

Fall 2013

# Intra-annual changes in population parameters as indicators of humpback whale (*Megaptera novaeangliae*) migratory behavior

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INTRA-ANNUAL CHANGES IN POPULATION PARAMETERS AS INDICATORS  
OF HUMPBACK WHALE (*MEGAPTERA NOVAEANGLIAE*) MIGRATORY  
BEHAVIOR

A Thesis

Presented to

The Faculty of Moss Landing Marine Laboratories

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Casey Clark

December 2013

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The Designated Thesis Committee Approves the Thesis Titled

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by

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## ABSTRACT

### INTRA-ANNUAL CHANGES IN POPULATION PARAMETERS AS INDICATORS OF HUMPBACK WHALE (*MEGAPTERA NOVAEANGLIAE*) MIGRATORY BEHAVIOR

by Casey Clark

Many techniques used to study animal migrations rely on observations of specific animals, which provide valuable information about individuals studied but do not capture population variability. By examining changes in population parameters, researchers may gain a better understanding of migratory behaviors. In this study, intra-annual changes in population parameters were used to study migratory behaviors of humpback whales off central California in 2011/2012. Data were compared with a historic dataset from 2004/2005. Parameters measured included sex ratio, pregnancy rate, mitochondrial DNA haplotype frequencies and mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values. Weighted moving averages of the sex ratio were moderately effective at revealing deviations from expected values. Progesterone assays successfully determined pregnancy in humpbacks and revealed a previously undocumented intra-annual decrease in pregnancy rate in 2011. Analyses of mitochondrial DNA haplotype frequencies indicated greater prevalence of haplotypes associated with British Columbia and Washington late in the year; however, the origin of these animals was unclear. Stable isotope ratios proved ineffective for measuring a fasting effect in humpbacks early in the year. The effectiveness of these parameters for investigating migratory behaviors varied, but used in conjunction with traditional methods of study, they may help create a broader understanding of animal migrations.

## ACKNOWLEDGEMENTS

This project would not have been possible without the support of a multitude of helpful people and organizations. I am especially grateful for the guidance and mentoring of my advisor, Dr. Jim Harvey. His instruction and experience have helped to shape me into a more thoughtful scientist. I would also like to thank the members of my committee, Dr. Leslee Parr and Dr. Phil Clapham, for the time and energy they put into my thesis. My special thanks go to John Calambokidis, who helped shape this project from its inception. The complete list of those who helped is too long to include here, but the following people were vital to the completion of my thesis: Dr. Alyson Fleming, Liz Mchuron, Stephanie Hughes, Deasy Lontoh, Mike Johns, Alex Olson, Drew Burrier, Katie Schmidt, John Douglas, Liza Schmidt, Alexis Howard, Ben Weitzman, Traci Kendall, Dr. Nick Kellar, Dr. Scott Baker, Dr. Karin Forney and Dr. Bree Witteveen. Finally, I would like to thank my family for making it possible for me to come this far.

Thanks to the Ocean Foundation and Pacific Life for providing the bulk of the funding for this project. I would also like to thank the Monterey Bay Chapter of the American Cetacean Society, the Dr. Earl and Ethel Myers Oceanographic and Marine Biology Trust, the CSU COAST program, the Dierks-Morgan Scholarship, the Archimedes Scholarship, the Sonia Linnik Hamilton Scholarship and the Captain Lee Bradford Memorial Scholarship for providing the remainder of the financial support. The following organizations were critically important to the completion of this project: Moss Landing Marine Laboratories, Cascadia Research, National Marine Fisheries Service Southwest Fisheries Science Center, as well as the NMFS Office of Protected Resources.

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## INTRODUCTION

Species from every major branch of the animal kingdom migrate. Dingle and Drake (2007) defined migration as “a seasonal to-and-fro movement of populations between regions where conditions are alternately favorable or unfavorable (including one region where breeding occurs).” For animals with a great degree of mobility, these seasonal movements allow the exploitation of food in polar, sub-polar, or temperate areas during summer and subsequent movement during winter to areas with more hospitable climates for reproduction, including sub-tropical and tropical areas (Newton 2003, Calvert *et al.* 2009).

Research on animal migrations typically relies on observations of the movements of specific animals. Individually identifying characteristics and artificially attached identifiers (such as leg bands in birds) allow researchers to apply capture-recapture methodologies and connect separate sightings of individual animals (Seber 1982, Nichols 1992). The deployment of telemetric tags, using short-range very high frequency (VHF) and satellite-based long-range ultra high frequency (UHF) radio transmissions, allows researchers to track fine-scale animal movements (LeMunyan *et al.* 1959, Kenward 1987, Fancy *et al.* 1988). Although these methods are extremely valuable for gaining a detailed understanding of the behavior of specific animals, they are unlikely to provide results that encompass the great degree of variability within animal populations. Only through repeated observations of many individual animals will these methods begin to accurately describe populations. Use of such techniques may be feasible for relatively inexpensive methods like photo-identification, but such repetition represents an impractical goal for

telemetric tagging, which is often costly, labor intensive and may be stressful for the tagged animals.

Situations where traditional methods fail to accurately capture the migratory behaviors of an animal population warrant a different approach. Instead, it may be possible to study the migratory behaviors of a population by measuring seasonal changes in the demographic and physiological parameters of a population. Such investigations provide minimal information about individual movements and behaviors but have the advantage of providing a larger-scale description of migratory behaviors of a population of animals.

The humpback whale (*Megaptera novaeangliae*) is an example of an animal exhibiting migratory behaviors that have not been accurately described by traditional methods. Studying the migration of marine mammals is challenging, as these animals live in an environment that is not easily accessible and that hinders use of many methods commonly used to study terrestrial migrations. This is particularly true for cetaceans, which spend the majority of their lives underwater. Thus, it is difficult to procure repeated observations of individual animals and the challenges of deploying long-term telemetric tags are greatly increased. For these animals, changes in population parameters may provide a useful alternative to traditional methods of study.

Humpback whales undertake the longest migration of any marine mammal, with some individuals migrating more than 16,000 km/yr (Rasmussen *et al.* 2007). These whales inhabit every ocean basin (Clapham 1996), spending summers in temperate or near-polar waters and migrating to low-latitude areas in the winter for breeding

(Chittleborough 1965, Mackintosh 1965, Dawbin 1966). It is assumed that all humpback whales within a population migrate annually; however, there is evidence that this may not be true. Living and stranded (dead) humpback whales have been regularly observed in feeding areas during winter, when the population is expected to be in the breeding area (Ingebrigtsen *et al.* 1929, Williamson 1961, Winn 1982, Straley 1990, Christensen *et al.* 1992, Clapham *et al.* 1993, Swingle *et al.* 1993, Wiley *et al.* 1995). Researchers have documented strongly male-biased sex ratios in humpback breeding areas (Chittleborough 1965, Calambokidis *et al.* 2008), a surprising finding, given that humpback whales have an approximate 1:1 sex ratio at birth and in the feeding areas (Chittleborough 1965). During migration, Brown *et al.* (1995) found 2.4 males for each female sampled. These researchers explained the apparent overabundance of migrating males in their study by suggesting that many females forego the annual migration.

For humpback whales, migration to the breeding area carries a substantial energetic cost, primarily because they do not feed during the migration or in the breeding area (Nishiwaki 1959, Corkeron and Connor 1999). This investment is especially great for females, which incur additional energetic expenditures during pregnancy, birthing and lactation. Female baleen whales may expend as much as 25% of their annual energy budget during migration (Lockyer 1981). The costs of reproduction and migration are likely greater for humpbacks, which lactate for 3 months longer than many other whale species (10.5 to 11 months; Chittleborough 1958, Lockyer 1984).

The great energetic costs of migration create a clear trade-off between reproduction and maintenance of body condition for female whales. These animals often rest for one or

two years between successive pregnancies (Chittleborough 1965, Dawbin 1966), presumably using this period to improve their body condition before future reproduction. For non-reproductive females, the costs of migration likely outweigh the benefits and they may remain in the feeding area through autumn, winter and early spring (hereafter referred to as the “late season”). Though prey biomass decreases during the late season (Marinovic *et al.* 1984), reduced competition with other whales would increase prey availability for animals in the feeding area during this period.

By examining seasonal changes in the demographic and physiological parameters of humpback whale populations, it may be possible to learn more about their migratory behaviors and to test the hypothesis that some animals forego the annual migration to the breeding area. Changes in the observed sex ratio and pregnancy rate of a humpback population would provide insight into which segments of the population are present in the feeding area at different times of the year. Similarly, examinations of the genetic population structure would allow for investigations of temporal changes of foraging habitat use by different, related groups of whales, or to detect the presence of migrants from other populations. Finally, investigations of intra-annual changes in the stable isotope ratios of these whales may reveal important information about fasting and foraging behaviors and provide insight into the factors driving migratory movements.

Ideally, I would have drawn direct comparisons between observations made in the feeding season and in the late season; however, inclement weather and lesser whale densities made late season studies of humpbacks in the feeding area impractical. The same questions may instead be addressed by focusing on the migratory periods at the beginning and end of

the feeding season. Examining changes in the sex ratio during the migratory periods is; however, more complicated than a simple comparison of sex ratios during the late and feeding seasons. Migration in humpback whales is segregated by age, sex and reproductive status (Craig *et al.* 2003). Female humpback whales in the late-stages of pregnancy are the last to arrive in the breeding area (Nishiwaki 1959, Dawbin 1966, Dawbin 1997, Craig *et al.* 2003). This contrasts with gray whales, where pregnant females return to the breeding area first (Rice and Wolman 1971). The difference in migratory behaviors is likely due in part to the energetic costs associated with extended lactation in humpback whales. Humpbacks in the late stages of pregnancy may extend their stay in the feeding area to take advantage of reduced intra-specific competition for prey and build important energy stores. Thus, it would be important when examining changes in the sex ratio during the migratory periods to understand the expected fluctuations in pregnancy rate given this segregated migratory timing and to compare the pregnancy rate of female humpbacks observed in the feeding area late in the year to those encountered earlier in the feeding season.

The later portion of the feeding season may represent an especially valuable time for energy acquisition due to shifts in prey abundance and availability. Some species of schooling fish, especially Pacific sardines (*Sardinops sagax*) and northern anchovy (*Engraulis mordax*), are most abundant in nearshore waters during this timeframe (Monterey commercial landing data from Southwest Fisheries Science Center, NMFS). These fish are rich in energy compared with invertebrate prey such as krill and provide a valuable prey resource for foraging humpback whales (Davis *et al.* 1998, Anthony *et al.* 2000).

The prevalence of such shifts in the diet of these whales can be investigated by examining the stable nitrogen and carbon isotopic ratios in the whales' skin. Stable isotope analysis is a powerful tool for understanding dietary patterns, energy flow and trophic relationships within ecosystems. Carbon and nitrogen isotopes, in particular, can help researchers determine an animal's foraging behavior, geographically, temporally and trophically (Hobson 1999). This method provides diet information that is integrated over a period of time, unlike studies of stomach contents, which only provide information about what an animal was eating at the time it was collected (Teiszen *et al.* 1983). The ratio of the heavy  $^{13}\text{C}$  isotope to the more common  $^{12}\text{C}$  (hereafter referred to as  $\delta^{13}\text{C}$ ) varies geographically and is commonly examined to provide information about the area in which an animal has been foraging. The ratio of  $^{15}\text{N}$  to  $^{14}\text{N}$  (hereafter referred to as  $\delta^{15}\text{N}$ ) increases with increasing trophic level and thus reflects the relative trophic position of the animal. Investigations of intra-annual changes in carbon and nitrogen stable isotope ratios might help test the hypothesis that animals remaining in the feeding area during the later portion of the feeding season are foraging on higher trophic level prey than earlier in the year.

Stable isotopes have the potential to provide insight into fasting by humpback whales. Like many other migratory animals, humpback whales undergo extended periods of fasting and are forced to rely on stored energy reserves to survive. Fasting animals often exhibit increased stable nitrogen isotope ratios, as they are essentially feeding on their own tissues, thus they appear to occupy a higher trophic position (Hobson *et al.* 1993). Although it has been widely demonstrated in other species, this fasting effect has

not been directly investigated in humpback whales. If a measurable fasting effect does exist for these animals, it could present a source of error for past and future isotopic diet studies of these whales. Additionally, it would provide an opportunity to investigate whether some individual whales feed during the late season, as these whales would be expected to have a lower proportion of  $^{15}\text{N}$  in their tissues than whales undergoing fasting. Detections of non-fasting animals might indicate the presence of non-migrating animals and would at least challenge the suggestion that all humpbacks fast during the breeding season. Stable carbon isotope values might then help to determine the region in which these animals had been feeding, providing further information about their migratory movements.

The California-Oregon (CA-OR) feeding area is unique in the North Pacific in that it lies on the migratory route of animals from another feeding herd. The southern British Columbia-Washington (SBC-WA) feeding area is located immediately north of CA-OR. Animals that forage in SBC-WA breed primarily in the waters off Mexico and central America (Calambokidis *et al.* 2008). Thus, humpbacks belonging to the SBC-WA feeding herd are likely to migrate through CA-OR early and late in the year. It may be possible to detect these northerly migrants by examining the frequency of mitochondrial DNA (mtDNA) haplotypes. The feeding herds in the North Pacific are defined by geographic variation in genetic population structure (Baker *et al.* 1998, Baker *et al.* In Press) thus there are substantial differences in the haplotype frequencies of whales from different feeding herds. Increased prevalence of haplotypes more commonly



associated with SBC-WA than with CA-OR during the migratory periods might indicate the presence of northerly migrants traveling through CA-OR at these times.

The objectives of this study were to: 1) measure the sex ratio of humpback whales in Monterey Bay and to detect any consistent intra-annual trends in this ratio, 2) measure the pregnancy rate of female humpback whales within Monterey Bay and examine intra-annual variability in pregnancy rate, 3) examine intra-annual variability in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values and to investigate the existence of a fasting signal in the early portion of the feeding season, 4) investigate the presence of animals from more northerly feeding areas migrating through the California-Oregon (CA-OR) feeding area and 5) test whether data collected from whales within the Monterey Bay region were representative of the entire CA-OR feeding area.

I hypothesized that the overall sex ratio of humpback whales in Monterey Bay would not differ significantly from parity (1:1, M:F) and that this sex ratio would trend significantly towards a female majority during the migratory periods. I hypothesized that the pregnancy rate of humpbacks in Monterey Bay would be significantly greater than previous reproductive estimates calculated from abundance of calves off CA-OR,  $4.1\% \pm 1.8\%$  (mean  $\pm$  SD, Steiger and Calambokidis 2000) and that pregnancy rate would be greatest at the end of the feeding season. I hypothesized that a fasting signal would be detectable early in the feeding season, characterized both by a significant linear relationship with Day of Year and greater variability in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values in the early part of the year. Additionally, I hypothesized that intra-annual variability in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  would remain consistent across years, with more depleted values earlier in the year

and more enriched values toward the end of the feeding season. I hypothesized that mtDNA haplotypes that are rare in CA-OR, but common in SBC-WA would be more frequently encountered early and late in the year than during the middle of the feeding season, reflecting the seasonal migration of animals from the SBC-WA feeding herd through CA-OR. Finally, I hypothesized that stable isotope ratios, the sex ratio and mtDNA information collected within central California would be representative of the greater California-Oregon feeding area.

## METHODS

Within the California-Oregon feeding area, my study area encompassed the coastal waters off central California, between Point Sur (36.31 N, -121.90 W) and Point Reyes (38.00 N, -123.00 W). This region includes Monterey Bay and the Gulf of the Farallones and serves as an important foraging area for humpback whales. This region is located in a highly productive upwelling system (Pennington and Chavez 2000). During spring and summer, southerly winds drive coastal upwelling, bringing cold, nutrient-rich water to the surface (Hickey 1979). This system is highly productive and supports large populations of grazers such as pelagic zooplankton and fish (Marinovic *et al.* 2002). These grazers include krill of the genera *Euphausia* and *Thysanoessa*, Pacific herring (*Clupea harengus pallasii*) and northern anchovy (*Engraulis mordax*) (Cailliet *et al.* 1979, Marinovic *et al.* 2002), all of which are important prey for humpback whales (Clapham *et al.* 1997).

Humpbacks that feed off California and Oregon generally breed off Mexico and Central America, with a great degree of interchange occurring between these breeding areas (Calambokidis *et al.* 2008). This population contains 2,043 individuals (Carretta *et al.* 2010). Though the study area may not be large enough to be considered representative of the entire California-Oregon feeding area, the vagile nature of humpback whales means that individuals likely move throughout the feeding area within a season (J. Calambokidis, pers. corr.), increasing the probability that samples collected in central California were representative of the population. Historical data collected throughout the CA-OR feeding area were used to test the validity of this assumption.

Tissue samples and photographs were collected from a 5.8 meter *Zodiac Rigid Hull Inflatable Boat*. Skin and blubber biopsies were obtained from free-ranging whales using a *Barnett RX-150* crossbow and 25-mm Ceta-Dart tips (Ceta-Dart, Copenhagen, Denmark), following the protocol described by Lambertsen (1987). Photographs of the ventral surface of the flukes of individual humpbacks were collected for use in photo-identification using a Canon EOS 20D or Canon EOS 7D Digital SLR camera. All samples and photographs were collected under National Marine Fisheries Service (NMFS) Permit No. 15271, issued to Dr. James T. Harvey and San José State University Institutional Animal Care and Use Committee (IACUC) Protocol #937.

Skin and blubber biopsies ( $n = 131$ ) were collected from 128 individual whales during two field seasons: 64 from May-November 2011 and 67 from April-July 2012. Three whales were unintentionally biopsied twice within a given season, two in 2011 and one in 2012. Sampling was conducted opportunistically, with the exact timing and location of sampling determined by weather and anticipated whale abundance. After collection, tissue samples were stored in a  $-80\text{ }^{\circ}\text{C}$  freezer pending analysis.

All statistical analyses were conducted using IBM SPSS Statistics (version 20.0, IBM, 2011) and Arlequin (version 3.5, Excoffier and Lischer 2010). Unless otherwise stated, assumptions for parametric tests were met before analysis. All results are presented on untransformed data.

Photographs of the ventral surface of humpback whale flukes were sent to Cascadia Research (Olympia, WA) for identification and matching. Cascadia Research maintains a database of photo-ID and sighting history information for humpback whales

in the North Pacific. Fluke photographs collected in this study were compared with Cascadia's catalog. Previously identified whales were linked to their individual sighting histories, whereas animals that had not been previously photographed were given a unique identifier and added to the catalog. Fluke photographs and sighting histories were used to identify repeat samples of individual whales.

A dataset including sex, stable isotope and mtDNA data from humpback whales sampled off CA-OR in 2004 and 2005 was used to compare the results of this study with historic observations of animals in this region. Historic data were collected under NMFS Permits 540-1811 and 774-1714-03.

A 3-mm<sup>2</sup> subsample of skin from each biopsy was sent to the Cetacean Conservation Genetic Laboratory (CCGL) at the Marine Mammal Institute, Oregon State University, for analysis of mtDNA and sex identification. Multiplex amplification of the male-specific *Sry* gene and ZFY/ZFX positive were used to identify the sex of individual sampled whales (Gilson *et al.* 1998).

Sex ratios, defined as the ratio of males to females (M:F) in a population or group of samples, were calculated for the 2011 and 2012 sampling periods. Intra-annual trends in sex ratio were investigated by calculating the percentage of samples identified as female within a given sampling day and examining how these percentages varied with Day of Year. A weighted moving average was calculated using the method described by Krebs (1999), with number of samples as the weighting factor. When generating the weighted moving average, the optimal time-span was selected such that largest possible number of samples was used to generate the statistic, while still allowing fine-scale

changes in the average to be observed. Calculations of the moving averages were conducted such that the average was always generated from at least two sampling days. For the 2011/2012 data, the moving average spanned a 53-day window. Weighted 95% confidence intervals were calculated for the weighted moving average (Krebs 1999). This process was repeated for data from the historic dataset (2004 and 2005). The weighted moving average for the 2004/2005 data spanned a 59-day window.

For the purposes of this study, the feeding season was defined as May through September. The expected sex ratio during the beginning, middle and end of the feeding season was calculated using information on sex-related differences in migratory timing from the literature (Craig *et al.* 2003). Mean dates of last identification in the breeding area were used to determine expected differences in timing of arrival in the feeding area for animals of different sex (male or female), age class (immature and mature) and reproductive status (pregnant or non-pregnant). Mean dates of first identification in the breeding area were used to determine expected differences in timing of departure from the feeding area for these same groups. The sex ratio was assumed 1:1 during the time between migratory periods (Fig. 1).

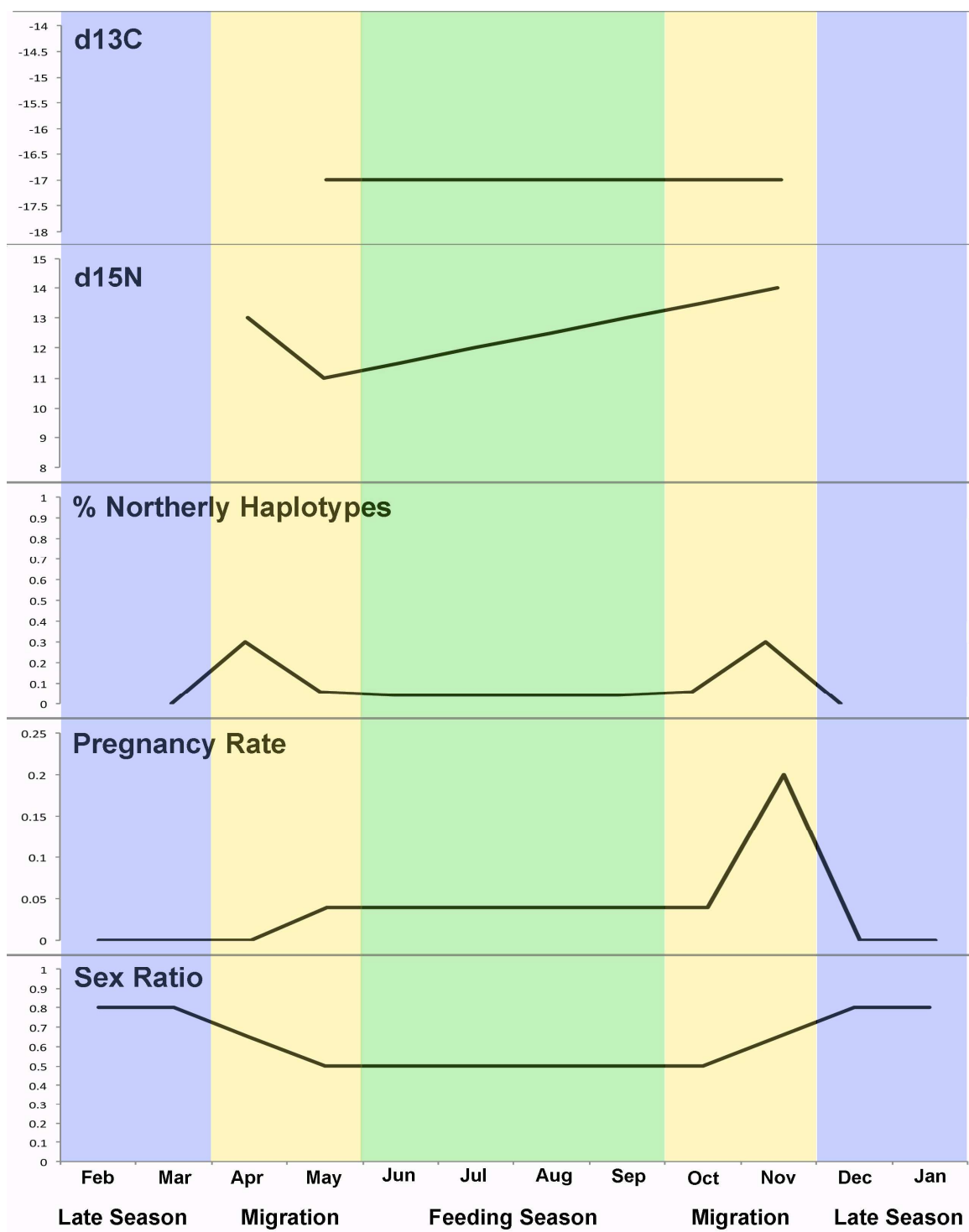


Figure 1. Approximate expected values for population parameters ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , percent mtDNA haplotypes identified as northerly, pregnancy rate and sex ratio) during late season, feeding season and migrations.

Females without calves are the first to leave the breeding area, followed by juveniles of both sexes, adult males and finally females with calves (Craig *et al.* 2003). The expected sex ratio thus was expected to tend toward a female majority early in the year, become closer to parity with the arrival of the juveniles, become male-dominated for a brief period as adult males arrive in the feeding area and approach 1:1 as females with calves reached the feeding area.

Juvenile humpbacks are the first to arrive in the breeding area, followed by females with no calves, adult males and females with calves (Craig *et al.* 2003). The sex ratio at the beginning of the fall migratory period thus was expected to be 1:1, tend toward a male majority as females without calves departed for the breeding area. This ratio was then expected to become female dominated as adult males migrate to the breeding area, leaving females in the late stages of pregnancy as the last whales to depart the feeding area.

For these calculations, population size was rounded to 2,040 animals, half the individuals in the population were considered to be mature and calving rate was assumed to be 38% (Nishiwaki 1959, Herman and Antinaja 1977, Baker *et al.* 1987) . The expected sex ratio was plotted alongside the weighted moving averages and 95% confidence intervals and examined visually. Deviations of the weighted moving averages from the expected values were analyzed qualitatively.

Blubber progesterone assays were conducted for all female whales sampled in 2011 ( $n = 31$ ) and a subset of females ( $n = 10$ ) sampled in 2012. Sighting histories of female humpbacks sampled in 2012 were examined to confirm maturity status of sampled



individuals. Female humpbacks previously sighted with a calf or sighted a minimum of six years before sampling were classified as sexually mature. Seven whales were classified as adult females and selected for progesterone analysis. The remaining three individuals were selected haphazardly. The tissue samples collected from two females in 2011 contained only small amounts of blubber and were not analyzed for pregnancy. Two individuals were sampled twice within a short period (<1 day, 6 days), thus the second samples were excluded from progesterone analyses.

Progesterone assays were conducted on 150mg of blubber in the laboratory of Dr. Nick Kellar at the NMFS Southwest Fisheries Science Center, La Jolla, California. The blubber was hormone extracted following the methods of Trego *et al.* 2013. The resulting residue was frozen at -20 °C until analyzed. An enzyme immunoassay was conducted according to the methods described in Trego *et al.* 2013. The assay was capable of detecting progesterone concentrations between 15 and 500 pg/ml. Samples with concentrations greater than the upper detection limit were diluted further and the assay rerun. Blubber progesterone concentrations were presented in ng progesterone/g of blubber.

An exact binomial test was used to determine whether the pregnancy rate of animals sampled in the early and middle portions of the 2011 feeding season (May – August) differed significantly from the pregnancy rate of animals sampled in the later portion of the feeding season (October – November). Linear regression analysis was used to test whether total blubber progesterone concentrations varied with Day of Year in pregnant and non-pregnant female humpbacks.

Laboratory preparation for stable isotope analysis was conducted at the NMFS Southwest Fisheries Science Center in La Jolla, California. A subsample of skin of approximately 10 mg wet mass was separated from each biopsy and dried for 24 hours in a VirTis benchtop lyophilizer (SP Industries, Warminster, Pennsylvania, USA). The dried subsamples were loaded into a Dionex Accelerated Solvent Extractor (Thermo Electron Corporation, Waltham, Massachusetts, USA) and tissue lipids removed using petroleum ether. After lipid extraction the subsamples were further divided and 0.5 to 1.0 mg of skin sealed in a tin capsule. Samples were then sent to the University of Florida, Gainesville, Stable Isotope Geochemistry Laboratory, where they were analyzed for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  by combustion using a Carlo Erba NA 1500 CNS Elemental Analyzer. This device was linked to a ConFlo II interface coupled with a Finnigan\_MAT252 isotope ratio mass spectrometer (Thermo Electron Corporation, Waltham, Massachusetts, USA). Vienna Pee Dee Belemnite (VPDB) and atmospheric nitrogen gas were used as reference materials for these analyses. USGS40 L-glutamic acid was run through the machine at regular intervals to calibrate the system. Results were reported as per mille using delta notation, determined from the equation:

$$\delta X = [(R_{\text{SAMPLE}}/R_{\text{STANDARD}}) - 1] * 1000$$

where X is  $^{15}\text{N}$  or  $^{13}\text{C}$  and R is the ratio of  $^{15}\text{N}/^{14}\text{N}$  or  $^{13}\text{C}/^{12}\text{C}$  in the sample and standard. Precision for these measurements was 0.05‰ for  $\delta^{13}\text{C}$  and 0.1‰ for  $\delta^{15}\text{N}$ . Mean  $\delta^{13}\text{C}$

and  $\delta^{15}\text{N}$  values were calculated for 2011 ( $n = 63$ ) and 2012 ( $n = 66$ ) samples and the two sampling periods were compared using independent samples  $t$ -tests.

Carbon and nitrogen stable isotope ratios from samples collected in the early portion of the feeding season were examined to investigate the existence of a fasting signal. Levene's Tests for Equality of Variances were used to determine whether the isotope ratios of samples collected in beginning of the feeding season (May – June) in 2011 exhibited greater variability than samples collected in the remainder of the feeding season (July – November). Linear regression analyses were conducted to examine the relationship between  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  and Day of Year in the beginning of the feeding season (May – June) in 2011 and 2012.

Linear regression analysis was used to examine how  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  varied in relation to Day of Year in 2011. Samples from this study were compared with a historical dataset containing  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  from samples collected in the study area in 2005 ( $n = 27$ ) to investigate whether intra-annual trends in carbon and nitrogen isotope ratios were consistent between years. An Analysis of Covariance (ANCOVA) was used to compare the historical isotope data and the 2011 data from this study to test the hypothesis that intra-annual trends in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  would be consistent between years. Day of Year was used as a covariate and year as a fixed factor for this analysis. A significant interaction between Day of Year and year would mean that the intra-annual trends differed among the two years. In this case, additional linear regressions would be used to further investigate the trends in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  within each year.

Mitochondrial DNA analyses were conducted at the CCGL, Marine Mammal Institute, Oregon State University. A section of the mtDNA control region approximately 800 base pairs in length was amplified using the primers light-strand tPro-whale Dlp-1.5 and heavy-strand Dlp-8G, using the methods of Garrigue *et al.* (2004). A 500 base pair segment was used to define the haplotypes. Visual assessments were conducted at each variable site to confirm haplotype identity.

Individual mitochondrial DNA haplotypes were selected for investigation of the occurrence of animals from SBC-WA migrating through CA-OR in the early and late portions of the feeding season. These haplotypes were chosen for being common in SBC-WA, but relatively rare in CA-OR. The frequency of each haplotype in CA-OR was calculated using pooled 2004/2005 and 2011/2012 data and subtracted from the frequency of that haplotype in SBC-WA (Baker *et al.* In Press). Negative values denoted haplotypes that were more frequently observed in CA-OR, whereas positive values represented haplotypes that were more common in SBC-WA. Haplotypes with positive frequency differences greater than 10% were designated “northerly” haplotypes.

Data from both this study (2011/2012:  $n = 130$ ) and the historic dataset (2004/2005:  $n = 124$ ) were used for these analyses. The monthly counts of these three haplotypes were summed, then divided by the total number of samples collected in that month. The resulting value was the percentage of total monthly samples classified as northerly haplotypes. Linear regression analysis was used to examine how this value changed through the year.

To examine whether the results of this study might be considered representative of the entire CA-OR feeding herd, historical data from samples collected off central California were compared with historical data from the entire feeding area. For these analyses, central California was defined as the area between Cambria, CA (~35.5° N) and Point Arena, CA (~39.0°N). Historical sex ratio, stable isotope, mtDNA data collected in central California in 2004 and 2005 were compared with the entire dataset of samples collected off CA-OR in these years. Significant differences between the central California data and the CA-OR data would indicate that the central California data were not representative of the entire feeding area, whereas non-significant differences would indicate the opposite.

Weighted moving averages and weighted 95% confidence intervals were calculated for whales sampled in 2004 and 2005 in central California and in the entire CA-OR feeding area. These values were then plotted together and differences between the two examined visually. Periods where the 95% confidence intervals overlapped were treated as similar, whereas periods where confidence intervals did not overlap were interpreted as different.

To test whether the genetic structure of the two regions differed, population pairwise genetic distances were calculated in Arlequin and used to generate pairwise F statistics ( $F_{st}$ ). This allowed the comparison of the haplotype frequencies from animals sampled in central California and the entire CA-OR feeding area. The F statistics were used to generate a p-value ( $\pm SE$ ) for this test.

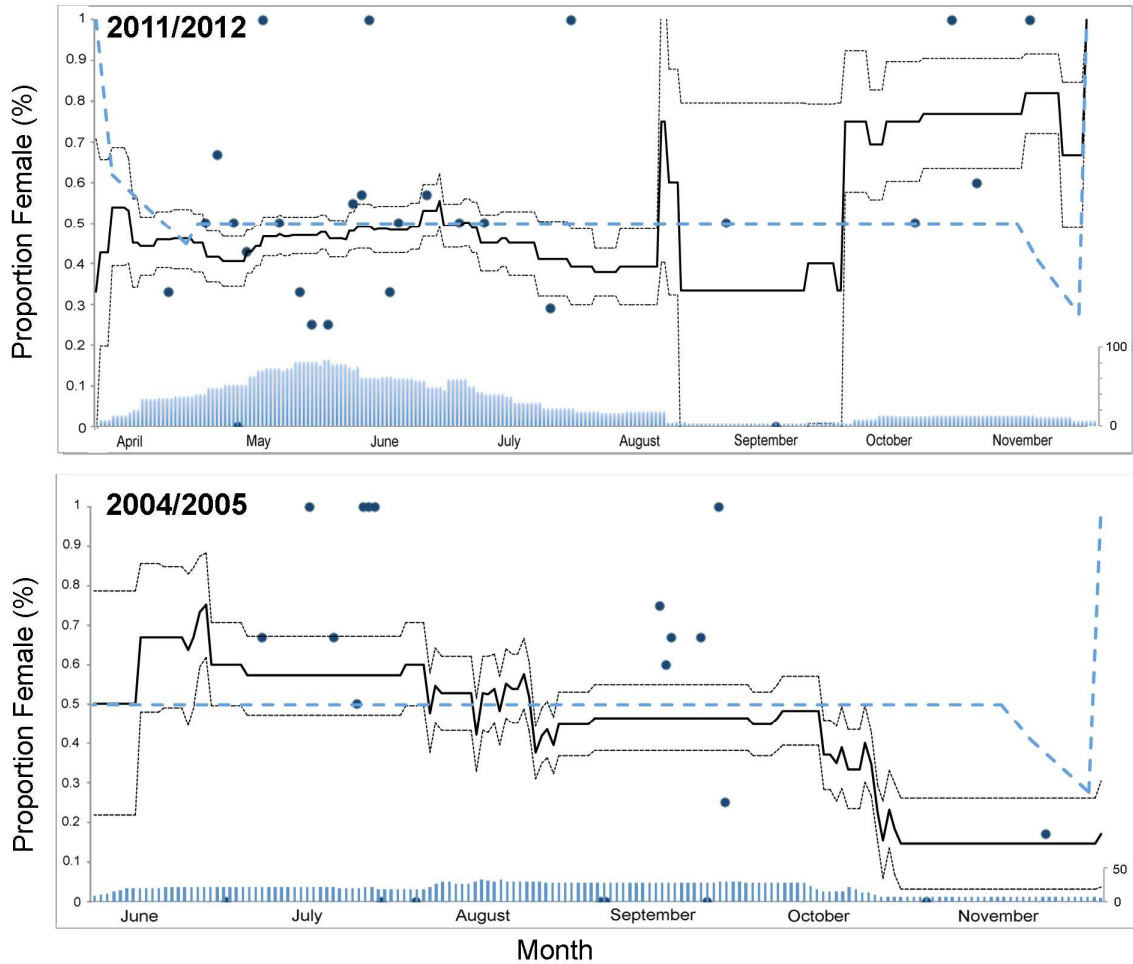
Mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values from 2005 were calculated and compared between central California and CA-OR using independent samples *t*-tests. ANCOVAs were used to determine whether intra-annual trends in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  differed among the two regions in 2005. For each ANCOVA, the stable isotope ratio was the dependent variable, Day of Year was the covariate and Region (central California or CA-OR) was a fixed factor. A significant interaction between Day of Year and Region would indicate that intra-annual trends in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  differed between central California and CA-OR in 2005.

## RESULTS

Sex was successfully assigned to 130 tissue samples from 128 individual whales. Two animals were double-sampled and the second sample of each individual was removed from further analyses. Of the 128 tissue samples analyzed, 66 were male and 62 were female. Thus, the overall sex ratio (M:F) across the two sampling years was 1.06:1 and was not significantly different from 1:1 (exact binomial test,  $P = 0.400$ ). Twenty-nine male and 33 female humpbacks (M:F = 0.88:1) were sampled from May-November 2011, which was not significantly different from parity (exact binomial test,  $P = 0.352$ ). Thirty-seven male and 29 female humpbacks (M:F = 1.28:1) were sampled from April-July 2012, which also was not significantly different from 1:1 (exact binomial test,  $P = 0.195$ ). The historical dataset contained the sex of 30 animals sampled in central California in 2004 (13 males, 17 females, sex ratio = 0.76:1) and 26 animals sampled in this area in 2005 (17 males, 9 females, sex ratio = 1.89:1). Neither of these ratios differed significantly from parity (exact binomial test, 2004:  $P = 0.292$ ; 2005:  $P = 0.084$ ), though the surplus of males in 2005 neared significance. Thus, pooled historic data from central California in 2004 and 2005 had a sex ratio 1.15:1, which did not differ significantly from parity (exact binomial test,  $P = 0.344$ ).

Visual assessment of the weighted moving average of 2011/2012 sex ratio (May – November) revealed deviations from the expected sex ratio early (May) and late (October – November) in the feeding season (Fig. 2). The sex ratio in the early portion of the feeding season tended towards a male majority, whereas the expected sex ratio was a

strong female majority. In October and November the sex ratio tended toward a female majority earlier than predicted by the expected values.



*Figure 2.* Weighted moving average of the sex ratio (solid black line) plotted with weighted 95% confidence intervals (dashed black line) and expected sex ratio (dashed blue line) for 2011/2012 and 2004/2005. Solid blue bars at the bottom of the figure represent the number of samples used to calculate the weighted moving average. Blue data points (circles) represent sex ratios of individual sampling days.

The 2004/2005 moving average covered a shorter span of time than the 2011/2012 data, beginning in June and ending in November (Fig. 2). Deviations from the



expected sex ratio occurred in October and November of these years. During these months, the sex ratio tended toward a male majority, whereas the expected value had a strong female majority.

Pregnancy status was determined for 41 individuals. Nineteen whales were classified as pregnant, 15 in 2011 and 4 in 2012. One animal was biopsied twice in one day in 2011 and the second sample was removed from further analyses. Thus, 14 pregnant whales from 2011 were included for analysis. Twenty-two whales were classified as non-pregnant, 16 sampled in 2011 and 6 sampled in 2012. The pregnancy status of one individual sampled in 2011 was unable to be classified. Its blubber progesterone concentration was 21.92 ng progesterone/g blubber, greater than the values associated with non-pregnant animals, but less than values typically associated with pregnant animals.

Mean blubber progesterone concentrations for female humpbacks classified as pregnant was  $129.55 \pm 14.23$  ng progesterone/g blubber (mean  $\pm$  SE). Mean blubber progesterone concentration for mature female humpbacks classified as non-pregnant was  $0.28 \pm 0.02$  ng/g. For female humpbacks of unknown maturity status classified as non-pregnant, the mean blubber progesterone concentration was  $0.26 \pm 0.03$  ng/g (Table 1).

Table 1. Blubber progesterone concentrations for pregnant, non-pregnant mature and non-pregnant humpback whales of unknown maturity status. Averages are presented as mean  $\pm$  SE.

Status	<i>M. novaeangliae</i>
Pregnant	
Average	129.55 $\pm$ 13.73
Minimum	46.05
Maximum	286.53
<i>n</i>	18
Non-pregnant/mature	
Average	0.28 $\pm$ 0.02
Minimum	0.23
Maximum	0.32
<i>n</i>	5
Immature and non-pregnant/mature	
Average	0.26 $\pm$ 0.03
Minimum	0.13
Maximum	0.68
<i>n</i>	16

The pregnancy rate of 61.9% for whales sampled in the early/middle portions of the feeding season (May – August,  $n = 21$ ) was significantly greater (exact binomial test,  $P = 0.003$ ) than the 11% the pregnancy rate for whales sampled in the late portion of the feeding season (October – November,  $n = 9$ ). Neither pregnant nor non-pregnant female humpbacks exhibited a significant linear relationship between blubber progesterone concentration and Day of Year in 2011 (Pregnant:  $P = 0.882$ ,  $R^2 = 0.002$ ; Non-pregnant:  $P = 0.830$ ,  $R^2 = 0.003$ ).

Stable carbon and nitrogen isotope ratios were analyzed for subsamples of skin from 131 biopsies, 64 from 2011 and 67 from 2012. Two individuals were sampled twice within one sampling day, one in May 2011 and the other in July 2012. Mean  $\delta^{13}\text{C}$

and  $\delta^{15}\text{N}$  values were calculated for the double samples and entered into the dataset to avoid over-representation of those individuals. Thus, for these analyses, there were 63 data points in 2011 and 66 in 2012. In 2011, mean ( $\pm\text{SE}$ )  $\delta^{13}\text{C}$  was  $-17.45 \pm 0.06$  and mean  $\delta^{15}\text{N}$  was  $12.93 \pm 0.10$ . In 2012, mean ( $\pm\text{SE}$ )  $\delta^{13}\text{C}$  was  $-18.14 \pm 0.07$  and mean  $\delta^{15}\text{N}$  was  $12.42 \pm 0.15$ . Both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were significantly different between the two years ( $p < 0.001$ ,  $P = 0.004$ ).

Levene's Tests of Equal Variances confirmed that neither  $\delta^{13}\text{C}$  nor  $\delta^{15}\text{N}$  had unequal variances among the early (May – June) and later (July – November) portions of the 2011 feeding season ( $P = 0.200$ ,  $P = 0.239$ ). Neither  $\delta^{13}\text{C}$  nor  $\delta^{15}\text{N}$  exhibited a significant relationship with Day of Year during 2011 ( $P = 0.747$ ,  $R^2 = 0.004$ ;  $P = 0.662$ ,  $R^2 = 0.007$ ) or 2012 ( $P = 0.712$ ,  $R^2 = 0.002$ ;  $P = 0.087$ ,  $R^2 = 0.048$ ).

$\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values increased slightly, but significantly with Day of Year ( $P = 0.001$ ,  $R^2 = 0.156$ ;  $P < 0.001$ ,  $R^2 = 0.214$ ; Fig. 3). Historical isotope data from samples collected off central California ( $35.5^\circ\text{N}$  to  $39.0^\circ\text{N}$ ) in 2005 ( $n = 27$ ) were entered into an ANCOVA along with isotope data from the 2011 sampling season of this study. The interaction term for Year\*Day of Year was non-significant for the ANCOVA on the  $\delta^{13}\text{C}$  data ( $P = 0.075$ ), thus the intra-annual variability in  $\delta^{13}\text{C}$  was similar in 2005 and 2011. There was a significant interaction of  $\delta^{15}\text{N}$  between Year and Day of Year ( $P = 0.002$ ), indicating that within-season trends in nitrogen isotope ratios were not consistent in 2005 and 2011 (Fig. 4). Additional linear regression analyses were used to examine within-season variability in stable isotope ratios for the historical data. Day of Year did not explain a significant amount of variability in either  $\delta^{13}\text{C}$  ( $P = 0.789$ ,  $R^2 = 0.003$ ), or  $\delta^{15}\text{N}$

( $P = 0.175$ ,  $R^2 = 0.072$ ) in 2005 (Fig. 5).

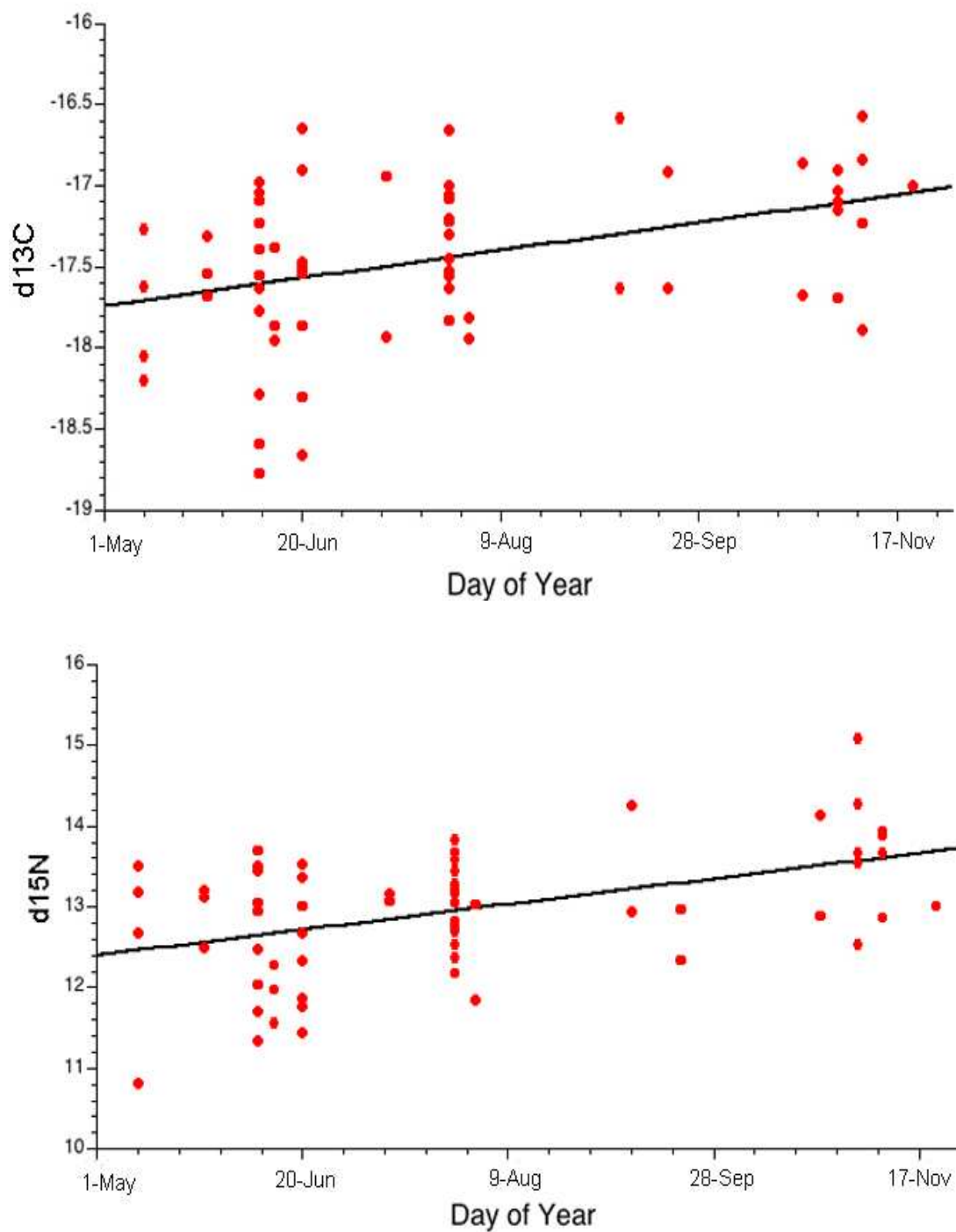


Figure 3. Stable isotope values ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) from 2011 plotted against Day of Year

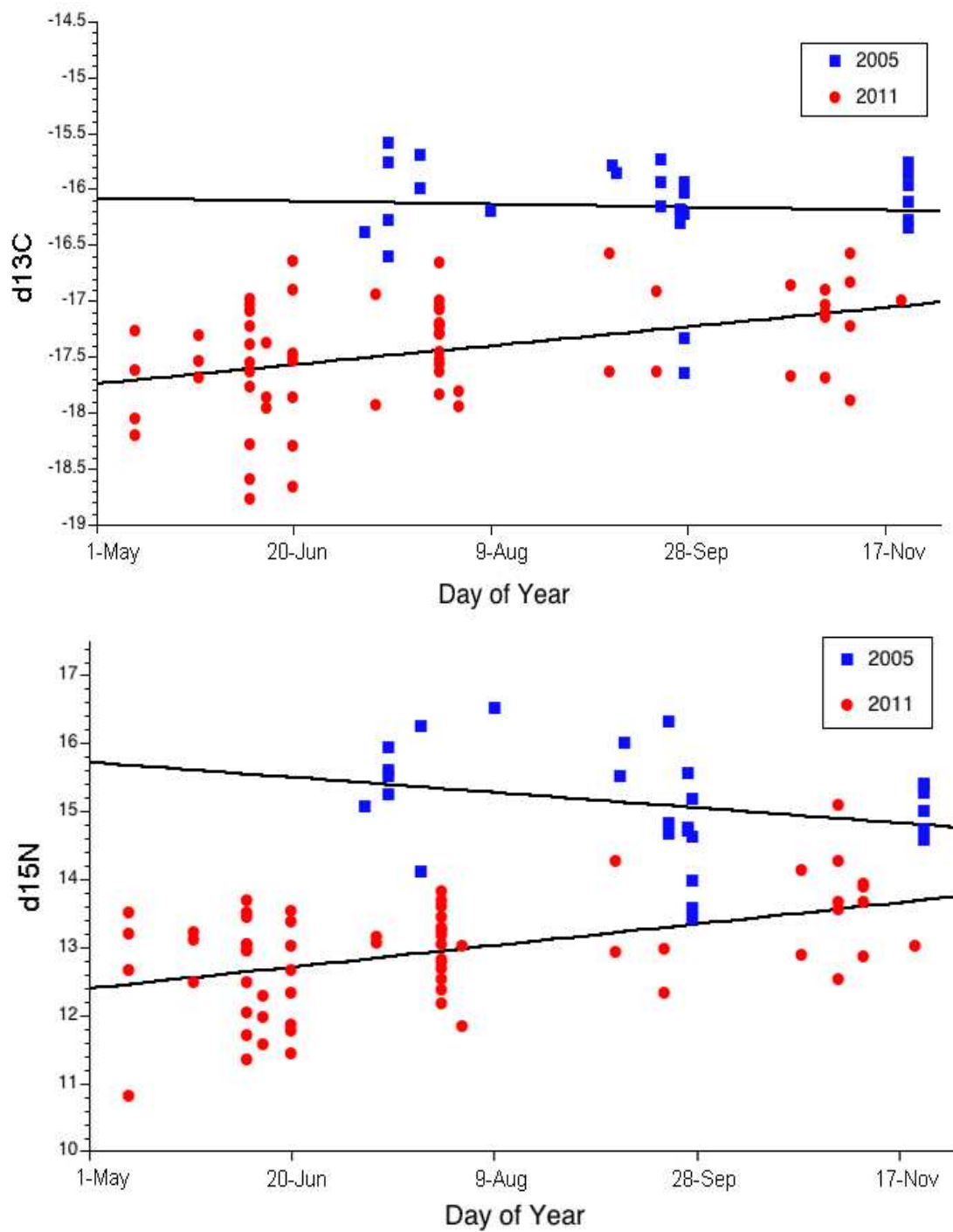


Figure 4. 2005 and 2011 stable isotope values ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) plotted against Day of Year for ANCOVA

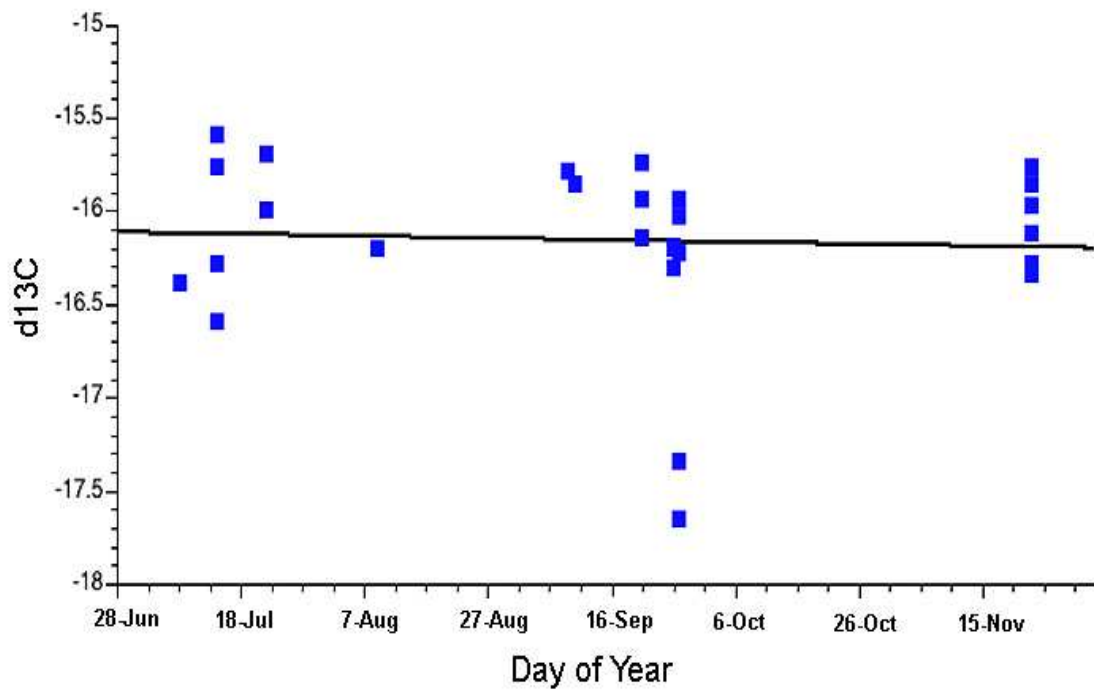
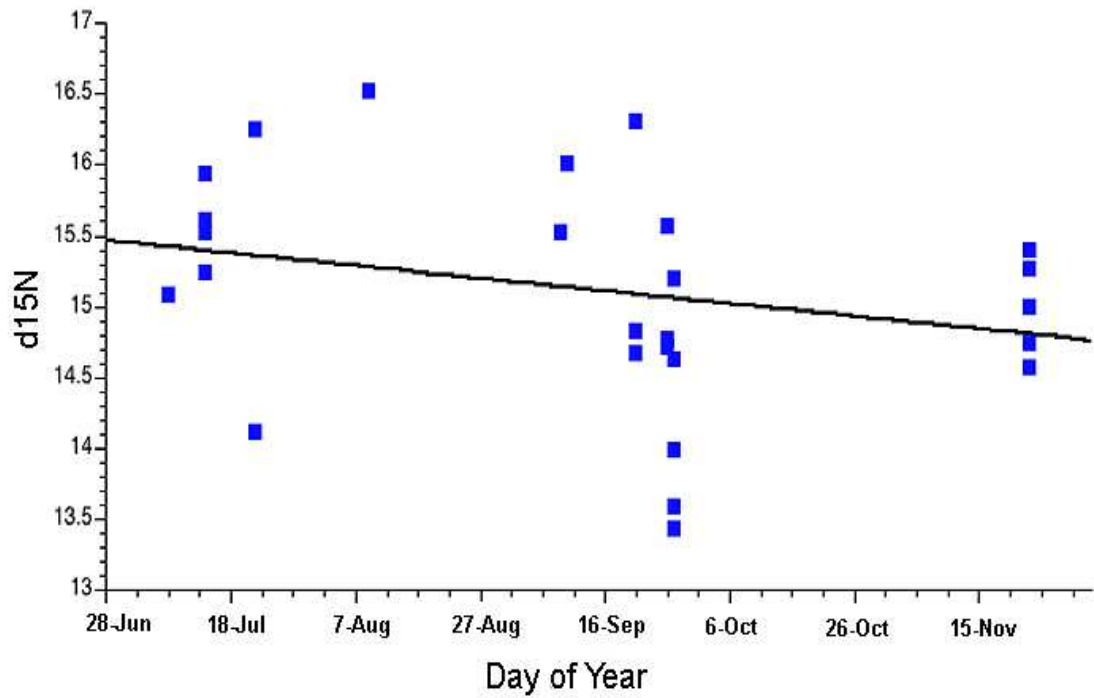


Figure 5. Stable isotope values ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) from 2005 plotted against Day of Year

Mitochondrial DNA haplotypes were determined for 130 biopsy samples. As with the sex ratio analyses, one sample was not used for mtDNA haplotype determinations and two individuals were biopsied twice within the 2011 sampling season, thus their duplicate samples were excluded from further analyses. In total, 128 samples were used for the following analyses. Eleven distinct haplotypes were identified between the two sampling years (Table 2). Six mtDNA haplotypes that were previously found in whales from this region were not present in samples from either 2011 or 2012 (Baker *et al.* in press).

Three haplotypes (A+, A- and E7) were more prevalent in SBC-WA than in CA-OR and were identified as “northerly” (Table 3). Percentage of monthly samples identified as northerly was plotted for the combined 2004/2005 and 2011/2012 data. There was a significant positive relationship between percentage of monthly samples identified as northerly and month ( $P = 0.002$ ,  $R^2 = 0.88$ ; Fig. 6).

Table 2. mtDNA haplotype frequencies for animals sampled in this study (2011 and 2012)

Year/Haplotype Code	A-	A+	A4	E1	E5	E6	E2	E3	E10	E13	F1	F2	F6	F3	F4	F8	A3	E4	E7	Total
2011	0	1	0	10	5	0	0	0	1	5	0	35	0	0	0	0	5	1	1	64
2012	0	1	0	23	0	1	0	4	1	3	0	29	0	1	0	0	2	0	1	66
All Years	0	2	0	33	5	1	0	4	2	8	0	64	0	1	0	0	5	1	2	130

Table 3. mtDNA haplotype frequencies for animals sampled in 2004 and 2005 in CA-OR and SBC-WA. Differences in haplotype frequencies were used to select “northerly” haplotypes. Frequencies presented as percentages. Haplotypes with frequency differences greater than 10% were classified as northerly (bolded values).

Haplotype Code	A-	A+	E1	E5	E6	E3	E10	E13	F1	F2	F6	F3	F8	A3	E4	E7	Total
SBC-WA	11	18	7	0	2	0	1	1	0	1	0	0	0	0	8	8	57
<i>Frequency</i>	<i>19.3</i>	<i>31.6</i>	<i>12.3</i>	<i>0.0</i>	<i>3.5</i>	<i>0.0</i>	<i>1.8</i>	<i>1.8</i>	<i>0.0</i>	<i>1.8</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>0.0</i>	<i>14.0</i>	<i>14.0</i>	
CA-OR	3	9	25	2	3	2	3	5	6	48	3	4	1	6	10	1	131
<i>Frequency</i>	<i>2.3</i>	<i>6.9</i>	<i>19.1</i>	<i>1.5</i>	<i>2.3</i>	<i>1.5</i>	<i>2.3</i>	<i>3.8</i>	<i>4.6</i>	<i>37.6</i>	<i>2.3</i>	<i>3.1</i>	<i>0.7</i>	<i>4.6</i>	<i>7.6</i>	<i>0.7</i>	
Frequency Difference	<b>17.0</b>	<b>24.7</b>	-6.8	-1.5	1.2	-1.5	-0.5	-2.1	-4.6	-34.9	-2.3	-3.1	-0.7	-4.6	6.4	<b>13.3</b>	



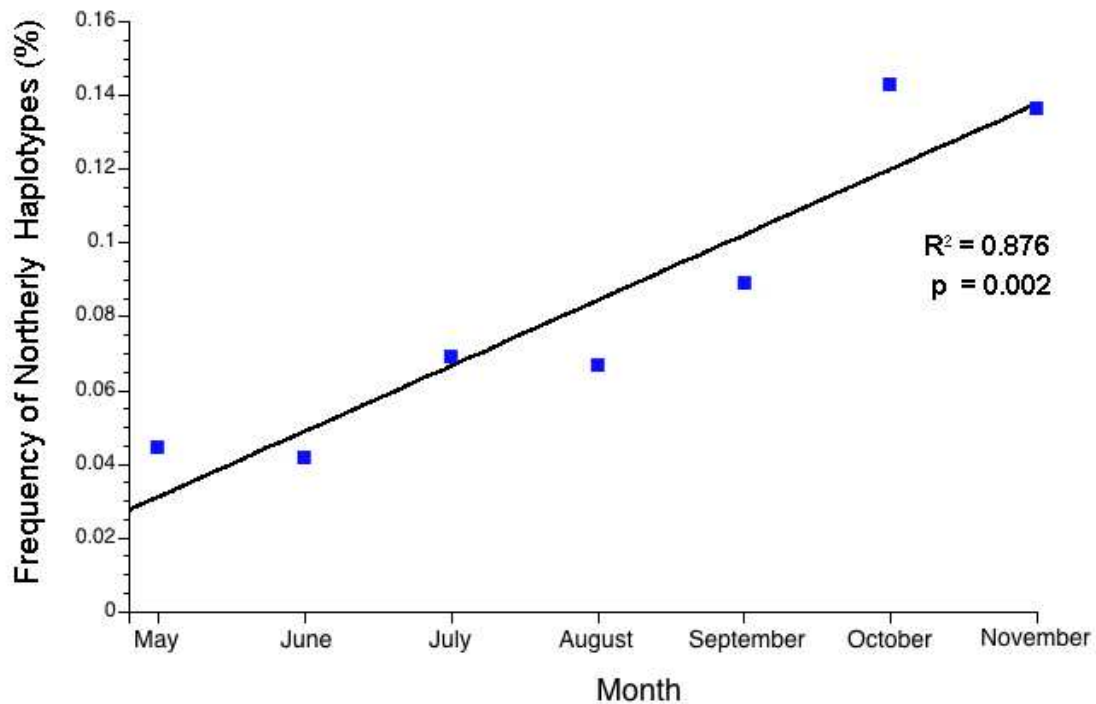


Figure 6. Relationship between observed frequency of northerly mtDNA haplotypes (A+, A-, E7) and month

It was not clear whether sex ratio in central California differed from sex ratio of the CA-OR feeding herd in 2004 ( $P = 0.053$ ). In 2005, the central California sex ratio did not differ significantly from that of the CA-OR feeding herd ( $P = 0.255$ ).

Visual comparisons of the weighted moving averages calculated for central California and CA-OR indicated no differences between the two regions (Fig. 7). The 95% confidence intervals of the weighted moving averages overlapped throughout the year, with one brief exception in early July.

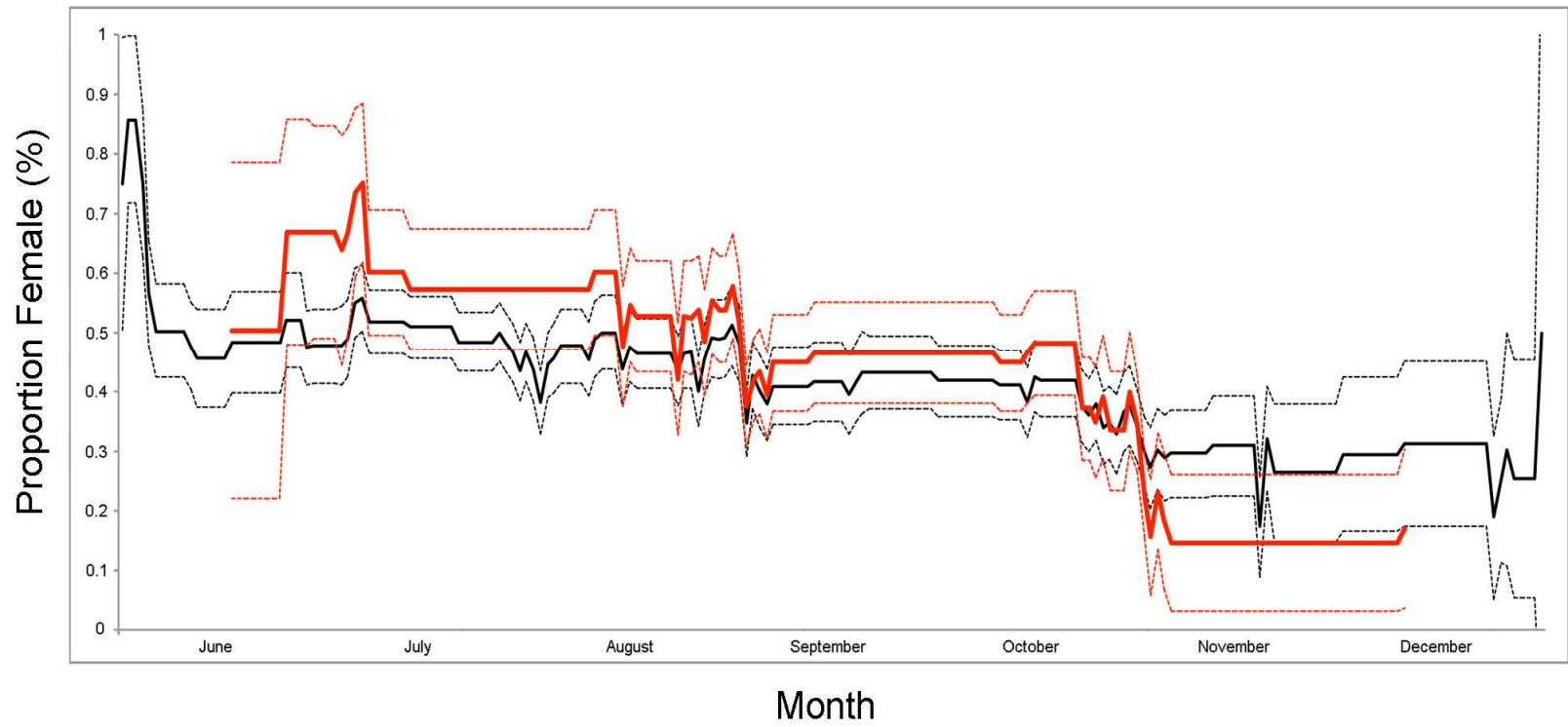


Figure 7. Weighted moving average of the sex ratios for central California (solid red line) and CA-OR (solid black line) with weighted 95% confidence intervals (dashed lines)

Population pairwise F statistics indicated that mtDNA haplotype frequencies of central California and CA-OR did not differ significantly ( $F_{st} = -0.002$ ,  $P = 0.715$ ). The assumption of equal variances was violated for the  $\delta^{13}\text{C}$  data (Levene's Test for Equality of Variances,  $P = 0.021$ ). Thus, equal variances were not assumed for the  $t$ -test for this variable. Mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values did not differ significantly between central California ( $P = 0.099$ ) and the entire CA-OR feeding area ( $P = 0.607$ ) in this year. The interaction between Region and Day of Year was non-significant for  $\delta^{13}\text{C}$  ( $P = 0.822$ ) and  $\delta^{15}\text{N}$  ANCOVAs ( $P = 0.519$ ), indicating that intra-annual trends in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values were similar within central California and across the entire CA-OR feeding area in 2005 (Fig. 8).

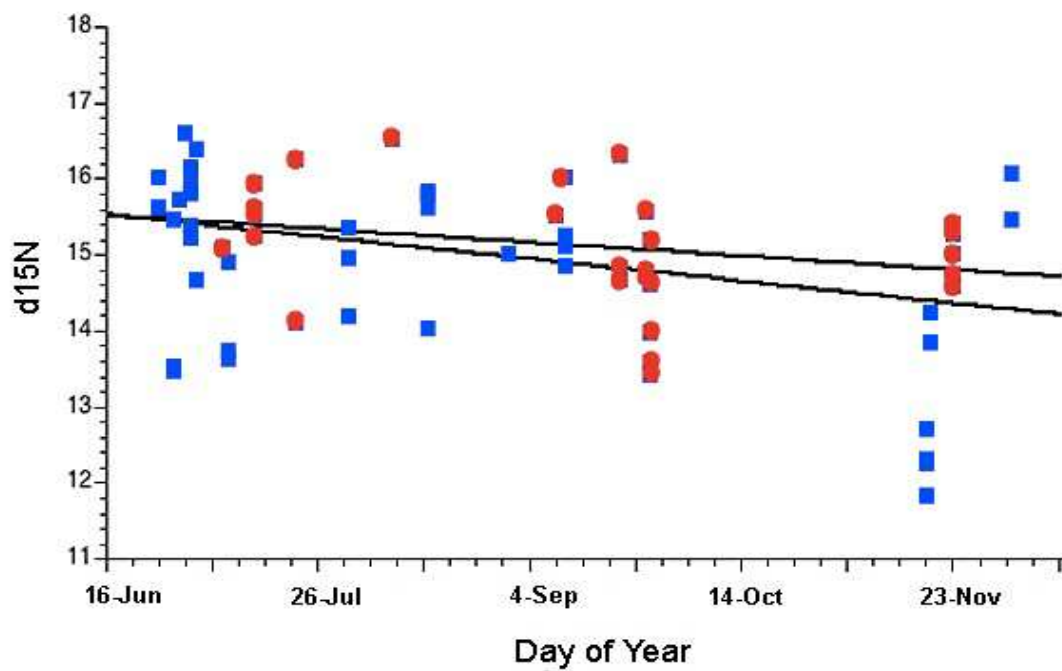
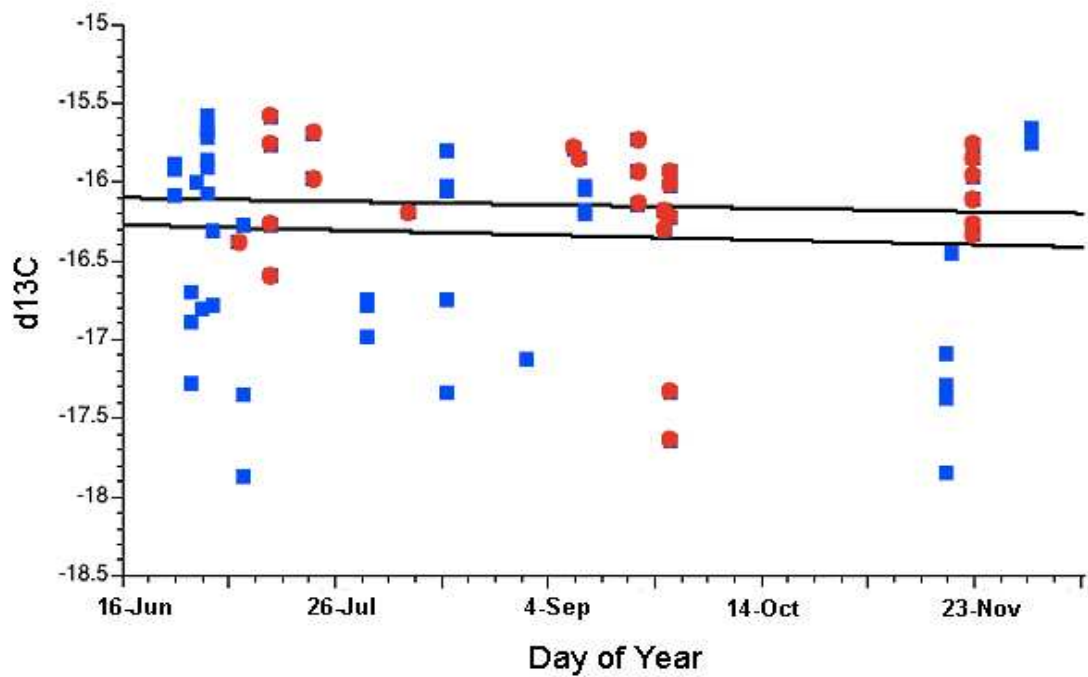


Figure 8. Central California (red circles) and CA-OR (blue squares)  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values plotted against Day of Year for ANCOVA

## DISCUSSION

### *Sex Ratio and Pregnancy*

The sex ratio of humpbacks is nearly 1:1 (parity) at birth and in the feeding areas (Chittleborough 1965). Repeated observations of male-dominated sex ratios in breeding areas around the world have led researchers to hypothesize that female humpbacks may exhibit different migratory behaviors than males, potentially shortening or foregoing migration in some years (Chittleborough 1965, Calambokidis *et al.* 2008). This study was designed to investigate the same phenomenon by examining intra-annual changes in the sex ratio of whales in a feeding area off central California. Few whales and inclement weather limited sampling success during winter and early spring, preventing direct comparison of the sex ratio between the feeding and late seasons. The focus was shifted, instead, to the migratory periods in the early and late portions of the feeding season. Expected values for the sex ratio were calculated from the literature and the observed variation from these expected values was used to provide evidence for or against the hypothesis that many female humpbacks remain in the feeding area during the late season.

Intra-annual changes in sex ratios observed in this study were generally consistent with the expected values, though some deviations were observed early and late in the feeding season. In the pooled 2011/2012 data, the sex ratio differed from expected values in April, tending toward a male majority when a female majority was expected and in October and November, tending toward a female majority when parity was expected.

The deviation from expectations in April occurred during a period with poor sample size and with broad confidence intervals. These factors are likely responsible for the apparent trend toward a male majority. The data do not; however, indicate a strong tendency for a female majority during the early portion of the feeding season, as expected. It is difficult to draw firm conclusions due to the degree of uncertainty in the estimate of the mean.

The two most likely explanations for the observed trend are that the changes in the sex ratio during the early portions of the year were not captured in the data due to incomplete sampling, or that these changes did not exist. Also, it is possible that the first animals arriving in Monterey Bay were not representative of the first animals arriving in the CA-OR feeding area and that the sex ratio did not reflect that of the entire region during the early portion of the feeding season. For example, if the first animals to arrive in CA-OR stayed in the waters off southern California for two or three weeks before heading north, this delay would be enough to allow other migrants to arrive. In this scenario, whales arriving in central California would likely be of mixed sex and age and any sex-related differences in migratory timing that may have existed in southern California would no longer be detectable.

The expected values calculated from the literature predicted a female majority at the end of the feeding season. In the 2011/2012 data, the sex ratio tended toward a female majority, but sooner than expected. This may have resulted from the expected values being poorly matched with the observed data. The expected sex ratio was plotted such that the first and last expected values coincided with the first and last observed values. It is possible that the last observed value did not represent the end of the

migratory period and that the animals observed in October/November were non-migrants remaining in the feeding area in the late season. This would explain the observed female majority in October, despite the expectation that this would not occur until late November.

The weighted moving average of the 2004/2005 sex ratio was generally similar to the trends observed in 2011/2012. These data only represented June to November, thus were unlikely to give insight into changes in sex ratio occurring early in the feeding season. The primary deviation from expected values in the 2004/2005 data occurred during October and November. In these months, the moving average of the sex ratio trended toward a strong male majority, whereas the expected values tended toward a female majority. This is likely the result of a lesser sample size late in the year in 2004/2005, though it is possible that this represents a real effect that is not explained by our current understanding of humpback whale migration.

It is difficult to draw concrete conclusions from the data in this study, primarily due to poor sample coverage and a great degree of variability in estimates of sex ratio. The results from autumn 2011/2012 provided support for the hypothesis that some female whales delay or forego the migration to the feeding area; however the results from 2004/2005 indicate the opposite. Though there are deviations from expected values in both the contemporary and historic datasets, it is difficult to conclude whether they represent a real effect, or are simply an artifact of incomplete sampling.

The results of the blubber progesterone assays support the use of this method as a tool for determining pregnancy status in humpback whales. The most important factor

for judging the efficacy of the assay for determining humpback pregnancy was that the signal needed to be strong enough to clearly differentiate between pregnant and non-pregnant animals (Kellar *et al.* 2006, Trego *et al.* 2013). Only one individual had a blubber progesterone concentration that was ambiguous and did not allow pregnancy status to be clearly assigned. Mean concentrations of blubber progesterone for whales classified as pregnant were, on average, three orders of magnitude greater than animals classified as non-pregnant. The differences in progesterone concentrations among the two groups were of a similar magnitude to those observed in other cetacean species (Mansour *et al.* 2002, Kellar *et al.* 2006, Perez *et al.* 2011), lending support to the assertion that this method is appropriate for use on humpbacks.

The marked decrease in the observed pregnancy rate throughout the feeding season in 2011 was unexpected. Previous studies of humpback whale migration indicated that pregnant females were the last to leave for the breeding area (Craig *et al.* 2003), thus it was expected that the pregnancy rate would be greatest in the later portion of the feeding season. The results of this study were contrary to this expectation, with the lesser pregnancy rates late in the year. These intra-annual changes in pregnancy rate make it difficult to draw conclusions about the overall pregnancy rate of humpback whales in CA-OR. Thus, it is unclear whether the pregnancy rate observed in October/November 2011 was greater or lesser than expected; however it was apparent that the majority of female humpbacks were not pregnant during these months, as was predicted in the literature.



One explanation for the intra-annual changes in the pregnancy rate is that some of the animals classified as pregnant during the early portion of the year were false positives. It is possible that all mature females undergo great changes in hormone concentrations during the breeding season and that the aftereffects of these changes were captured in the samples collected during the early portion of the feeding season. This explanation is implausible for two primary reasons: the magnitude of the differences in blubber progesterone concentrations between pregnant and non-pregnant animals were greater than seasonal changes in hormone concentrations in other cetacean species (Atkinson *et al.* 1999, Robeck *et al.* 2005), though it is difficult to make comparisons as previous researchers measured progesterone concentration in the blood serum or urine, not in blubber. The differences between mean progesterone concentrations of non-pregnant and pregnant animals in this study were at least an order of magnitude greater than the seasonal differences in captive cetaceans. Second, shifts in blubber progesterone concentration associated with changes in reproductive status typically occur over short time-spans in cetaceans. Most of these shifts occur over days or weeks (Kellar *et al.* 2006), thus it is unlikely that they would be observed in animals observed off CA-OR, especially in July.

Another explanation for the observed changes in pregnancy rate within the 2011 feeding season is a change in habitat use or likelihood of sampling pregnant whales. If pregnant females tend to exploit a particular type of habitat or exhibit similar migratory behaviors, it is possible that changes in pregnancy rate in this study reflect actual movements by these whales. There is evidence that humpback whales use different

habitats based on sex, age and reproductive status on a small scale in the breeding area (Smultea 1994, Craig and Herman 2000, Ersts and Rosenbaum 2003). Within the feeding area, there are indications that female humpbacks with dependent calves may use slightly different habitats than other whales (Steiger and Calambokidis 2000), but otherwise, habitat stratification or segregation has not been reported for this species (Robbins 2007). Further study would be required to determine whether pregnant humpbacks move together, or exploit different resources and habitats than non-pregnant animals.

Finally, the observed changes in pregnancy rate could reflect an actual decrease in number of pregnant animals across the feeding season. Some mammals reabsorb a fertilized embryo or abort a fetus in response to decreases in serum progesterone concentrations (Huck *et al.* 1988). These progesterone concentrations fluctuate in response to a number of factors including illness, age and nutritional stress. It is possible that humpback whales use a previously undocumented reproductive strategy, in which the majority of mature females become pregnant during the breeding season, but only whales in optimal body condition carry the pregnancy to term. This strategy would be energetically favorable, as environmental variability makes it impossible for female humpbacks to predict resource availability in the coming year. Pregnancy may act as an insurance policy, giving humpbacks the option to have a calf in good years, thereby allowing them to skip reproduction in years when there are decreased food resources. The costs of the first months of pregnancy are relatively small, especially when compared with the costs of lactation and care of young (Lockyer 1984). It follows, then, that many

female humpbacks might become pregnant in the breeding area, but that relatively few of these animals carry their pregnancy to term.

If humpbacks were using this reproductive strategy, birth rates would be expected to vary from year to year, based on the environmental conditions of the previous year. Further study is required to confirm a connection between environmental conditions and birth rate, but there does appear to be a great degree of inter-annual variability in the reproductive rates of these whales (Steiger and Calambokidis 2000). Other animal species, especially seabirds, use such reproductive “insurance policies” (Forbes 1990). The use of such a strategy might help to explain why humpbacks have recovered from commercial whaling at a much greater rate than other large whale species (Zerbini *et al.* 2010).

The results of this study do not support the hypothesis that blubber progesterone concentrations of humpback whales increase throughout pregnancy, as in other mammal species (Ishwar 1995, Spencer and Bazer 2002). This finding is in agreement with the conclusions reached by other researchers, who suggested that progesterone concentrations remain relatively constant in pregnant cetaceans (Cornell *et al.* 1987, Robeck 1996), but this study is among the first to investigate this question using blubber samples and the only one to do so in a baleen whale.

Only one animal was unable to be classified as pregnant or non-pregnant. This sample had a blubber progesterone concentration of 21.92 ng/g, falling between the values associated with pregnant and non-pregnant animals. There are a number of possible explanations for this value. It is possible that this animal was pregnant and

exhibiting low progesterone values, as has been observed in sick and nutritionally stressed animals (Williams and Cumming 1982, Sugino *et al.* 1991, McAndrews *et al.* 1994). Some lipids may have been lost during sample preparation and processing, resulting in an observed progesterone concentration that was less than typically associated with pregnant animals (N. Kellar pers. corr.). Finally, these intermediate progesterone levels might be associated with an animal that was sampled just as it was beginning or terminating a pregnancy (Kellar *et al.* 2006), though the latter seems more likely given that the sample was collected in August.

Alternatively, this animal may not have been pregnant, but may have been exhibiting elevated progesterone levels for another reason. Researchers have observed increases in progesterone levels associated with non-fertile ovulation (Brook *et al.* 2004) and pseudo-pregnancy (Robeck *et al.* 2001). These phenomena increase progesterone concentrations in animal tissues, though typically not to the levels associated with pregnant animals. It remains unclear why the animal sampled in this study exhibited intermediate levels of blubber progesterone concentration, but the possible phenomena responsible for this anomalous value merit further investigation.

#### *Fasting Effect and Trends in Stable Isotope Ratios*

Stable isotope ratios are a valuable tool for studying animal migratory movements, especially for animals that move great distances or for which attachment of a telemetric tag is impractical (Graham *et al.* 2010). Studying isotope data is, in many

ways, similar to studying data from a tag. Both methods provide information about an animal's movements and indicate the regions in which the animal has likely foraged (Hobson 1999, Hobson 2010). Laboratory and field studies have recently established the utility of stable isotope analysis as a tool for studying fasting and nutritional stress in animals (Hobson *et al.* 1993, Cherel *et al.* 2005b). The effects of fasting and nutritional stress have been primarily observed in  $\delta^{15}\text{N}$  values (Hare *et al.* 1991, Habran *et al.* 2010), though some researchers reported changes in  $\delta^{13}\text{C}$  in response to these factors (Hatch *et al.* 1995, Boag *et al.* 2006, McCue and Pollock 2008). Whole body  $\Delta^{15}\text{N}$  is predicted to increase with increasing duration of fasting or nutritional stress (Martinez del Rio and Wolf 2005), resulting from the preferential retention of  $^{15}\text{N}$  during amino acid and protein synthesis (Steele and Daniel 1978). Changes in  $\delta^{13}\text{C}$  are more difficult to predict, with some organisms exhibiting enriched values as a result of fasting (Oelbermann and Scheu 2002, Doi *et al.* 2007) and others having more depleted values (Gaye-Siessegger *et al.* 2004).

Tissue samples from humpback whales returning to central California early in the year in 2011 and 2012 did not show evidence of fasting. If a detectable fasting effect existed, variability in  $\delta^{15}\text{N}$  and/or  $\delta^{13}\text{C}$  would be expected to be greater early in the year, reflecting the staggered arrival of whales in the feeding area. Samples from this period would contain a mix of feeding and fasting animals, thus isotope ratios would be expected to be more variable than later in the year when all animals had been feeding. Animals sampled early in the feeding season in 2011 did not exhibit increased variability in stable isotope ratios. By definition, a fasting effect would cause changes in  $\delta^{15}\text{N}$

and/or  $\delta^{13}\text{C}$  values in the tissue. Regardless of the direction of these changes, the isotope ratios would return to levels indicative of foraging, resulting in a relationship between isotope ratios and Day of Year. Neither  $\delta^{15}\text{N}$  nor  $\delta^{13}\text{C}$  had a significant relationship with Day of Year during the early portion of the feeding season in 2011 or 2012, indicating that a fasting effect did not exist or was not detectable.

A number of confounding factors make it difficult to draw conclusions from the results of these analyses. The foremost of these limitations is that it was not possible to obtain repeat samples from individual animals, as has been done in previous studies of fasting effects (Hobson *et al.* 1993, Cherel *et al.* 2005b). This means that the fasting signal would need to be pronounced enough to be detectable through the variability in stable isotope signals in the population. There was a great degree of variability in isotope ratios of humpback whales sampled in this study, thus a fasting signal would likely be difficult or impossible to detect. Studies in king penguins (*Aptenodytes patagonicus*) and Nile Tilapia (*Oreochromus niloticus*) indicated the effect of fasting on  $\delta^{15}\text{N}$  was observable and significant, but that the magnitude of this effect was small ( $< 1\text{‰}$ ; Cherel *et al.* 2005a, Gaye-Siessegger *et al.* 2007). Both linear regressions and the Levene's Test of Equal Variances used in this study would have difficulty detecting a small effect size, given the great degree of variability among individuals.

Additionally, these analyses may be limited by the relatively rapid turnover rate of humpback whale skin. Turnover rates are a measure of how quickly the tissue of interest is regenerated and replaced and the span of time described by an isotope ratio depends directly on this value (Tieszen *et al.* 1983). Some tissues, such as blood, turn

over quickly (0.9 days, Hobson and Clark 1992) and are useful for studying short-term changes in isotope ratios. Other tissues take much longer to turn over or become metabolically inert and do not turn over at all, thus are useful for studying long-term diet of an animal (Schell *et al.* 1989). Stable isotope ratios in humpback whale skin represent the animal's diet for the past two weeks to one month (Todd 1997). If the whales fasted for the duration of the breeding season, but began feeding on the migration two weeks or a month before they were sampled in Monterey Bay, their tissues would reflect the recent feeding. Any fasting signal that was present in their tissues upon leaving the breeding area would then be removed, making detection impossible.

Alternatively, humpback whales may not exhibit effects of fasting in the stable isotope ratios of their tissues. The enrichment of  $\delta^{15}\text{N}$  values as a result of nutritional stress is a direct result of protein catabolization within the fasting animal; however, the oxidation of lipids does not produce the same response (Martinez del Rio and Wolf 2005, Hatch 2012). Animals such as humpback whales that rely primarily on energy stored in fat reserves during fasting periods might not show effects of the annual fast in their tissues. Thus, the lack of an observable fasting effect in this study may be an indication that humpbacks generally do not exhaust their fat stores during the annual migration and fasting period.

Changes in  $\delta^{13}\text{C}$  as a result of fasting are poorly understood as compared with changes in nitrogen stable isotope signatures. In previous studies, some species exhibited increased  $\delta^{13}\text{C}$  values after fasting (Doucett *et al.* 1999, Doi *et al.* 2007, McCue and Pollock 2008), whereas others had no response or decreased  $\delta^{13}\text{C}$  values (Hobson *et al.*

1993, Focken 2001, Kempster *et al.* 2007, Williams *et al.* 2007). The  $\delta^{13}\text{C}$  values in the skin of humpbacks sampled as part of this study did not vary significantly with time during the early portion of the feeding season, nor did they exhibit increased variability during this time. In 2011; however, all of the most depleted  $\delta^{13}\text{C}$  values occurred within the early portion of the feeding season (Fig. 3). It is unclear whether this is a byproduct of fasting, changes in diet, or random chance alone.

If a fasting effect did exist and was detectable, it could potentially be used as a tool for detecting non-migratory whales. Such an effect of fasting would be expected to cause consistent changes in the ratios of  $\delta^{13}\text{C}$  and/or  $\delta^{15}\text{N}$ . Animals that had shortened or foregone the annual migration to the breeding area would likely have been feeding; thus, their tissues would not show signs of fasting. Depending on the magnitude of the fasting effect, these  $\delta^{13}\text{C}$  and/or  $\delta^{15}\text{N}$  in the tissues of these animals might appear different from animals that had recently returned from the breeding area, allowing the non-migratory individuals to be identified. As there was no detectable fasting effect in the tissues of animals sampled for this study; however, this method could not be used.

Trends in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were consistent with the hypothesis that the diet of humpback whales sampled for this study shifted towards higher trophic-level prey later in the feeding season. The magnitude of these changes was not as great as would be expected for a complete step up in trophic level (~3-4%, Peterson and Fry 1987); thus, these results may reflect a partial shift from a diet consisting of krill to a larger proportion of higher trophic level prey (e.g. fishes). Alternatively, the changes observed in the tissue of humpback whales could have resulted from seasonal shifts in baseline  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$



during the sampling period associated with environmental forces such as upwelling or changes at the base of the food web (Goering *et al.* 1990). The relationship between  $\delta^{13}\text{C}$  and Day of Year was similar in 2005 and 2011, whereas that of  $\delta^{15}\text{N}$  and Day of Year varied between those two years. Analyses would need to include a greater number of years before concrete conclusions could be drawn regarding the consistency of intra-annual trends in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  through time.

#### *Investigating the Presence of Northerly Migrants in CA-OR*

The CA-OR feeding area is unique in that it is the only feeding area in the North Pacific that is likely to lie directly on the migratory route of whales from an adjacent feeding herd. North America's coastline is shaped in such a way that the shortest path for animals migrating to SBC-WA from Mexico is directly through CA-OR. Therefore, it would be expected that migrants from the SBC-WA feeding herd could be observed in CA-OR during the migratory periods at the beginning and end of the feeding season, though this has not been demonstrated.

The presence of animals with mtDNA haplotypes that are rare in CA-OR, but common in SBC-WA may be useful as a tool for investigating the presence of these northerly migrants. Analyses of the proportion of northerly haplotypes present in CA-OR throughout the feeding season revealed a much greater proportion of these animals at the end of the feeding season in October and November. No such increase was observed early in the feeding season, contrary to expectations. This is likely a result of incomplete

sampling during the early months, which may have allowed these animals to move through CA-OR unsampled. An alternative hypothesis is that northerly migrants travel farther offshore or are more difficult to sample during the early portion of the feeding season.

Sighting histories were available for five individuals with northerly mtDNA haplotypes. None of these animals were seen in other feeding areas, including SBC-WA; however, this is likely due to greater sampling effort in CA-OR than in SBC-WA. Two of these whales were only observed in CA-OR late in the feeding season (October and November), whereas the remaining three had all been seen during the middle of the feeding season (late June – August). These mixed results make it difficult to draw conclusions, especially given the small sample size.

The presence of northerly migrants in CA-OR, though not unexpected, has the potential to impact research, conservation and management of humpbacks in both CA-OR and SBC-WA. Studies of humpback whales in CA-OR are unlikely to consider the presence of migrants from other feeding areas. Thus, estimates of abundance for CA-OR generated from surveys conducted early and late in the year may be artificially inflated. Additionally, analyses of genetic stock structure may include whales from other regions, leading to decreased estimates of differentiation among feeding herds. Similarly, management plans are unlikely to take into account the use of the CA-OR habitat by whales from SBC-WA, which could lead to improper or ineffective management of this endangered species.

### *Applicability of Results to CA-OR Feeding Herd*

Humpback whales in the North Pacific have historically been managed as a single stock (Donovan 1991). Contemporary research; however, has used photo-ID records and genetic analyses to investigate and characterize sub-groups, referred to as “feeding herds” within the North Pacific basin (Baker *et al.* 1990, Palumbi and Baker 1994, Baker *et al.* 1998). Sighting histories and tests of genetic relatedness have led researchers to hypothesize that the individuals within these feeding herds are genetically and behaviorally similar. Moreover, the great degree of mobility exhibited by these whales allows them relatively easy access to all parts of the area in which they feed. Thus, data collected from animals sampled in one region of the feeding area are hypothesized to be representative of the entire feeding herd to which they belong.

To test this hypothesis, data collected from whales sampled in the waters off central California (35.5° N – 39.0° N) were compared with data collected within the entire CA-OR feeding area (32.5° N – 42.0° N). Parameters used for this comparison included sex ratio, mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values and mtDNA haplotype frequencies.

Many species of marine mammal exhibit sex segregated habitat use. Some of these species, such as sperm whales (*Physeter macrocephalus*) exhibit strong segregation over large spatial scales (Rice 1989). Other species have sex related differences in habitat use that occur over a smaller scale. For example, grey seals (*Halichoerus grypus*) exhibit sex segregation just before and after breeding, with males primarily using areas along the shelf break and females occupying habitat over the shelf (Breed *et al.* 2006).

Humpback whales do not segregate by sex within the feeding areas, thus it was not unexpected that the sex ratio of animals sampled off central California in 2004 and 2005 did not differ from that of the entire CA-OR feeding herd. Both the overall sex ratio and the intra-annual changes in the observed sex ratio were similar between the two regions.

It is worth noting that the weighted moving average of the sex ratio for 2004/2005 from all of CA-OR, had greater temporal coverage than the data that was available from central California, spanning the months of May – December. The moving average for this larger dataset tended to follow the expected values more closely than the values from only central California. This serves to highlight the importance of greater temporal coverage and sample size when conducting these analyses.

The factors driving patterns in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  are more complex than those affecting the sex ratio. Stable carbon isotopes ratios observed in animal tissue are heavily influenced by the primary producers at the bottom of the food web in which the animal has foraged (Graham 2010). Phytoplankton communities tend to differ greatly among coastal and offshore habitats and  $\delta^{13}\text{C}$  commonly decreases with increasing distance from shore (Graham 2010). Stable isotope data collected from whales off central California would, therefore, be expected to be similar to signatures from samples collected across the CA-OR feeding area. This would not hold true if animals in central California, as a whole, tended to forage at a different distance from shore than animals in other portions of the CA-OR feeding area. There is; however, no evidence to suggest that this is the case and the mean  $\delta^{13}\text{C}$  values observed in this study were similar to those of the entire CA-OR feeding herd.

Stable nitrogen isotope ratios exhibit a positive relationship with latitude (Graham *et al.* 2010). As central California lies close to the geographic center of the CA-OR feeding area, it is likely that  $\delta^{15}\text{N}$  values from samples collected in this area would lie close to the mean of  $\delta^{15}\text{N}$  values for the entire region. However,  $\delta^{15}\text{N}$  also is influenced by the trophic level at which the sampled animal has been foraging (Hobson 1999). Therefore, a difference in the mean  $\delta^{15}\text{N}$  values of humpbacks sampled off central California from the entire CA-OR feeding area could indicate a difference in diet among animals inside and outside of central California. The year 2005 was the only one in the historical dataset with enough samples inside and outside of central California to make a strong comparison. In this year, the mean  $\delta^{15}\text{N}$  values in central California and CA-OR were similar, supporting the conclusion that samples collected within the smaller area were representative of the greater feeding area.

Whereas mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values might be expected to be similar within central California and CA-OR, finer scale processes such as intra-annual trends in isotope ratios were more difficult to predict. Such trends are driven by more complex, smaller-scale processes such as upwelling, which affect primary producer community composition and growth rates (Graham *et al.* 2010). Though CA-OR is, as a whole, subject to many of the same environmental drivers (large-scale weather and oceanographic patterns, day length and other seasonal factors), it is possible that the highly variable local factors might cause intra-annual changes in stable isotope ratios to vary geographically. In 2005, these intra-annual trends did not differ significantly between central California and CA-OR for either  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$ . The analyses contained in

this study were limited, in that only one year was sampled completely enough to examine these intra-annual shifts both within central California and CA-OR as a whole. These results; however, provide evidence that, at least in some years, the seasonal trends in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  measured in central California are representative of CA-OR as a whole.

The mtDNA haplotype frequencies did not differ significantly among central California and the entire CA-OR feeding area, a result that matches expectations. The current delineations of the feeding herds in the North Pacific were created through analyses of microsatellite and mitochondrial DNA (Baker *et al.* 1998). It would have been surprising if the genetic structure of humpbacks off central California differed greatly from that of the greater CA-OR feeding herd.

While not unexpected, these results provide strong evidence that samples collected within central California are representative of the CA-OR feeding area. This, in turn, supports the assertion that conclusions drawn from this study are relevant to the feeding area as a whole and not only to animals sampled in central California.

### *Conclusions*

A variety of population parameters were evaluated for use as tools for studying migratory behavior in humpback whales. Analyses of intra-annual changes in these parameters yielded mixed results, with some parameters proving more effective as tools for examining migratory behavior than others. Sex ratio and accompanying pregnancy rate information proved moderately effective in capturing sex-related differences in

migratory timing of humpback whales in CA-OR. The great degree of variability inherent in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of humpback whales made it difficult to draw concrete conclusions from analyses of isotope data, thus were not particularly effective as tools for assessing migratory behavior at the population level. Mitochondrial DNA haplotype frequencies proved useful for distinguishing specific groups of animals that were likely from a separate population, though distinguishing between migrants from outside populations and individuals from within the population that have rare mtDNA haplotypes was difficult or impossible.

Taken together, these parameters may help to provide a more complete description of the migratory behaviors of the population of humpback whales that feed off CA-OR. These tools do not provide particularly detailed information, nor do they give any indication of the behaviors of specific individuals. If used in tandem with more traditional methods of study; however, these parameters have the potential to provide a much more comprehensive understanding of humpback whale migratory behaviors.

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