.* 2005, Westgate and Read 2007).

Important for biopsy sampling, we found that BT concentration varies significantly across different anatomical sampling sites. In our study, the samples taken from the dorsal fin had significantly lower concentrations of testosterone than those collected from other sites. This result is possibly due to the relatively low levels of lipid per mass of sample in the dorsal fin relative to other body sites. Thus, if mature males were sampled in the dorsal fin, they would be more likely to be misclassified as nonmature, which could lead to an underestimate of the proportion of mature animals sampled. Because we found relatively low concentrations of testosterone in the dorsal fin tissue, we recommend eliminating such samples from the analysis. Thus, when biopsing, the anatomical site of the sample should be noted whenever possible. In the laboratory, most dorsal fin tissue can be readily identified by its durable elasticity when probed with forceps. 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 the differences in sampling location and increase the utility of this method.

A high-quality reference sample collection is necessary before it is possible to accurately interpret data collected from biopsies of wild populations, especially if the ultimate goal is to estimate the fraction of mature males within a population. The reference collection should contain specimens collected throughout the year to capture any seasonal changes in BT concentration (*i.e.*, those corresponding to mating). For instance, we are currently gathering additional specimens during spring and summer months to more precisely capture the annual rise of our dolphins' BT concentration. Once a robust reference collection is established, one can model the relationship between BT, maturity state, and season and estimate the proportion of mature males that are biopsied. Note that biopsy collection may be selective relative to male maturity state, and depending on the degree of this selectivity, the proportion of mature males that are biopsied may or may not be representative of the population itself. Currently, to our knowledge, there is no information regarding the selectivity of biopsing relative to maturity state.

Also important when using biopsy samples is to identify multiple samples from single individuals. This is most easily accomplished *via* an iterative or progressive genetic fingerprinting. In this process, sets of genetic markers are assayed in steps such that each additional marker set is only employed on sample pairs (or larger aggregates) that have shown identical genotypes (or near-identical to allow for typing errors) in proceeding assays. The process continues until all samples have been shown to be unique or the probability of identity is acceptable (which can then be included in the final demographic estimate). This process can minimize the additional labor and expense of acquiring complete genetic genotypes for every sample.

Another important caveat is that it is unknown how this approach will work for nonseasonal reproducers. Their BT may stay substantially elevated in individual mature males throughout the year such that no matter when they were sampled, maturity status could be determined. Or it may be that at any one time, only a fraction of the mature males are producing high levels of testosterone, in which case, this approach would have markedly less utility. Or it may be neither of these. In any case, whether your population/species of interest is a seasonal or nonseasonal

reproducer, again it is very important to have a robust inclusive reference collection. These samples from animals of known maturity state will help not only characterize the expected BT concentration related to each maturity condition but also elucidate the seasonal dynamics of testosterone production.

When interpreting testosterone concentrations of any kind, one should consider that testosterone levels could be affected by physiological and social conditions other than those associated with sexual maturation and sperm production. Increases in steroid stress hormones (glucocorticoids) are correlated with higher concentrations of androgens (including testosterone) in cetaceans (Hunt *et al.* 2006), perhaps as part of the generalized stress response. Although in other mammals this relationship appears to be more complex (Bubenik *et al.* 1999, Apfelbach *et al.* 2005), it is found that endocrine stress response affects testosterone concentration in many cases. In addition, there are a number of other factors that influence testosterone concentrations, including social status, limited resources, and nutritional state (Bubenik *et al.* 1999, Pineda 2003, Apfelbach *et al.* 2005, Robinson and Kruuk 2007).

Although there are several obstacles to the measurement and interpretation of cetacean BT concentration, it appears to have potential management utility. The approach delineated here uses samples that are relatively easy to obtain and are already being obtained from numerous cetacean species for many different types of studies. Although the substantial overlap found in BT between mature and nonmature males during the nonbreeding season suggests that BT by itself is not consistently effective for the maturity diagnosis of all sampled individuals, it can provide information regarding the seasonal reproductive behavior of cetaceans, highlighting periods in which animals may be more sensitive to anthropogenic disturbance. Especially with additional reference samples and further elucidation of the relationship between maturity states as a function of season, this approach could also be used to estimate the proportion of mature males within a population; an index that when combined with other vital rate information (*e.g.*, proportion pregnant *via* blubber progesterone) can be used to assess abundance status relative to carrying capacity. Given that there are very few nonlethal ways to obtain demographic information from cetaceans, measuring testosterone in biopsies may prove to be a valuable tool for future studies.

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