Marine Mammal Science

Notes

MARINE MAMMAL SCIENCE, **(*): ***–*** (*** 2014)

DOI: 10.1111/mms.12191

Seasonal blubber testosterone concentrations of male humpback whales (*Megaptera novaeangliae*)

Elizabeth T. Vu,[1] Scripps Institution of Oceanography, University of California San Diego, 9500 Gilman Drive La Jolla, California 92093, U.S.A.; **Casey Clark**, Moss Landing Marine Laboratories, 8272 Moss Landing Road Moss Landing, California 95039, U.S.A.; **Krista Catelani**, and **Nicholas M. Kellar**, Southwest Fisheries Science Center, NOAA, 8901 La Jolla Shores Drive, La Jolla, California 92037, U.S.A.; **John Calambokidis**, Cascadia Research Collective, 218½ 4th Avenue West, Olympia, Washington 98501, U.S.A.

The study of endocrine hormones has accelerated knowledge of marine mammal physiology, health, and reproduction (Hunt *et al.* 2013). Although blood is most commonly used in hormone-related research (*e.g.*, in humans and captive animals), few studies use blood serum for baleen whale research with the exception of postmortem samples (Kjeld *et al.* 1992, 2003, 2004; Fukui *et al.* 1996; Mogoe *et al.* 2000; Watanabe *et al*. 2004). Cetacean researchers have typically used other matrices—feces (Rolland *et al.* 2005, Hunt *et al.* 2006), respiratory samples (Hogg *et al.* 2009), cerumen (Trumble *et al.* 2013), baleen (Hunt *et al.* 2014), and blubber (Mansour *et al*. 2002, Kellar *et al.* 2013*b*)—to study baleen whale endocrinology. Novel applications of techniques used in measuring reproductive hormones in the blubber of free-ranging cetaceans has allowed for analyses of samples collected over wide spatial and time scales, thus providing information about population-wide reproductive status. For example, studies of progesterone concentrations in female odontocetes (Kellar *et al.* 2006, Trego *et al.* 2013) and testosterone concentrations in male delphinids (Kellar *et al.* 2009) have shown significant differences in hormone concentrations between pregnant and nonpregnant females and mature and immature males. Similarly, Mansour *et al.* (2002) and Kellar *et al.* (2013*a*) were able to quantify significant progesterone values in pregnant minke and bowhead whales, respectively. There are no published studies of blubber testosterone concentrations in male baleen whales to date.

A historic study from the commercial whaling era showed that Southern Hemisphere humpback whales (*Megaptera novaeangliae*) had a seasonal peak in testes

[1]Corresponding author (e-mail: jeepurs@gmail.com).

size from July to October that corresponded with the austral breeding season (Chittleborough 1955). Testes size in whales of the corresponding austral feeding season was reported to be one-third smaller and lacked spermatozoa. It is not known whether testosterone concentrations mirror these seasonal changes in testes size. To investigate seasonality in hormone concentrations, we measured blubber testosterone concentrations in 35 opportunistic biopsy samples from male North Pacific humpback whales over several seasons in different habitats (Fig. 1). We tested the expectation that the highest concentration of testosterone occurred during their breeding season, the lowest concentrations during their feeding season, and intermediate values during the fall shoulder season. We also investigated the change in testosterone concentration throughout the year.

To cover several seasons, we selected blubber biopsy samples from a large archive of tissue samples collected by Cascadia Research Collective as part of the SPLASH[2] (Structure of Populations, Levels of Abundance, and Status of Humpbacks) project of 2004–2006 (Barlow *et al.* 2011). Twelve blubber samples were selected for analysis: four samples from the winter breeding season (January–March) collected off Central America, four samples from the summer feeding season (May–September), and four samples from the fall shoulder season (October–November) collected off Washington and California. Past photo-identification and genetics have demonstrated the migration of humpback whales between Central America wintering areas and summer feeding areas off the west coast of the United States (Baker *et al.* 2013). An additional 23 samples from a summer field season conducted in Monterey Bay in 2011 were also analyzed. To investigate individual variation, we selected biopsies that were collected from the same individual based on photo-identification in different locations and times of the year. In the archive, we were able to find three individuals who were each sampled twice during different seasons for a total of six samples.

The hormonal extraction and measurement methods were modified from those described in Kellar *et al.* (2006). Frozen tissues samples with at least 150 mg of blubber were subsampled and prepped for hormone extraction. Samples were homogenized in 1,400 μL 100% ethanol using an automated, multi-tube Omni Bead Ruptor (Omni International, Kennesaw, GA) and processed for six 45 s periods in 2 mL reinforced lysing tubes with 0.70 mm garnet beads (Omni International, Kennesaw, GA). The homogenates were mixed individually and transferred through three wash steps of 500 μL 100% ethanol. Two milliliters of ethanol:acetone (4:1) were added to the homogenate, then mixed and centrifuged. Supernatants were aspirated and evaporated. Volumes of acetonitrile and hexane were added and thoroughly vortexed, centrifuged, and evaporated, resulting in a final residue. Samples were then applied to the enzyme immunoassay (EIA) kit ADI-901-065 (Enzo Life Sciences, Farmingdale, NY) with a standard curve range between 1.95 and 2,000 pg/mL. The reported interassay coefficient of variation (CV) ranged from 0.093 to 0.146 and intra-assay CV ranged from 0.078 to 0.108 for the two assays run on 35 samples. Extraction efficiency was determined by spiking select subsamples from 0 to 5 ng according to Kellar *et al.* (2009). The resulting extraction efficiency rate was estimated as the percentage of testosterone recovered in the final quantification after

[2]For detailed information on the SPLASH project see Calambokidis, J., E. A. Falcone, T. J. Quinn, *et al.* 2008. SPLASH: Structure of populations, levels of abundance and status of humpback whales in the North Pacific. Final report for Contract AB133F-03-RP-00078 prepared by Cascadia Research for the U.S. Department of Commerce. 57 pp.

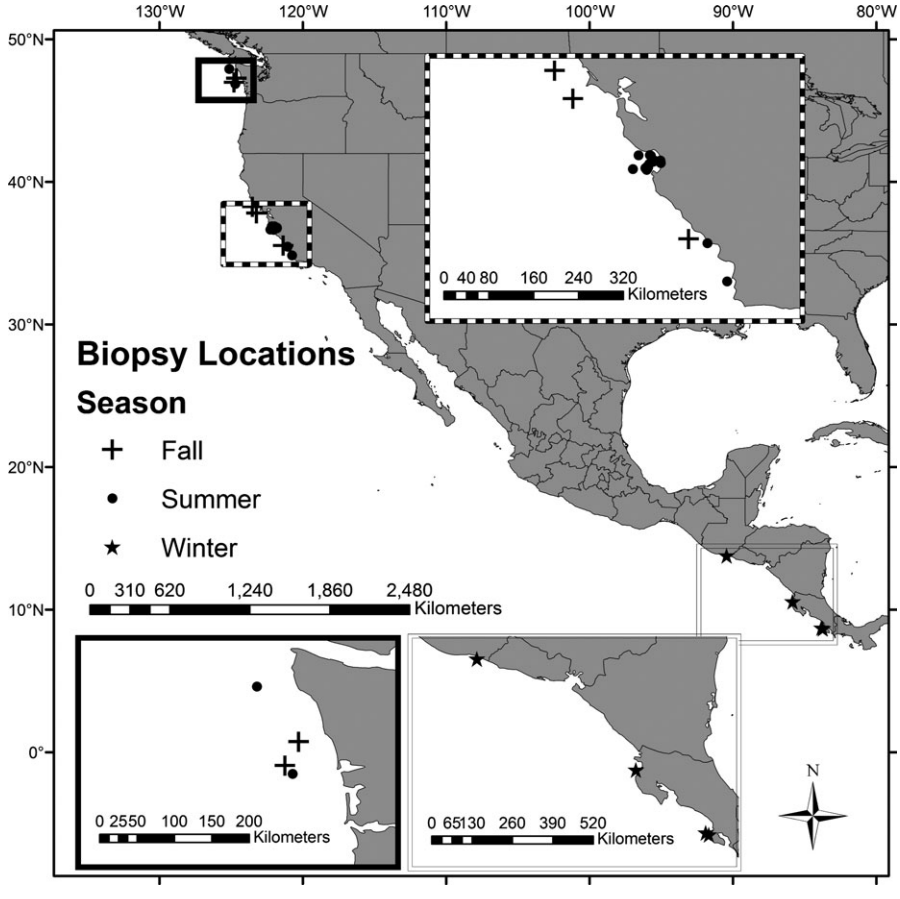

Figure 1. Humpback whale (*Megaptera novaeangliae*) biopsy sample locations during the winter, summer, and fall seasons from 2004 to 2011.

correcting for the intrinsic amount measured in the nonspiked samples. The averaged extraction efficiency across all extractions was 96%.

Due to the small sample size in our study, randomization tests were performed on the samples, testing the expectation that summer concentrations are different from winter and fall concentrations. Additionally, a polynomial regression was conducted to investigate how testosterone concentrations change over time. Male humpback whale testosterone concentrations were fit with a third-order polynomial regression, with Julian calendar day as a significant predictor ($r^2 = 0.672$, $P < .001$; Fig. 2). The value of 365 was added to "Julian day" only for winter samples in order to have the winter values occur directly after the fall values and thereby excluding the large gap in samples during spring. Randomization tests of testosterone concentrations calculated from permutated samples (with replacement, 1,000 times) revealed significantly lower summer values than the winter breeding and fall shoulder seasons (Table 1). Moreover, fall and winter concentrations represented the highest values of testosterone within this study with no difference between the two seasons. These significantly greater concentrations of testosterone in North Pacific humpback whale blubber

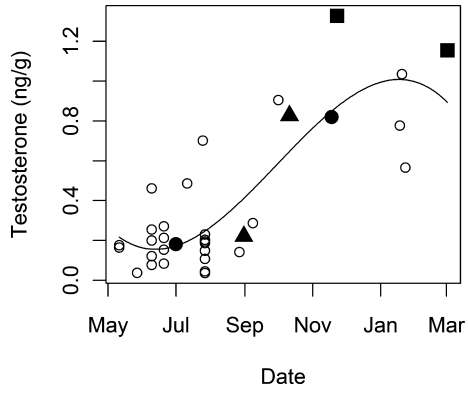

Figure 2. Testosterone extraction concentrations (ng/g) *vs.* time of year of biopsy collection (Julian day). The three matching symbols refer to three individuals who were each sampled twice during difference seasons of the year. The curve represents the polynomial fit: Testosterone = $-0.0000163x^3 + 0.00133x^2 - 0.0305x + 2.29$ where x represents the Julian calendar day. The value of 365 was added to the winter Julian day samples to have the winter values occur directly after the fall values and thereby excluding the large gap in samples during spring.

during the winter breeding season correspond with Chittleborough's (1955) findings of a seasonal peak in testes size during the austral breeding season. Taken in context with terrestrial studies of testosterone's known role in testis development and spermatogenesis (Dixson and Anderson 2004), the cooccurrence of peak testis size and testosterone concentration supports the existence of a positive feedback loop within the pituitary-gonadal axis as seen in other marine mammalian taxa (Atkinson 1997).

Increased mean testosterone concentrations in blubber observed during the shoulder fall season were greater than the expected. The expectation of intermediate testosterone values was based on Kjeld *et al.* (1992, 2003, 2004), who showed steady increasing blood testosterone concentration in fin (*Balaenoptera physalus*), minke (*Balaenoptera acutorostrata*), and sei (*Balaenoptera borealis*) whales as the season progressed from summer to fall. Elevated fall testosterone values support the idea that the physiological conditioning for reproductive behavior occurs prior to the breeding season

Table 1. *P*-values from randomization tests of humpback whale blubber testosterone concentrations over three seasons. Summer concentration values were significantly different from fall, winter, and pooled fall and winter samples. Fall and winter concentrations were not significantly different from each other.

	Number of samples	Mean (SD) ng/g	*P*-value		
			Winter	Summer	Fall
Winter	4	0.88 (0.26)	–	<0.001	0.72
Summer	27	0.21 (0.15)	–	–	–
Fall	4	0.97 (0.24)	–	<0.001	–
Fall + Winter	8	0.92 (0.24)	–	<0.001	–
All samples	35	0.37 (0.35)	–	–	–

and outside of the geographical breeding regions, likely as preparation for the coming breeding season.

Of note, the three individuals who were each sampled two times were sampled in different seasons (Fig. 2). One individual showed an increase in hormone values from summer to fall within the same year. Another individual showed a decrease in testosterone concentration from fall of 2004 to the summer of 2005. For the third individual, sampling from fall to winter showed a slight decrease in testosterone concentration. The results from these three multisampled individuals emphasize intra-individual variation while also reflecting the overall sample population's seasonal trend.

Age-class is particularly important to note due to the significant differences in testosterone concentrations between immature and mature males seen in killer whales (Robeck and Monfort 2006). Of the presumed 32 individual whales sampled in this study, 11 whales were not photographically identifiable. Of the 21 whales that were given a photo-identification number, eight were confirmed to be fully mature adults at the time of biopsy, determined from previous photo-id records spanning eight years. The whales that were not confirmed to be fully mature were at least juvenile, if not fully mature, males. No samples were taken from calves.

Despite the detectable seasonal trend, there are many factors which may contribute to the variability seen in this study. In addition to the aforementioned age-class and development stage of the individual males, other sources of variability include the location of the biopsy on the body of the animal as well as the depth of the blubber biopsy sample. Kellar *et al.* (2009) were able to investigate the relationship between biopsy depth, body site, and testosterone in odontocetes. They found no significant effect of biopsy depth on testosterone concentration. Additionally, they found significantly lower values of testosterone only in the dorsal fin and caudal tail regions. However, these analyses were done on bycaught odontocetes and have yet to be investigated for larger baleen whales. As biopsy effort increases and biopsy archives grow in size, researchers can select for samples which minimize these sources of variability. Thus, alternative analyses can be performed on larger samples sizes than what was possible for this study.

Our results have shown that the minimally invasive collection of biopsies can yield and detect testosterone concentrations from blubber that generally reflect what we expect from humpback whale seasonal reproductive physiology. We have also shown that elevated testosterone concentrations during the fall season while animals are still on their high latitude feeding areas are unexpected but support the notion that reproductive conditioning starts months before peak breeding time. In light of previous findings that singing, another conferred reproductive behavior, is not limited to breeding grounds (Norris *et al.* 1999, Clark and Clapham 2004, Vu *et al.* 2012), the hormonal patterns reported in this study may hold implications about breeding, singing behavior, or migration that we have yet to understand.

Acknowledgments

We would like to acknowledge John Hildebrand, Ana Sirovic, Sara Kerosky, Alyson Fleming, and conversations from the Southern California Marine Mammal Conference, from which the collaboration for this project was born. Support for the collection of samples from 2004 to 2006 came in part through support for the SPLASH project from the National Marine

Fisheries Service, Pacific Life, and Marisla Foundation. Paulina Godoy, Erin Falcone, Greg Schorr, and Jim Harvey assisted with the collection of samples under NMFS permits #15271, 540-1811, and 540-1502. Funding for the lab analyses was subsidized by the Kellar Lab of the Southwest Fisheries Science Center, Pacific Life, The Ocean Foundation, and the American Cetacean Society. This study was done while ETV was supported by the National Defense Science and Engineering Graduate Fellowship (NDSEG). We would like to thank three anonymous reviewers who greatly contributed to this study.

Literature Cited

Atkinson, S. 1997. Reproductive biology of seals. Reviews of Reproduction 2:175–194.

Baker, C. S., D. Steel, J. Calambokidis, *et al*. 2013. Strong maternal fidelity and natal philopatry shape genetic structure in North Pacific humpback whales. Marine Ecology Progress Series 494:291–306.

Barlow, J., J. Calambokidis, E. A. Falcone, *et al*. 2011. Humpback whale abundance in the North Pacific estimated by photographic capture-recapture with bias correction from simulation studies. Marine Mammal Science 27:793–818.

Chittleborough, R. G. 1955. Aspects of reproduction in the male humpback whale, *Megaptera nodosa* (Bonnaterre). Marine and Freshwater Research 6:1–29.

Clark, C. W., and P. J. Clapham. 2004. Acoustic monitoring on a humpback whale (*Megaptera novaeangliae*) feeding ground shows continual singing into late spring. Proceedings of the Royal Society of London 271:1051–1057.

Dixson, A. F., and M. J. Anderson. 2004. Sexual behavior, reproductive physiology and sperm competition in male mammals. Physiology and Behavior 83:361–371.

Fukui, Y., T. Mogoe, Y. G. Jung, *et al*. 1996. Relationships among morphological status, steroid hormones, and post-thawing viability of frozen spermatazoa of male minke whales (*Balaenoptera acutorostrata*). Marine Mammal Science 12:28–37.

Hogg, C. J., T. L. Rogers, A. Shorter, K. Barton, P. J. O. Miller and D. Nowacek. 2009. Determination of steroid hormones in whale blow: It is possible. Marine Mammal Science 25:605–618.

Hunt, K. E., R. M. Rolland, S. D. Kraus and S. K. Wasser. 2006. Analysis of fecal glucocorticoids in the North Atlantic right whale (*Eubalaena glacialis*). General and Comparative Endocrinology 148:260–272.

Hunt, K. E., M. J. Moore and R. M. Rolland, *et al*. 2013. Overcoming the challenges of studying conservation physiology in large whales: A review of available methods. Conservation Physiology 1:cot006.

Hunt, K. E., R. Stimmelmayr, C. George, C. Hanns, R. Suydam, H. Brower and R. M. Rolland. 2014. Baleen hormones: A novel tool for retrospective assessment of stress and reproduction in bowhead whales (*Balaena mysticetus*). Conservation Physiology 2:cou030.

Kellar, N. M., M. L. Trego, C. I. Marks and A. E. Dizon. 2006. Determining pregnancy from blubber in three species of delphinids. Marine Mammal Science 22:1–16.

Kellar, N. M., M. L. Trego, C. I. Marks, S. J. Chivers, K. Danil and F. I. Archer. 2009. Blubber testosterone: A potential marker of male reproductive status in short-beaked common dolphins. Marine Mammal Science 25:507–522.

Kellar, N. M., J. Keliher, M. L. Trego, K. N. Catelani, C. Hanns, J. C. C. George and C. Rosa. 2013*a*. Variation of bowhead whale progesterone concentrations across demographic groups and sample matrices. Endangered Species Research 22:61–72.

Kellar, N. M., M. L. Trego, S. J. Chivers and F. I. Archer. 2013*b*. Pregnancy patterns of pantropical spotted dolphins (*Stenella attenuata*) in the eastern tropical Pacific determined from hormonal analysis of blubber biopsies and correlations with the purse-seine tuna fishery. Marine Biology 160:3113–3124.

Kjeld, M., J. Sigurjonsson and A. Arnason. 1992. Sex hormone concentrations in blood serum from the North Atlantic fin whale (*Balaenoptera physalus*). Journal of Endocrinology 134:405–413.

Kjeld, M., G. A. Vikingsson, A. Alfredsson, O. Olafsson and A. Arnason. 2003. Sex hormone concentrations in the blood of sei whales (*Balaenoptera borealis*) off Iceland. Journal of Cetacean Research and Management 5:233–240.

Kjeld, M., A. Alfredsson, O. Olafsson, M. Tryland, I. Christensen, S. Stuen and A. Arnason. 2004. Changes in blood testosterone and progesterone concentrations of the North Atlantic minke whale (*Balaenoptera acutorostrata*) during the feeding season. Canadian Journal of Fisheries and Aquatic Sciences 61:230–237.

Mogoe, T., T. Suzuki, M. Asada, Y. Fukui, H. Ishikawa and S. Oshumi. 2000. Functional reduction of the southern minke whale (*Balaenoptera acutorostrata*) testis during feeding season. Marine Mammal Science 16:559–569.

Mansour, A. A., D. W. Mkay, J. Lien, J. C. Orr, J. H. Banoub, N. Olen and G. Stenson. 2002. Determination of pregnancy status from blubber samples in minke whales (*Balaenoptera acutorostrata*). Marine Mammal Science 18:112–120.

Norris, T. F., M. McDonald and J. Barlow. 1999. Acoustic detections of singing humpback whales (*Megaptera novaeangliae*) in the eastern North Pacific during their northbound migration. Journal of the Acoustical Society of America 106:506–514.

Robeck, T. R. and S. L. Monford. 2006. Characterization of male killer whale (*Orcinus orca*) sexual maturation and reproductive seasonality. Theriogenology 66:242–250.

Rolland, R. M., K. E. Hunt, S. D. Kraus and S. K. Wasser. 2005. Assessing reproductive status of right whales (*Eubalaena glacialis*) using fecal hormone metabolites. General and Comparative Endocrinology 142:308–317.

Trego, M. L., N. M. Kellar and K. Danil. 2013. Validation of Blubber progesterone concentrations for pregnancy determination in three dolphin species and a porpoise. PLOS ONE 8(7):e69709.

Trumble, S. J., E. M. Robinson, M. Berman-Kowalewski, C. W. Potter and S. Usenko. 2013. Blue whale earplug reveals lifetime contaminant exposure and hormone profiles. Proceedings of the National Academy of Sciences of the United States of America 110:16922–16926.

Vu, E. T., D. Risch, C. W. Clark, *et al*. 2012. Humpback whale (*Megaptera novaeangliae*) song occurs extensively on feeding grounds in the western North Atlantic Ocean. Aquatic Biology 14:175–183.

Watanabe, H., T. Mogoe, M. Asada, *et al*. 2004. Relationship between serum sex hormone concentrations and histology of seminiferous tubules of captured baleen whales in the western North Pacific during the feeding season. Journal of Reproduction and Development 50:419–427.

Received: 28 April 2014
Accepted: 29 October 2014