Population genetic structure of living and dead southern right whales (*Eubalaena australis*) off Península Valdés, Argentina.

Luciano O. Valenzuela[1, 2], Mariano Sironi[2,3,4], Victoria J. Rowntree[1,4,5], Luciano La Sala[4], Luciana Pozzi[4,6,7], Nadia Mohamed[4], Luciana Musmeci[4,6,7], Julian Andrejuk[4], Marcela Uhart[4,8], Andrea Chirife[4], Carina Maron[1,2], Jon Seger[1]

[1] Department of Biology, University of Utah. 257 South 1400 East, Salt Lake City, UT 84112 USA.

[2] Instituto de Conservación de Ballenas. Miñones 1986, CP 1428, Ciudad de Buenos Aires, Argentina.

[3] Universidad Nacional de Córdoba. Av. Vélez Sársfield 299, 5000 Córdoba, Argentina.

[4] Programa de Monitoreo Sanitario Ballena Franca Austral, Triunvirato 56, Puerto Madryn, Chubut 9120, Argentina.

[5] Ocean Alliance/Whale Conservation Institute. 191 Weston Rd, Lincoln, MA 01773 USA.

[6] Fundación Patagonia Natural. Marcos A. Zar 760. CP 9120, Puerto Madryn, Argentina

[7] Centro Nacional Patagónico, Consejo Nacional de Investigaciones Científicas y Técnicas. Bv. Brown 2825, CP 9120, Puerto Madryn, Argentina.

[8] Global Health Program, Wildlife Conservation Society, Bronx, NY USA

ABSTRACT

Strong site fidelity to breeding or feeding grounds may diminish an animal's ability to find and use better areas when its current habitat becomes unsuitable. Southern right whale populations are recovering from near extinction, and as their numbers have increased they have begun to reutilise former habitats. An assessment of intra- and inter-population genetic heterogeneity is needed to reveal how dispersal influences survival and reproduction in this species. Here we address several aspects of genetic structure in the Península Valdés population, using sequence data from a 630bp region of the mitochondrial genome. We examine genetic differentiation between areas, age-sex classes, calving cohorts, and living versus dead whales, and we reassess the large-scale population structure of Eubalaena australis throughout its range using all available mitochondrial data. There is significant genetic heterogeneity among years for stranded (dead) individuals but not for living individuals at Península Valdés. The pattern of genetic differentiation previously described between the subpopulations breeding off South America, South Africa, Australia and New Zealand is seen in the enlarged mitochondrial data set.

KEYWORDS: POPULATION GENETIC STRUCTURE; BREEDING GROUNDS; SOUTHERN RIGHT WHALE; STRANDINGS

INTRODUCTION

Genes and genotypes are often not randomly distributed between and within populations (Futuyma, 1986; Hartl and Clark, 1989). Factors such as habitat selection and site fidelity can produce genetic structure even in highly mobile species such as migratory birds and marine mammals (Hoelzel, 1998; Webster and Marra, 2005). Genetic structure of mitochondrial DNA (mtDNA) among populations as a consequence of maternally directed site fidelity is a recognized characteristic of many species of baleen whales (Hoelzel, 1998). However, the extent of genetic structure within populations and its consequences for survival and reproduction has not been fully explored (Rosenbaum *et al.*, 2002). Southern right whales (*Eubalaena australis*) are found in all the oceans of the Southern Hemisphere where they migrate between winter coastal nursery grounds and offshore, mostly unknown, summer feeding grounds (IWC, 2001). Nursery grounds are used primarily by adult females for raising their calves during their first three months of life (Payne, 1986; Best, 1994).

Long-term photo identification studies have shown that females return to the same nursery ground in their calving years, and that there is little exchange of animals between nursery grounds, despite the lack of obvious geographic barriers (Best *et al.*, 1993; IWC, 2001). MtDNA analyses have confirmed that all major nursery grounds are genetically differentiated and that the differentiation is probably maintained by maternally directed site fidelity (Portway, 1998; Baker *et al.*, 1999; Patenaude *et al.*, 2007). A recent discovery of genetic heterogeneity on the nursery ground at Península Valdés appears to be driven by population structure on the feeding grounds (Valenzuela *et al.*, 2009). However, a thorough assessment of intra-population heterogeneity and its consequences for survival and reproduction of southern right whales is still lacking.

The overall genetic differentiation among southern right whale nursery areas has been well documented (Portway, 1998; Baker *et al.*, 1999; Patenaude *et al.*, 2007). Portway (1998) and Baker *et al.* (1999) found population differentiation within the South Atlantic and the Indo-Pacific ocean basins respectively, and Patenaude *et al.* (2007) reported genetic differentiation between these two ocean basins. Together, these studies document significant genetic differentiation at the haplotype and nucleotide levels among the four known calving grounds: Península Valdés and South Africa in the South Atlantic basin, and south-western Australia and New Zealand in the Indo-Pacific basin. The limited gene flow reported in all of these studies is thought to result from maternal fidelity to nursery grounds (Schaeff *et al.*, 1993; Hoelzel, 1998). Within ocean basins, no genetic differentiation has been found between nursery grounds and feeding grounds (Portway, 1998; Baker *et al.*, 1999; Patenaude *et al.*, 2007). This could be explained by mixing of lineages from neighbouring nursery grounds on common feeding grounds (Patenaude *et al.*, 2007). At the level of ocean basins, differentiation is higher, with only one mtDNA haplotype shared by both basins (Patenaude *et al.*, 2007).

More sampling and new types of analyses are revealing population genetic structure on the feeding grounds and the existence of within-population substructure on the nursery grounds. Recently, Valenzuela *et al.* (2009) combined genetic and stable isotope analyses of skin samples to show that mtDNA haplotypes of adult females from Península Valdés are not randomly distributed along an isotopic range. Furthermore, whales from the same matriline showed isotopic values more similar to each other than expected by chance (Valenzuela *et al.*, 2009). In addition, the mean isotope value for each haplotype was structured along a gradient of carbon and nitrogen isotopes, indicating feeding along a latitudinal gradient (Valenzuela *et al.*, 2009). Because isotope ratios in animal tissues are known to reflect the animal's diet and migratory origins (Hobson, 1999), the authors interpret this result as evidence of genetic structure on the feeding grounds. This is the first indication of genetic heterogeneity within a nursery-area subpopulation of southern right whales. The North Atlantic right whale (*Eubalaena glacialis*) has a similar pattern: a subgroup of females shows female directed site fidelity to the Bay of Fundy feeding ground, while a second group uses unknown feeding areas (Schaeff *et al.*, 1993; Malik *et al.*, 1999). The existence of a similar pattern in other southern right whale winter aggregations is unknown, as is the occurrence of any other internal substructuring.

The consequences of such substructuring within a breeding population are unknown; however, a likely result is that the effects of changes in food abundance within a particular feeding ground might not be spread across an entire breeding population, but instead focused on particular genetic lineages. On the nursery ground this could be reflected in changes in the haplotype frequencies of calving mothers in particular years (calving cohorts) or in the haplotype frequencies of stranded whales.

Although little is known about other substructuring within southern right whale populations, there is evidence of internal substructuring on the breeding grounds of the ecologically similar grey whale (*Eschrichtius robustus*). Grey whales, in their calving lagoons along the Pacific coast of Baja California show significant genetic differentiation between calving females and mating females, as well as low, but not significant, differentiation between calving females using two different lagoons (Goerlitz *et al.*, 2003). This pattern is thought to result from a fine scale site fidelity to natal lagoons, especially during calving years (Goerlitz *et al.*, 2003). Similarly, on the shores of Península Valdés, southern right whales congregate primarily in two gulfs, Golfo San José (GSJ) and Golfo Nuevo (GN), with similar numbers of mother-calf pairs in each gulf (Rowntree *et al.*, 2001). In the 1970s and early 1980s, different proportions of age-sex categories were found in different areas of Península Valdés (Payne, 1986). Adult males and single females predominated in GN, all age-sex classes were found in GSJ but with a higher proportion of juveniles, while the largest proportion of mother-calf pairs was found off the outer coast. With time, the distribution and proportion of animals in each area have changed. Females with calves abandoned the location that had the highest concentration of mother-calf pairs (the outer coast; Rowntree *et al.*, 2001). Although the observed change in distribution seems to work against the establishment of genetic structure due to site fidelity within sub-areas of the nursery ground, some other processes that produce and maintain genetic heterogeneity within an otherwise homogenous population might be taking place (e.g. kinship based social structuring).

Here we describe a preliminary assessment of genetic substructuring of southern right whales on the Península Valdés nursery ground, using sequence data from a 630 bp region of the mitochondrial genome; we compare gulfs, age-sex classes, reproductive states and calving cohorts, and living versus dead whales. We also reanalyse the large-scale structure of southern right whales by combining our samples with those previously reported for other populations (Portway, 1998; Baker *et al.*, 1999; Malik *et al.*, 2000; Patenaude *et al.*, 2007).

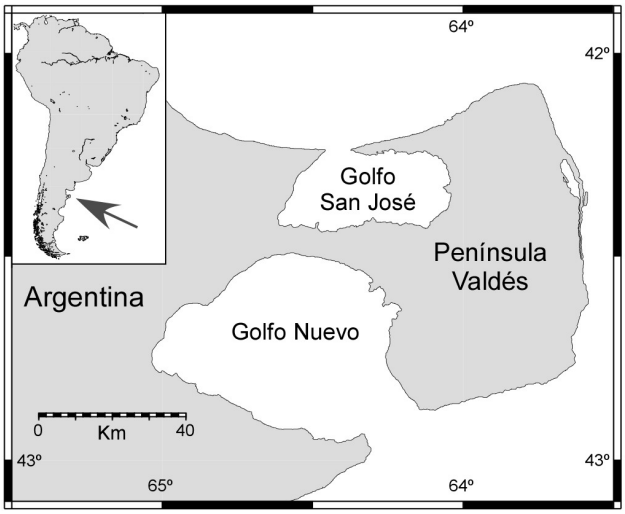
MATERIALS AND METHODS

Sample collection

Live whales. Skin samples were obtained from 219 whales by biopsy darting live animals off Península Valdés (42º 30' S, 64º 00' W; Fig. 1), Argentina. Sample collection was concentrated in the northern gulf, Golfo San Jose (GSJ) though some skin samples were collected in the southern gulf, Golfo Nuevo (GN). In GSJ, biopsy collection was carried out over four consecutive years (2003 – 2006) at the time of peak whale abundance (September and October; Payne, 1986). In GN sampling took place in 2005 and 2006, from August to October. To avoid resampling of whales, individuals were photographed for identification based on callosity patterns and other natural marks (Payne *et al.*, 1983). Samples were preserved in saturated NaCl with 20% DMSO for long-term storage (Amos and Hoelzel, 1991). Age classes (adults and juveniles) were identified primarily based on body size; adult females were recognized by the close proximity of a calf over an extended period of time. Gender of juveniles and single adults was determined whenever possible by observation of the genital area; otherwise, gender was identified by PCR amplification and electrophoresis of Z*fx* and Z*fy* introns following Shaw *et al.* (2003).

Dead whales. Two sets of skin samples from dead animals were used in our analyses. The first set (*set A*) was a group of 43 skin samples from whales that diead at Península Valdés between 2003 and 2006, the same period that skin samples were collected from live whales. The second set (*set B*) includes a total of 155 skin samples and includes the above mentioned samples collected from dead whale in the 2003-2006 period (n = 43) and an additional 112 samples from whales that died between 2007 and 2009. *Set A* was previously analyzed as part of the first author's Ph.D. dissertation (Valenzuela, 2008); *set B* was obtained and analysed later. All skin samples from dead whales were provided by the Programa de Monitoreo Sanitario Ballena Franca Austral (PMSBFA), which is active from June through December (Uhart *et al.*, 2008, 2009). At each stranding, body measurements and skin tissue were collected; all samples were preserved in 70% EtOH and the dead animals were tagged to avoid resampling. Whales were classified by age class (calves, juveniles and adults) based on their length and sex class based on observations of external or internal reproductive anatomy.

Figure 1: Map of Península Valdés, Argentina showing the two gulfs where samples were collected. Inset shows the position of Península Valdés on the South America coast.



MtDNA sequences.
DNA was extracted using a standard phenol-chloroform procedure for cetacean skin as described in Amos and Hoelzel (1991). A 630 base pairs region of the mitochondrial genome was amplified by polymerase chain reaction (PCR) using primers AB6617 and H00034 (Malik *et al.,* 1999). This sequence starts at nucleotide 40 of t-Thr, and includes t-Pro and the first 530 bp of the control region. The purified PCR product was sequenced in both directions and sequences were assembled using Sequencher 4.5 software (Gene Codes Corp.) and deposited at GenBank with accession numbers EU290462-EU290592 and GQ389687-GQ389690. Sequence alignment was conducted using Clustal X 1.8 software (Thompson *et al.*, 1997). Haplotypes were named following the format used in previous publications (Patenaude *et al.,* 2007), using a three-letter abbreviation of the author followed by an alphabetical code corresponding to individual haplotype identity.

Phylogenetic relationships

A phylogeny of the mtDNA haplotypes was reconstructed using neighbour-joining and maximum likelihood methods in the program PAUP (Swofford, 2003). The tree was rooted with a homologous sequence extracted from the complete mitochondrial genome of a North Pacific right whale (*E. japonica*); GenBank accession number AP006474 (Sasaki *et al.,* 2005).

Genetic diversity and within-population differentiation.

Haplotype (*h*) and nucleotide (π) diversity (Nei, 1987) were estimated using Arlequin 2.0 (Schneider *et al.,* 2000). The degree of differentiation among groups (e.g. among areas, years, age and gender classes) was estimated by analysis of molecular variance (AMOVA; Excoffier *et al.,* 1992) as implemented in Arlequin 2.0, using both haplotype frequencies (*Fst*; Wright, 1951) and molecular distances (Φst; Excoffier *et al.,* 1992).

Southern Hemisphere population differentiation

The population structure of southern right whales was reanalysed by incorporating the 262 samples reported by L.O.V. in his PhD dissertation (219 live + 43 stranded) to the 146 samples reported by Patenaude *et al.* (2007). The populations used were Argentina (AR, n = 282), South Africa (SA, n = 41), South Georgia feeding ground (SGF, n = 8), New Zealand (NZ, n = 42), south-western Australia (SWA, n = 20) and south-western Australia feeding ground (SWAF, n = 5). Haplotype (h) and nucleotide (π) diversity, and the degree of differentiation were calculated as described above.

We reconstructed the haplotypes reported by Patenaude *et al.* (2007) using the 37 variable sites from their paper (Table 2, page 150), and as templates, the haplotypes reported by Malik *et al.* (2000; GenBank accession numbers AF395044-AF395053). The resulting haplotypes are 275 bp long, and the first nucleotide corresponds to base number 119 in the 630 bp sequences. Where these reduced sequences are identical to those from previous studies, haplotypes keep the names used by Patenaude *et al.* (2007).

RESULTS

Sequence analysis and mtDNA phylogeny

Sequence analysis of a 630 base pair region of the mitochondrial genome from 374 whales (live + *set B*) revealed 37 unique haplotypes (Table 1). These haplotypes are defined by 55 polymorphic sites; of those, 47 are transitions, 6 are transversions and 2 are insertions (Table 1). The insertions represent an extra adenine within a small region of four consecutive adenines (from position 100 to 103). Because it is impossible to determine the exact position of the insertions, we arbitrarily placed them at position 100 for the clade with haplotypes O and Y, and at position 104 for the clade with haplotypes R, FF and W (Fig. 2). The 37 haplotypes are not equally represented in the sample; for example, one third (n=12) of the haplotypes account for almost 75% of the sample, while another third account for just 4% of the sample (Table 1). The overall haplotype (*h*) and nucleotide (π) diversity were 0.95 ± 0.01 and 1.63 ± 0.82% respectively.

Phylogenetic reconstruction of these 37 haplotypes revealed two main clades that correspond to the previously known "A" and "W" clades (Fig. 2; Baker *et al.*, 1999; Patenaude *et al.*, 2007). Clade A and W are supported by 72% and 63% bootstrap values respectively when rooted with a North Pacific right whale sequence. Clade A contains 16 haplotypes and 54% (n = 201) of the total sample, while clade W has 21 haplotypes and 51% (n= 189) of the total sample (Table 2). When the samples from live whales and from *set A* were analyzed together, the haplotype diversity was similar between the two clades (clade A $h = 0.91 \pm 0.01$, clade W $h = 0.88 \pm 0.01$) while nucleotide diversity was 3 times higher in clade W ($\pi = 1.45 \pm 0.74\%$) than in clade A ($\pi = 0.48 \pm 0.28\%$). This analysis has not been repeated using the total sample (Live + *set B*).

Within-population differentiation

Significant differentiation was found between live (n = 219) and stranded whales in *set A* (n = 43) for the period 2003-2006 (Table 2). Stranded whales were more abundant in clade A (Table 2). The *Fst* statistic, based on haplotype frequencies, shows that 1% of the variance was accounted for by these two groups ($P < 0.05$). At the nucleotide level, the *Φst* statistic shows that 4% of the molecular variance is explained by the separation between samples from live and stranded whales ($P < 0.005$). However, when the samples in *set B* were compared with live whales no differentiation was found (*Fst* = 0.001, $P > 0.1$). Haplotype and nucleotide diversity were similar between live and the *set A* of stranded whales (live whales, $h = 0.94 \pm 0.005$, $\pi = 1.6\% \pm 0.8$; *set A* stranded whales, $h = 0.95 \pm 0.01$, $\pi = 1.6\% \pm 0.8$).

The stranded whales (*set B*) show significant genetic differentiation among years (Fst = 0.008, P = 0.03), and this contrasts sharply with the live whales, which show no differentiation among years (*Fst* = -0.001, $P > 0.1$). No genetic differentiation among other groups of live whales was detected. When live whales were used to test for genetic differentiation between Golfo Nuevo (n = 21) and Golfo San José (n = 198), *Fst* was low and almost significant (*Fst* = 0.015, $P = 0.051$), while Φ_{ST} was also low and not significant ($\Phi_{ST} = 0.001$, $P > 0.1$). No genetic differentiation was detected among age-sex groups of live whales at the haplotype or nucleotide level (overall *Fst* = 0.002, overall *Φst* = -0.008, $P > 0.1$ for both), or between calving females and single females (*Fst* = 0.001, *Φst* = 0.007, $P > 0.1$). These comparisons (i.e., areas, age-sex groups) have not been repeated for stranded animals.

Southern Hemisphere population structure (275 bp haplotypes)

Sequence alignment of the 35 haplotypes discovered in this study using live whales and stranded whales from the 2003-2006 period (*set A*) with the 37 haplotypes previously published (Patenaude *et al.*, 2007) revealed 45 unique haplotypes (of length 275 bp) for the whole Southern Hemisphere. This represents eight newly discovered haplotypes for the entire species, seven of which belong to clade W. With these additions, the overall haplotype diversity (h) for southern right whales is 0.955 (±0.003) and the overall nucleotide (π) diversity is 2.80% (±1.45%). The Península Valdés sample now includes of 33 haplotypes of which 14 are new for this population. Haplotype and nucleotide diversities in the Península Valdés whales are high and similar to values for the whole Southern Hemisphere ($h = 0.94 \pm 0.005$, $\pi = 2.83\% \pm 1.47$).

Significant differentiation at the haplotype and nucleotide level was found among the six subpopulations analysed (overall *Fst* = 0.166, $P < 0.001$; overall *Φst* = 0.158, $P < 0.001$). Argentina shared haplotypes with all populations (Table 3); however, no single haplotype was present in all the populations and only two haplotypes were shared by the South Atlantic and Indo-Pacific ocean basins (BakHapA and BakHapE). Pairwise comparisons at the haplotype level (*Fst*) revealed differences between Argentina and all other populations ($P < 0.01$; Table 3) except the South Georgia feeding ground (*Fst* = 0.007, $P > 0.05$; Table 3). In comparisons at the nucleotide level (*Φst*), the differentiation was statistically significant when compared to other nursery areas ($P < 0.001$; Table 3) but not to either feeding ground (*Φst*= -0.016 for SG, *Φst* = 0.078 for SWA F; $P > 0.05$ for both comparisons), although the sample sizes for these two areas are too small for reliable statistical comparisons (Table 3).

Table 1: Polymorphic sites in 630 bp region of mtDNA of 374 southern right whales (live + *set B*). Similarities are noted by "·", and differences are noted by proper nucleotide changes. The grey area represents the variable sites found in the same region studied by Patenaude *et al.* (2007); position 119 in our study corresponds to position 1 in Patenaude *et al.* (2007). "N" represents the haplotype frequency.

Haplotype	N	1 2	1 3	1 6	4 8	5 0	0 0	1 4	1 6	1 7	1 8	1 2	1 3	1 5	1 6	1 4	1 5	2 2	2 3	0 1	2 1	2 9	2 2	3 4	3 6	3 2	3 5	3 3	3 5	4 0	4 7	4 2	4 3	4 3	4 3	4 4	5 3	5 6	5 7	5 8	6 4	6 9	6 1	Synonyms[1]
valhapM	44	C	A	C	T	T	-	-	A	T	C	T	A	T	C	G	T	T	A	G	G	T	C	T	C	C	C	T	G	A	G	C	T	C	A	A	T	T	T	C	A	G	T	MalHapF
valhapF	36	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	T	C	.	T	T	.	A	G	A	.	.	.	.	.	C	C	.	T	.	.	.	G	.	PorHap4
valhapJ	31	.	.	.	.	.	.	.	.	.	.	.	.	C	T	A	C	C	G	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	T	.	.	C	.	T	MalHapJ
valhapK	27	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.	.	T	.	.	.	G	.	PorHap16
valhapD	21	.	.	.	.	C	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	T	C	T	T	.	.	A	G	A	.	.	.	.	.	C	C	.	T	.	.	.	G	.	PorHap7
valhapL	21	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.	.	T	.	.	.	G	.	MalHapH
valhapB	19	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	T	C	.	T	.	.	A	G	A	.	.	.	.	.	C	C	.	T	.	.	.	G	.	MalHapD
valhapA	17	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	T	C	.	T	.	.	A	G	A	.	.	.	.	.	C	C	.	T	.	.	.	G	.	PorHap3
valhapE	16	.	.	.	.	C	.	.	.	.	.	.	.	C	.	.	.	.	G	.	.	.	T	C	.	T	T	.	A	G	A	.	.	.	.	.	C	C	.	T	.	.	.	G	.	MalHapB
valhapQ	16	.	.	.	.	C	.	.	.	T	.	.	.	.	.	.	.	.	G	.	.	.	T	C	.	T	.	.	A	G	A	.	.	.	.	.	C	C	.	T	.	.	.	G	.	MalHapE
valhapZ	14	.	.	.	.	C	.	.	.	T	.	.	C	.	.	.	.	.	.	.	.	.	T	C	.	T	.	.	A	G	A	.	.	.	.	.	C	C	.	T	.	.	.	G	.	PorHap11
valhapH	12	.	.	.	.	C	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	.	T	C	.	T	T	.	A	G	A	.	.	.	.	.	C	C	.	T	.	.	.	G	.	BakHapA
valhapW	11	.	.	.	.	.	.	A	.	.	.	.	.	T	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	G	C	.	C	T	.	.	.	.	G	G	.	PorHap25
valhapC	10	.	.	.	.	C	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	T	C	.	T	.	.	A	G	A	.	.	.	.	.	C	C	.	T	.	.	.	G	.	PorHap5
valhapO	10	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	
valhapI	9	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	C	C	G	.	.	.	T	.	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.	C	.	T	PorHap12
valhapT	8	.	.	.	.	C	.	.	.	.	.	.	.	C	T	.	.	.	G	.	.	.	T	C	.	T	T	.	A	G	A	.	.	.	.	.	C	C	.	T	.	.	.	G	.	PorHap6
valhapP	7	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	C	.	G	.	.	.	T	.	.	.	.	.	.	A	T	C	.	.	.	C	.	.	T	.	.	C	.	T	.	
valhapX	7	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	T	C	.	T	T	.	A	G	A	.	.	.	.	.	C	C	.	T	.	A	.	G	.	PorHap4
valhapAA	6	.	.	.	.	C	.	.	.	T	.	.	C	.	.	.	.	.	.	.	.	.	T	C	.	T	.	.	A	G	A	.	.	.	.	.	C	C	.	T	G	.	.	G	.	PorHap11
valhapDD	5	.	.	.	C	.	.	.	.	T	.	.	C	T	.	C	C	.	A	.	.	T	.	.	.	.	.	.	.	.	.	T	G	.	C	C	.	.	.	.	.	.	C	G	T	.	G	.	.	
valhapY	4	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	G	.	.	
valhapBB	3	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	T	C	.	T	T	.	A	G	A	.	.	.	.	.	C	.	.	T	.	.	.	G	.	.	
valhapEE	2	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	G	G	.	.	
valhapG	2	.	.	.	.	C	.	.	.	.	.	.	.	C	T	.	.	.	G	.	.	.	T	C	.	T	T	.	A	G	A	.	.	.	.	.	C	C	.	T	.	.	.	.	.	PorHap6
valhapGG	2	.	.	.	.	.	.	.	G	.	T	C	G	C	T	.	.	C	.	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	.	T	.	.	T	.	.	MalHapI
valhapN	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	PatHap27
valhapR	2	.	.	.	.	.	.	A	.	.	.	.	.	T	.	C	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	T	.	.	G	C	.	.	T	.	.	T	.	T	G	G	.	.	
valhapCC	1	.	.	.	.	.	.	.	G	.	T	C	G	C	T	.	.	C	.	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	.	T	.	.	T	.	.	G	.	C	.	MalHapI
valhapFF	1	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.	T	.	T	.	.	.	.	.	.	.	T	.	.	G	C	.	.	T	.	.	T	.	T	G	G	.	.	
valhapHH	1	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	T	.	.	.	T	.	.	.	.	C	.	.	.	.	T	.	.	C	C	.	T	.	.	.	.	.	PorHap17
valhapII	1	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	MalHapF
valhapS	1	.	.	G	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	T	C	.	T	T	.	A	G	A	.	.	.	.	.	C	C	.	T	.	.	.	G	.	PorHap4
valhapU	1	.	.	.	.	.	.	.	.	.	.	.	.	T	.	C	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.	.	T	.	.	.	G	.	.	A	MalHapH
valhapV	1	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	MalHapF
valhapJJ[2]	2	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	not aligned
valhapKK[2]	1	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	T	C	T	T	.	.	A	G	A	.	.	.	.	.	C	C	.	T	.	.	.	G	.	.	not aligned

[1] Previously published haplotypes from Portway (1998), Baker *et al.* (1999), Malik *et al.* (2000) and Patenaude *et al.* (2007). [2] Discovered among the new samples added to *set B*; no alignment with previously published haplotypes (of length 275 bp) has been attempted.

Figure 2: Phylogenetic relationships among 37 mtDNA haplotypes of 630 bp length from southern right whales off Península Valdés, Argentina. The tree is rooted with a homologous sequence of a North Pacific right whale (Sasaki *et al.*, 2005). Bootstrap percentages after 10,000 replications are indicated next to clades A and W.

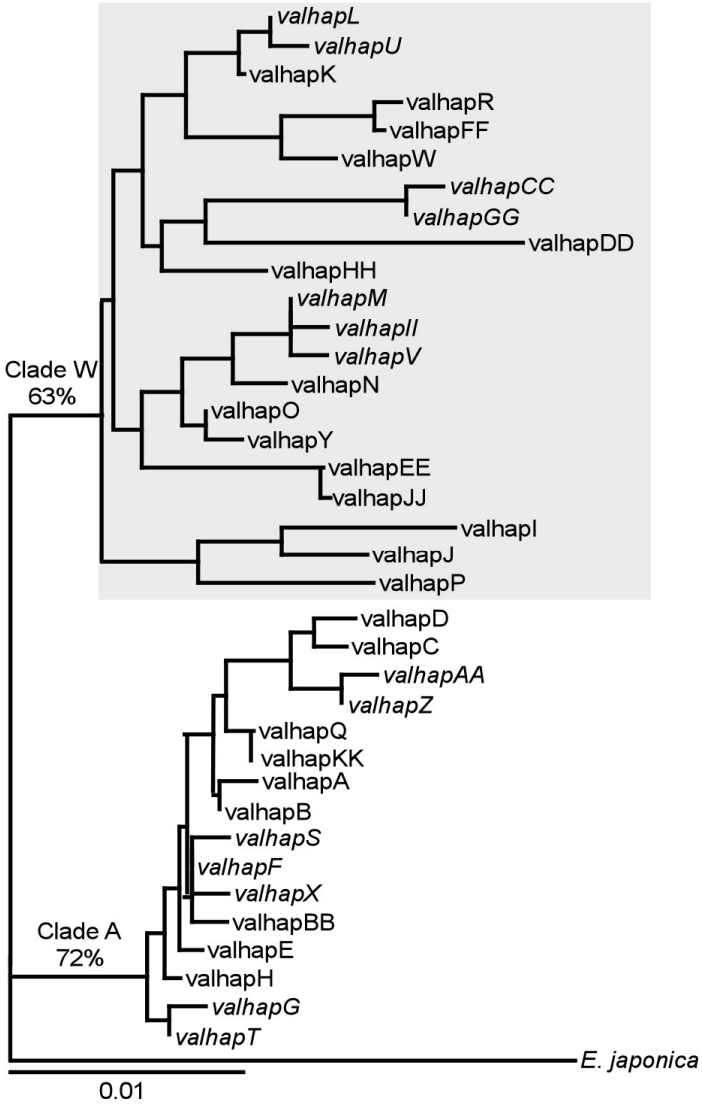


Table 2: Haplotype distribution by year of collection for live and stranded animals. Clades A and W correspond to the same clades in Fig. 2.

		Live					Stranded							
Clade	**Haplotyp**	**2003**	**2004**	**2005**	**2006**	**Total**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**Total**
W	valhapM	7	7	10	5	29			1		4	9	1	15
W	valhapJ	4	2	9	4	19		1	3	1	3	4		12
W	valhapK	2	6	7	3	18			1		3	3	2	9
W	valhapL	1	2	4	2	9				1	4	7		12
W	valhapW	2		1	2	5			2		1	3		6
W	valhapO	2	3	1	2	8					1		1	2
W	valhapI		6	1	1	8					1			1
W	valhapP		2	2	1	5						2		2
W	valhapDD							1	3			1		5
W	valhapY			3		3						1		1
W	valhapEE			1	1	2								
W	valhapGG				1	1							1	1
W	valhapN		2			2								
W	valhapR	1	1			2								
W	valhapJJ										2			2
W	valhapCC			1		1								
W	valhapFF			1		1								
W	valhapHH				1	1								
W	valhapII				1	1								
W	valhapU		1			1								
W	valhapV		1			1								
A	valhapF	5	4	6	3	18	2		2	1	5	5	3	18
A	valhapD	2	4	2	4	12		1	2	1		3	2	9
A	valhapB	1	2	2	6	11				2	2	4		8
A	valhapA	3	2	2	5	12			1	1	1	2		5
A	valhapE	2	4	3	3	12		1	1		1	1		4
A	valhapQ	1	2	1	4	8	1				2	3	2	8
A	valhapZ			2	3	5	1		3		3	2		9
A	valhapH	1	1	3	1	6	2	1			2	1		6
A	valhapC	2	1	1	2	6							4	4
A	valhapT	1	1	1		3	1		1			2	1	5
A	valhapX			3		3	1			1	1	1		4
A	valhapAA	1		1		2			1	1	1		1	4
A	valhapBB			1	1	2					1			1
A	valhapG		1			1						1		1
A	valhapS		1			1								
A	valhapKK											1		1
	TOTAL	38	56	69	56	219	8	5	21	9	38	56	18	155

Table 3: Pairwise genetic comparisons between Península Valdés, Argentina, and five seasonal subpopulations. For each group, the total number of haplotypes followed by the sample size is shown between parentheses.

Argentina (33/282)	***Fst***	***Φst***	**Shared haplotypes**
South Africa (21/41)	0.034***	0.056***	15
South Georgia F. (8/8)	0.007	-0.016	6
New Zealand (4/42)	0.192***	0.208***	1
Australia (5/20)	0.130***	0.113***	2
SWAustralia F (3/5)	0.125**	0.078	1

*** P <0.001, ** P <0.01

DISCUSSION

Genetic diversity and phylogeny

Although southern right whales in the South Atlantic were hunted for a long period of time (as recently as the 1960s), levels of genetic diversity do not seem to have been greatly affected. The haplotype diversity currently detected at Península Valdés is relatively high and similar to levels previously reported for this, and other, recovering southern right whale populations (Portway, 1998; Baker *et al.*, 1999; Malik *et al.*, 2000; Patenaude *et al.*, 2007). The nucleotide diversity appears lower than previously reported for this population, but this appears to be due to the use of a longer sequence interval, much of which is well conserved. When nucleotide diversity was recalculated using shorter sequences we found values similar to those previously reported (Portway, 1998; Patenaude *et al.*, 2007). As pointed out by Portway (1998), commercial whaling appears not to have reduced genetic diversity in the Península Valdés population to an extent that has affected its recovery.

The large number of samples and longer mtDNA sequences used in this study has revealed 37 haplotypes in the whales from Península Valdés, as many haplotypes as were previously known for the entire Southern Hemisphere (37 haplotypes presented by Patenaude *et al.*, 2007). The phylogenetic reconstruction of these 37 haplotypes reveals two well-supported clades that correspond to the previously reported A and W clades (Baker *et al.*, 1999; Patenaude *et al.*, 2007). The low-diversity clade A may represent a clade that historically had a smaller population size or suffered more depletion than clade W. However, this analysis needs to be repeated using a larger sample size (live + *set B*). We found that the Argentinean right whale population is divided equally between the two clades. In contrast, Patenaude *et al.* (2007) found 90% (n=18) of the samples from Península Valdés concentrated within clade W and only 10% (n = 2) within clade A. The large increase in the number of haplotypes within clade A is most simply explained by the larger sample size in our study. It is important to note that 19 of those 20 samples presented by Patenaude *et al.* (2007) were collected from *stranded* whales between 1994 and 1996 (original data in Portway, 1998). An alternative explanation for the equal frequency of both clades in our sample involves the influx of immigrants from areas that are rich in clade A (South Africa and New Zealand; Patenaude *et al.*, 2007), indicating contemporary gene flow between, or complete mixing of, formerly isolated populations (Portway, 1998; Patenaude *et al.*, 2007).

Within-population heterogeneity

The most unexpected finding in this study was the non-random distribution of mtDNA lineages among dead whales. The among-year differentiation of the stranded animals does not result from just one year being distinct from all the others; instead, most pairwise comparisons present positive values of *Fst*, and some of these are individually significant (data not shown). This pattern seems to suggest that at least some portion of the recent (2007-2009) increase in calf mortality at Península Valdés has been caused by processes that occurred away from the Peninsula, on feeding grounds where the population shows modest levels of mitochondrial genetic differentiation. The observed difference between live and stranded whales for the period 2003-2006, as well as the differential proportion of clade A and W in the stranded sample are intriguing, but the small sample size of some years preclude

a rigorous analysis and interpretation of the data. Furthermore, if the distribution of haplotypes discovered in all stranded whales (*set B*, 2003-2009) is compared against the overall distribution of haplotypes (live + *set B*) or just the distribution discovered in live whales we found no differentiation between stranded and live whales and no differences in the frequency of haplotypes and samples within clades. Unfortunately, there are no samples from live whales in the period 2007-2009 which could be compared directly to the samples of dead.

Differential susceptibility to environmental variability affecting food abundance on feeding grounds could explain the differential mortality for some haplotypes among years. Changes in krill abundance in the western South Atlantic are correlated with sea surface temperature anomalies and changes in the amount of sea ice around Antarctica (Trathan *et al.,* 2006; Murphy *et al.,* 2007). These anomalies affect the reproductive output of marine predators, including the southern right whale (Leaper *et al.,* 2006; Trathan *et al.,* 2006). Valenzuela *et al.* (2009) showed that southern right whales appear to have site fidelity to feeding grounds. If the climate, and resource, variability affect different areas in different years and months (e.g., early and late feeding season), as is the case for changes in sea ice extent and SST associated to the Antarctic Circumpolar Wave (Barbraud and Weimerskirch, 2001), then a pattern of genetic heterogeneity among years would appear in stranded animals. Future research should examine with more detail the population structure of southern right whales throughout their entire migratory range by taking skin samples and photographs on the feeding grounds or using indirect methods to infer migratory origins (such as stable isotopes and/or trace metals).

Other than the heterogeneity among stranded calves across years, no evidence of genetic heterogeneity was found within the Península Valdés population. The weak, yet not significant, differentiation in haplotype frequencies detected between Golfo Nuevo and Golfo San José is probably the result of the small number of samples collected from Golfo Nuevo. The lack of heterogeneity between gulfs is supported by observations of the same whales using different gulfs in different years as well as some whales moving between gulfs in the same season (Rowntree *et al.,* 2001). However, this comparison must be repeated including samples collected from stranded whales.

Southern Hemisphere population structure

Additional sampling substantially increased the genetic differentiation between Argentina and South Africa at the haplotype level, but decreased the genetic differentiation between Argentina and both nursery areas in the Indo-Pacific basin (Australia and New Zealand) at the haplotype and nucleotide levels. No genetic differentiation was found between Argentina and both feeding grounds (South Georgia and southwestern Australia); however the small sample sizes from these feeding grounds prevent further statistical analyses and conclusions. Although the use of a large number of samples is likely responsible for the changes in genetic differentiation among populations, the lower differentiation detected between Argentina and the Indo-Pacific basin could result from a recent increase in gene flow between populations. Further sampling on different feeding grounds would increase our understanding of the population structure of southern right whales across the Southern Hemisphere.

ACKNOWLEDGEMENTS

We thank the following individuals for their invaluable contribution to sample collection: Rafael Benegas, Moira Brown, Carole Carlson, Jose Carracedo, Sarah Haney, Julieta Martino, Maximiliano Oro, Martin Quadro, Roxana Schteinbarg, Sydney Stringham, Diego Taboada and Alejandra Varisco. We also thank R. Schteinbarg, D. Taboada and all Instituto de Conservación de Ballenas volunteers for their logistic and institutional support. We thank Armada Argentina and Guillermo Harris of Wildlife Conservation Society for permission to use the research station. We also thank all members, volunteers and collaborators of the Stranding Network from the Programa de Monitoreo Sanitario Ballena Franca Austral (PMSFBA, conducted by WCI/ICB, WCS, FPN and EC). Zofia A. Kaliszewska and Heather McGirk provided invaluable technical assistance during lab analyses. This work was supported by funding from Ocean Alliance/Whale Conservation Institute (OA/WCI), Canadian Whale Institute, Modeling the Dynamics of Life Fund (University of Utah), Department of Biology (University of Utah) and Associated Students of the University of Utah (ASUU) Senate Contingency Fund. We thank the Office of Protected Resources of the US National Marine Fisheries Service, National Ocean and Atmospheric Administration (Order #DG-133F-06-SE-5823), which provided the funds to develop and continue the PMSBFA. Biopsy collection from live and stranded whales was conducted under permits from Dirección de Fauna y Flora Silvestre (DFyFS) and Secretaría de Turismo

y Areas Protegidas del Organismo Provincial de Turismo (OPT), Chubut Province. Biopsy samples were imported under NMFS permit #751-1614 and CITES permits #03US819824/9, #04US082589/9, #05US819824/9. Samples from stranded animals were imported under permits NMFS permit# 932-1489-05 and CITES permit #04S020950/9, 05US082589/9 and 06US082589/9. This research was approved by the University of Utah Institutional Animal Care and Use Committee (IACUC) under assigned protocol number 05-01003.

LITERARUTE CITED

Amos, B. and Hoelzel, A.R. 1991. Long-term preservation of whale skin for DNA analysis. *Rep. int. Whal. Commn.* (special issue) 13:99-103.

Baker, C.S., Patenaude, N.J., Bannister, J.L., Robins, J. and Kato, H. 1999. Distribution and diversity of mtDNA lineages among southern right whales (*Eubalaena australis*) from Australia and New Zealand. *Mar. Bio.* 134:1-7.

Barbraud, C. and Weimerskirch, H. 2001. Emperor penguins and climate change. *Nature* 411(6834):183-186.

Best, P. 1994. Seasonality of reproduction and the length of gestation in southern right whales *Eubalaena australis*. *J. Zool. (Lond)* 232:175-189.

Best, P., Payne, R., Rowntree, V., Palazzo, J. and Both, M. 1993. Long-range movements of South Atlantic right whales *Eubalaena australis. Mar. Mamm. Sci.* 9:227-234.

Excoffier, L., Smouse, P.E. and Quattro, J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* 131:479-491.

Futuyma, D. 1986. *Evolutionary Biology*. Sinauer Associates, Sunderland, Massachusetts. 763pp.

Goerlitz, D.S., Urban, J., Rojas-Bracho, L., Belson, M. and Schaeff, C.M. 2003. Mitochondrial DNA variation among eastern north pacific gray whales (*Eschrichtius robustus*) on winter breeding grounds in Baja California. *Can. J. Zool.* 81:1965-1972.

Hartl, D.L. and Clark, A.G. 1989. *Principles of population genetics*. Sinauer Associates, Sunderland, Massachusetts. 542 pp.

Hobson, K. 1999. Tracing origins and migration of wildlife using stable isotopes: a review. *Oecologia* 120:314-326.

Hoelzel, A. 1998. Genetic structure of cetacean populations in sympatry, parapatry, and mixed assemblages: implications for conservation policy. *J. Hered.* 89:451-458.

International Whaling Commission. 2001. Report of the Workshop on the Comprehensive Assessment of right whales: a worldwide comparison. *J. Cetacean Res. Manage.* (special issue) 2:1-60.

Leaper, R., Cooke, J., Trathan, P., Reid, K., Rowntree, V. J. and Payne, R. 2006. Global climate drives southern right whale (*Eubalaena australis*) population dynamics. *Biol. Lett.* 2:289-292.

Malik, S., Brown, M.W., Kraus, S.D., Knowlton, A., Hamilton, P. and White, B. 1999. Assessment of mitochondrial DNA structuring and nursery use in the North Atlantic right whale (*Eubalaena glacialis*). *Can. J. Zool.* 77:1217-1222.

Malik. S, Brown, M.W., Kraus, S.D. and White, B.N. 2000. Analysis of mitochondrial DNA diversity within and between North and South Atlantic right whales. *Mar. Mamm. Sci.* 16:545-558.

Murphy, E.J, Trathan, P.N., Watkins, J.L., Reid, K., Meredith, M.P., Forcada, J., Thorpe, S.E., Johnston, N.M and Rothery, P. 2007. Climatically driven fluctuations in southern ocean ecosystems. *Proc. R. Soc. Lond. B. Biol. Sci.* 274:3057-3067.

Nei, M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York. 526pp.

Patenaude, N.J., Portway, V.A., Schaeff, C.M., Bannister, J.L., Best, P.B., Payne, R.S., Rowntree, V.J., Rivarola, M and Baker, C.S. 2007. Mitochondrial DNA diversity and population structure among southern right whales (*Eubalaena australis*). *J. Hered.* 98:147-157.

Payne, R. 1986. Long term behavioral studies of the southern right whale (*Eubalaena australis*). *Rep. int. Whal. Commn.* (special issue) 10:161-167.

Payne, R., Brazier, O., Dorsey, E., Perkins, J., Rowntree, V. and Titus, A. 1983. External features in southern right whales (*Eubalaena australis*) and their use in identifying individuals. *In*: R. Payne (ed.) *Communication and behavior of whales*. AAAS Selected Symposia Series 76, Westview Press, Boulder, CO. xii+643pp.

Portway, V. 1998. Genetic population structure of South Atlantic right whales (*Eubalaena australis*). Masters Thesis, American University.

Rosenbaum, H.C., Weinrich, M.T., Stoleson, S.A., Gibbs, J.P., Baker, C.S. and DeSalle, R. 2002. The effect of differential reproductive success on population genetic structure: Correlations of life history with matrilines in humpback whales of the Gulf of Maine. *J. Hered.* 93:389-399.

Rowntree, V., Payne, R. and Schell, D. 2001. Changing patterns of habitat use by southern right whales (*Eubalaena australis*) on their nursery ground at Península Valdés, Argentina, and in their long-range movements. *J. Cetacean Res. Manage.* (special issue) 2:133-143.

Sasaki, T., Nikaido, M., Hamilton, H., Goto, M., Kato, H., Kanda, N., Pastene, L., Cao, Y., Fordyce, R., Hasegawa, M., and Okada, N. 2005. Mitochondrial phylogenetics and evolution of mysticete whales. *Syst. Biol.* 54: 77-90.

Schaeff, C.M., Kraus, S.D., Brown, M.W. and White, B.N. 1993. Assessment of the population structure of western North Atlantic right whales (*Eubalaena glacialis*) based on sighting and mtDNA data. *Can. J. Zool.* 71:339-345.

Schneider, S., Roessli, D. and Excoffier, L. 2000. *Arlequin: A software for population genetics data analysis.* Ver 2.0. Genetics and Biometry Lab, Department of Anthropology, University of Geneva.

Shaw, C.N., Wilson, P.J. and White, B.N. 2003. A reliable molecular method of gender determination for mammals. *J. Mammal.* 84:123-128.

Swofford, D. 2003. *Paup* phylogenetic analysis using parsimony (*and other methods).* Version 4. Sinauer Associates, Sunderland, Massachusetts.

Thompson, J., Gibson, T., Plewniak, F., Jeanmougin, F. and Higgins, D. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 24:4876-4882.

Trathan, P., Murphy, E., Forcada, J., Croxall, J., Reid, K. and Thorpe, S. 2006. Physical forcing in the southwest Atlantic: ecosystem control. *In*: I. Boyd, S. Wanless and C.J. Camphuysen (eds.) *Top predators in marine ecosystems, their role in monitoring and management*. Cambridge University Press, New York. 378 pp.

Uhart, M., Rowntree, V.J., Mohamed, N., Pozzi, L., La Sala, L., Andrejuk, J., Musmeci, L., Franco, M., Sironi, M., Sala, J.E., McAloose, D., Moore, M., Touhey, K., McLellan, W.A., Rowles, T. 2008. Strandings of southern right whales (*Eubalaena australis)* at Península Valdés, Argentina from 2003-2007. SC/60/BRG15 presented to the International Whaling Commission Scientific Committee, Chile, June 2008 (unpublished). [Available from the IWC Office]. 9pp.

Uhart, M., Rowntree, V.J., Sironi, M., Chirife, A., Mohamed, N., Pozzi, L., Franco, M., and McAloose, D. 2009. Continuing southern right whale mortality events at Península Valdés, Argentina. SC/61/BRG18 presented to the International Whaling Commission Scientific Committee, Portugal, June 2009 (unpublished). [Available from the IWC Office]. 10pp.

Valenzuela, L.O. 2008. Population Structure and Foraging Ecology of Southern Right Whales (*Eubalaena Australis*): Insights from Isotopic and Genetic Analyses. Doctoral Thesis, University of Utah. 121 pp.

Valenzuela, L.O., Sironi, M., Rowntree,V.J. and Seger, J, 2009. Isotopic and genetic evidence for culturally inherited site fidelity to feeding grounds in southern right whales (*Eubalaena australis*). *Mol. Ecol.* 18:782-791.

Webster, M.S. and Marra, P.P. 2005. The importance of understanding migratory connectivity and seasonal interactions. *In*: R. Greenberg and P.P. Marra (eds.) *Birds of Two Worlds: The Ecology and Evolution of Migration.* Johns Hopkins University Press, Baltimore, Maryland. 288pp.

Wright, S. 1951. The genetical structure of populations. *Ann. Eugenics* 15:323-35.