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Abstract: A key goal for wildlife managers is identifying discrete, demographically independent conservation units. Previous genetic work assigned killer whales that occur seasonally in the Strait of Gibraltar (SoG) and killer whales sampled off the Canary Islands (CI) to the same population. Here we present new analyses of photo-identification and individual genotypes to assess the level of contemporary gene flow and migration between study areas, and analyses of biomarkers to assess ecological differences. We identified 47 different individuals from 5 pods in the SoG and 16 individuals in the CI, with no matches found between the areas. Mitochondrial DNA control region haplotype was shared by all individuals sampled within each study area, suggesting that pods have a matrifocal social structure typical of this species, whilst the lack of shared mitogenome haplotypes between the CI and SoG individuals suggests that there was little or no female migration between groups. Kinship analysis detected no close kin between CI and SoG individuals, and low to zero contemporary gene flow. Isotopic values and organochlorine pollutant loads also suggest ecological differences between study areas. We further found that one individual from a pod within the SoG not seen in association with the other four pods and identified as belonging to a potential migrant lineage by genetic analyses, had intermediate isotopic values and contaminant between the two study areas. Overall our results suggest a complex pattern of social and genetic structuring correlated with ecological variation. Consequently at least CI and SoG should be considered as two different management units. Understanding this complexity appears to be an important consideration when monitoring and understanding the viability

of these management units. Understand the viability will help the conservation of these threatened management units.

Dear Editor,

Please find attached the following manuscript: *Using a multi-disciplinary approach to identify a critically endangered killer whale management unit.*

In this study we identify at least two demographic independent management units of killer whales in Spain, through differences approaches, not only population structure by genetics analyses, first defining their social structure, genetic structure and chemical tracers, and finally through multiple regression quadratic assignment procedures we determine if all this measure were predictors of association within these killer whales. We found that there are at least two management units of killer whales at southern Iberian Peninsula, although previously they were defined as a unique population, that are not related socially, with low or zero temporary gene flow between them, and that they also have ecological differences.

These results are really important for this already endangered population. Because if they are considered as a unique population, different population trends could be masked it if they are treated as one, and consequently separately management actions should be implemented to ensure the conservation of these two small subpopulations of killer whales. This kind of multidisciplinary approach for defining management units could be used in other populations or species.

The work is all original research carried out by the authors. All authors agree with the contents of the manuscript and its submission to the journal. No part of the research has been published in any form elsewhere.

The research featured in the manuscript do not relates to any other manuscript of a similar nature that they have published, in press, submitted or will soon submit to *Ecological Indicators* or elsewhere. The manuscript is not being considered for publication elsewhere while it is being considered for publication in this journal. Any research in the paper not carried out by the authors is fully acknowledged in the manuscript. All sources of funding are acknowledged in the manuscript, and authors have declared any direct financial benefits that could result from publication. All appropriate ethics and other approvals were obtained for the research.

Thank you for your consideration

Best regards

Ruth Esteban

CIRCE (Conservation, Information and Research on Cetaceans)

Abstract

A key goal for wildlife managers is identifying discrete, demographically independent conservation units. Previous genetic work assigned killer whales that occur seasonally in the Strait of Gibraltar (SoG) and killer whales sampled off the Canary Islands (CI) to the same population. Here we present new analyses of photo-identification and individual genotypes to assess the level of contemporary gene flow and migration between study areas, and analyses of biomarkers to assess ecological differences. We identified 47 different individuals from 5 pods in the SoG and 16 individuals in the CI, with no matches found between the areas. Mitochondrial DNA control region haplotype was shared by all individuals sampled within each study area, suggesting that pods have a matrifocal social structure typical of this species, whilst the lack of shared mitogenome haplotypes between the CI and SoG individuals suggests that there was little or no female migration between groups. Kinship analysis detected no close kin between CI and SoG individuals, and low to zero contemporary gene flow. Isotopic values and organochlorine pollutant loads also suggest ecological differences between study areas. We further found that one individual from a pod within the SoG not seen in association with the other four pods and identified as belonging to a potential migrant lineage by genetic analyses, had intermediate isotopic values and contaminant between the two study areas. Overall our results suggest a complex pattern of social and genetic structuring correlated with ecological variation. Consequently at least CI and SoG should be considered as two different management units. Understanding this complexity appears to be an important consideration when monitoring and understanding the viability of these management units. Understand the viability will help the conservation of these threatened management units.

Keywords: Social structure, genetics, stable isotopes, pollutants, conservation.

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1. Introduction

Identifying populations using individual genotype data is not always straight forward, especially in natural populations for which isolation-by-distance, inbreeding or social philopatry can lead to a divergence from Hardy-Weinberg equilibrium (Waples and Gaggiotti, 2006). This can lead to a failure to detect subtle population structure such as when two populations have recently diverged and have led to arguments that the criteria for identifying and defining populations should not simply be a strong rejection of panmixia (Palsbøll et al., 2007; Taylor and Dizon, 1999; Taylor, 1997). For example, two populations could be identified and managed as one unit using genetic criteria which failed to reject panmixia due to historical gene flow. If contemporary migration between the two populations is low and anthropogenic mortality rates are high in one of these local populations, the level of recruitment can fall below the survival rate leading to a decline in this local population and its eventual extinction (Taylor, 1997). Therefore, methods able to distinguish between historic and contemporary gene flow and dispersal are needed to identify recently diverged population units for effective conservation management (Palsbøll et al., 2007; Taylor and Dizon, 1999; Taylor, 1997).

Management units (hereafter MUs) have been defined as geographical areas with restricted interchange of the individuals of interest with adjacent areas (Taylor and Dizon, 1999). Different geographical areas also potentially imply ecological differences between individuals. Consequently MUs could also be identified through the analysis of chemical tracers that reflect the ecosystem in which organisms live and feed (Borrell and Aguilar, 2007). These tracers can range from natural elements to man-made molecules that are released into the environment, where they persist over time. Here we used organochlorine compounds (OCs) and stable isotopes as both groups have been proposed as useful tools for discriminating population structuring in marine mammals (Aguilar, 1987; Born et al., 2003; Borrell and Aguilar, 2007; Dietz et al., 2000; Muir et al., 2000; Smith et al., 1996; Storr-Hansen and Spliid, 1993). OCs are a group of synthetic chemicals that were introduced in the 1950s and extensively used over the following decades in a wide range of agricultural and industrial applications. Although their production and use have been much reduced worldwide since the 1970-1980s, and in most cases totally banned, substantial amounts have remained in the ecosystem and are still being recycled by organisms, particularly at seas (Tanabe et al., 1988). OCs are lipophilic, extremely stable and difficult to

degrade, and they tend to accumulate through trophic webs. Because the principal source of OCs intake in mammals is diet, MUs inhabiting different geographical areas accumulate in their tissues pollutant loads that are characteristic of such areas and that often differ qualitatively and quantitatively (Aguilar, 1987). Stable isotopes of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) have been used to study animal ecology since the late 1970s, mostly as dietary tracers (Kelly, 2000). Environmental differences such as light intensity, nutrient concentrations and species composition affect the $\delta^{13}C$ and $\delta^{15}N$ values of primary producers in a region (Walker et al., 1999), so MUs from different geographic locations often display dissimilar isotopic signatures, even if they have similar diets.

The regular occurrence of killer whales in the Strait of Gibraltar (hereafter SoG) has been well-reported during the past century (Aloncle, 1964; Esteban et al., 2013). The first dedicated study of their distribution reported that they are seen during summer in the south-western part of SoG, where they interact with the Atlantic bluefin tuna (*Thunnus thynnus*) (hereafter ABFT) drop-line fishery (de Stephanis et al., 2008; Esteban et al., 2013). During spring, killer whales were observed to chase tuna for up to 30 min at a relatively high sustained speed, until the capture (Esteban et al., 2013; Guinet et al., 2007). The interactions with tuna fisheries have led to conflicts with local fishermen. So in addition to depleted prey resources, these whales are potentially also at risk from direct mortality, following several unconfirmed reports of killings by fishermen in recent years (Cañadas and de Stephanis, 2006). The killer whales in the SoG have been proposed for listing as a "Critically Endangered" subpopulation by ACCOBAMS-IUCN (Cañadas and de Stephanis, 2006). Likewise, the International Whaling Commission has recommended implementing a conservation plan for this subpopulation as soon as possible. In 2011, the Spanish Ministry of Environment catalogued these whales as "Vulnerable" in the Spanish Catalogue of Endangered Species (R.D. 139/2011). Currently, a Conservation Plan for these whales is being prepared by the Spanish Ministry of Environment. A priority research task identified by ACCOBAMS-IUCN was to clarify the relationship of these killer whales with others in the Northeast Atlantic (Cañadas and de Stephanis, 2006).

Foote *et al*., (2011) used a 'population-based' method to determine the number of populations within a dataset of 83 Northeast Atlantic killer whale individual multilocus genotypes and assign

individuals to a population. They found that the number of populations estimated by the software STRUCTURE (Pritchard et al., 2000) was $k = 5$. Using this estimate, the individuals sampled in the SoG were assigned to a different population to individuals sampled off the Canary Islands (hereafter CI). However, an *ad hoc* test as recommended by Evanno *et al*. (2005) suggested that the best estimate of the number of populations was $k = 3$. Under this scenario the individuals sampled off the SoG and the CI were assigned to the same population. There is therefore ambiguity over the degree of genetic isolation of the SoG and CI whales, a key question in determining its status as a proposed Critically Endangered population by the IUCN. An alternative approach to applying 'population-based' methods is to apply 'kinship-based' methods, which can perform better at determining subtle population structure and distinguishing between historic and current gene flow (Palsbøll et al., 2010).

Here we further investigate contemporary population structure of killer whales in the SoG and neighbouring waters by using four complimentary techniques: firstly, we use photo-identification records of naturally marked individuals spanning over a decade to determine their social structure; secondly, we assess kinship between sampled individuals using multilocus genotypes to determine the relationship between site-faithful individuals in the SoG and individuals sampled around the CI; and we used pollutants loads and stable isotopes as ecological differences to finally distinguished them into MUs.

2. Materials and methods

2.1 Surveys

Survey transects were conducted between 1999-2011 from the motorboat "Elsa" (11m) in the SoG by CIRCE (Conservation Information and Research on Cetaceans). In the CI the motorboat "Oso Ondo" (16.85m) was used by SECAC (Study of Cetaceans in the Canary Archipelago). Whenever killer whales were found, we approached to photo-identify them (Esteban et al. 2015, In review a). Identified individuals were compared in order to find matches between study areas. Skin biopsies were obtained using crossbows and modified darts with sterilized stainless-steel biopsy tips designed by Finn Larssen, following protocols described in Giménez *et al*. (2011).

Immediately after collection, skin was cut from blubber and skin portions were preserved in two different ways. One part was immediately put in a 2 ml tube containing a solution of 20% dimethylsulphoxide (DMSO) in saturated salt (NaCl) (Amos and Hoelzel, 1991) and frozen at -20ºC. This was used to perform genetic sexing of individuals and population structure analysis (Foote et al. 2011). The second part was frozen to -20ºC without any treatment, and was used to assay δ^{13}C and δ^{15}N values. Blubber samples were wrapped in solvent-washed aluminium foil and frozen at -20ºC for contaminant load analysis. All samples were collected under a special permit from the Spanish Ministry of Environment. Adults and subadults were the main target, no calf under 3 years-old was sampled.

2.2 Social structure

We calculated the strength of relationships between pairs of individuals, using the half-weight association index (HWI) to define pods (Cairns and Schwager, 1987; Ginsberg and Young, 1992). Modularity (Whitehead, 2008), was used controlling for gregariousness of individuals to define pods. To visualize their social structure, we defined a weighted social network by HWI matrix showing individuals (nodes) connected by their HWI (edges), using the Kamada-Kawai layout (Kamada and Kawai, 1989) using the STATNET package (Handcock et al., 2003) in the open-source statistical programming language R 3.1.2 (R Core Team, 2014).

HWI measures the proportion of time that individuals were seen together and ranges from 0 (two individuals never seen together) to 1 (never seen apart). Associated individuals were defined based on group membership, defined as animals within 10 body lengths of one another engaged in similar and/or coordinated behaviour (Williams & Lusseau 2006). Individuals photographed in the same group at least once during a day were considered associated for the day (sampling period). In the SoG only animals sighted ≤4 days were included; we also excluded calves and individuals that died. Modularity quantifies the tendency of nodes to cluster into cohesive sub-groups and identifies the most parsimonious network division; values ≤0.3 are considered appropriate. All above parameters were measured by SOCPROG 2.4 (Whitehead, 2009).

2.3Genetic structure

DNA extraction, amplification and sequencing methods details can be seen in Foote *et al*., (2011). The mtDNA control region (989-bp) was sequenced for all samples and complete mitogenomes were sequenced for a subset of individuals by three previously published studies (see Morin et al. 2010, 2015; Foote et al. 2011). The relationship among lineages based upon complete mitochondrial genomes was reconstructed using PhyML (Guindon and Gascuel, 2003) as per Foote et al. (2011).

Samples had been previously genotyped using polymorphic microsatellite loci by two different lab groups. Foote *et al*., (2011) genotyped 9 CI individuals and 8 SoG individuals using 17 loci, a further 3 individuals and 5 replicate samples from Foote *et al*. (2011) were genotyped using 10 loci by L. Barrett-Lennard at the University of British Columbia. In total 21 genotyped individuals were included in this study.

Five individuals were genotyped by both lab groups and using 7 of the same microsatellite loci: EV1, EV37 (Valsecchi and Amos, 1996), FCB5, FCB12, FCB17 (Buchanan et al., 1996), 417 (Schlötterer et al., 1991), KWM2a (Hoelzel et al., 1998). This allowed the normalization of the allele scores for these loci. In addition to the 7 shared loci, a further 10 loci FCB4, FCB11 (Buchanan et al., 1996), Ttru GT142, Ttru AAT44 (Caldwell et al., 2002), Ttr04, Ttr11 (Rosel et al., 2005), D08, D18, D22 (Shinohara et al., 1997), MK5 (Krützen et al., 2002) were used by Foote et al., (2011) and a further 2 loci 415, 464 (Schlötterer et al., 1991) were used by L. Barrett-Lennard. Therefore, where possible, data analyses were done twice, using just the 7 loci used by both labs and using all 19 loci with missing data for some individuals. Quality control measures of genotyping are given in Foote *et al*., (2011).

We estimated genetic differentiation between the SoG samples and the CI samples from allele frequencies of the 7 loci used for all individuals using Weir and Cockerham's, (1984) F_{ST} calculated in FSTAT 2.9.3 (Goudet, 1995), and 95% confidence intervals were estimated from 15,000 bootstrap resamplings. An estimation of short-term (the past 1-3 generations) gene flow was performed using BAYESASS+ (Wilson and Rannala, 2003). BAYESASS+ has the advantage of not assuming that populations are at mutation-drift equilibrium. Initial runs showed

that convergence was reached after 1 x 10^7 iterations, therefore we ran the program for 3 x 10^7 iterations, of which 1 x 10^7 were burn-in, and sampled every 2000th iteration.

We tested for a recent change in effective population size in the SoG pods using the software BOTTLENECK (Cornuet and Luikart, 1996; Piry et al., 1999). The test was performed with two different mutation models of microsatellite evolution: the infinite allele model and the stepwise mutation model. The parameters were set with 70% single-step mutations and 30% multiple-step mutations and a variance among multiple steps of 12. The significance was assessed with the Wilcoxon sign-rank test, a more powerful and robust test when used with few polymorphic loci ($n<20$).

We used the modified linkage disequilibrium based approach (Hill, 1981; Waples, 2006) as implemented in LDNE (Waples and Do, 2008) to estimate effective population size of the SoG pods. However, the results were negative which can occur due to a sampling bias, in this case sampling multiple individuals from within matrilineal pods, leading to greater detected linkage disequilibrium than the expected value.

Pairwise genetic relatedness among the individuals genotyped was estimated using Queller & Goodnight´s (1989) relatedness coefficient, *r*. The coefficient, ranging from -1.0 to 1.0 was calculated by comparing their alleles in the program RELATEDNESS 4.2 (Goodnight and Queller, 1998). Average genetic relatedness was then calculate *d* for each of the social pods identified, with standard errors obtained by jackknifing over all loci (Queller and Goodnight, 1989).

2.3 Chemical tracers

2.3.1 OCs

Lipid weight concentrations (mg/kg lipid) of 25 individual polychlorobiphenyl (PCB) congeners (IUPAC numbers: 18, 28, 31, 44, 47, 49, 52, 66, 101, 105, 110, 118, 128, 138, 141, 149, 151, 153, 156, 158, 170, 180, 183, 187, 194), dichlorodiphenyldichloroethlylene (pp'-DDE) and hexachlorobenzene (HCB) were generated using internationally standardized methodology (Law et al 2012). Concentrations below the limit of quantification (LOQ) were set to one-half of the

LOQ. Congener/isomer concentrations were normalized to the lipid content of individual blubber samples. Natural log transformation of summed concentrations was undertaken so as to stabilize the variance.

Non-metric multidimensional scaling (NMDS) ordination was applied on the basis of congener/isomer concentrations in each sample, with ecodist package (Goslee and Urban, 2007) in R 3.1.2 (R Core Team, 2014). Stress values were calculated as a measure of goodness-of-fit, where low values are optimal (i.e. <0.1 is considered a good ordination result and <0.05 is excellent) (Clarke, 1993; Kruskal, 1964).

2.3.2 Stable isotope

Skin samples were dried during 48 hours at 60ºC and powdered with a mortar and pestle. The analysis could be skew by high lipid concentration decreasing $\delta^{13}C$ content (DeNiro and Epstein, 1978), so chloroform:methanol solution (2:1) was used to extract lipids. Subsamples of powdered material (0.3mg) were weighed into tin capsules for $\delta^{13}C$ and $\delta^{15}N$ determinations.

CI samples were analysed by the Laboratorio de Isótopos Estables of Estación Biológica de Doñana (LIE-EBD, Spain; www.ebd.csic.es/lie/index.html). All samples were combusted at 1,020ºC using a continuous flow isotope-ratio mass spectrometry system by means of Flash HT Plus elemental analyser coupled to a Delta-V Advantage isotope ratio mass spectrometer via a CONFLO IV interface (Thermo Fisher Scientific, Bremen, Germany). Replicate assays of standards routinely inserted within the sampling sequence indicated analytical measurement errors of ±0.2 ‰ for $\delta^{15}N$. The standards used were: EBD-23 (cow horn, internal standard), LIE-BB (whale baleen, internal standard) and LIE-PA (feathers of Razorbill, internal standard). These laboratory standards were previously calibrated with international standards supplied by the International Atomic Energy Agency (IAEA, Vienna).

SoG samples were analysed in the Laboratory of Isotopic Mass Relationship Spectrometry of the Universdad Autónoma de Madrid, each sample was reduced to a purified gas (CO_2, N_2, SO_2, SH_6 and H_2) that was analysed by a mass spectrometer, a Micromass Cf-Isochrom of magnetic sector.

The isotopic relationship of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ were determined in CO_2 and N_2 using a continuous flow elemental analyser Carlo Erba 1108-Chns. The analytic precision was 0.1 and 0.2‰ for C and N, respectively. Results between laboratories were compared with a Paired t-test, using 5 samples of long-finned pilot whales (*Globicephala melas*) analysed in both laboratories.

We refer to the isotope ratios in terms of delta values (δ) per mil notation (‰), relative to atmospheric N_2 ($\delta^{15}N$) (Coleman and Frey, 2012). Results are expressed in delta (δ) notation, calculated as:

$$\delta X = [(R_{sample} / R_{standard})-1) \bullet 1000$$

where X is ^{13}C or ^{15}N, R_{sample} is the ratio of the heavy isotope to the light isotope of the sample, and $R_{standard}$ is the ratio of the heavy isotope to the light isotope in the reference.

Differences between sexes and study areas were checked, for OCs and stable isotopes, with Kruskall-Wallis' test or ANOVA, depending if samples values follow a normal distribution according to Shapiro's test, and assumption of homogeneity of variances was checked with Levene's test. To estimate overlap between study areas we delineated convex hull and a multivariate ellipse-based metrics enclosing OCs or isotopic values (Jackson et al., 2011; Quevedo et al., 2009), with SIAR package (Parnell and Jackson, 2013) in R 3.1.2 (R Core Team, 2014).

2.4 Management units

MRQAP regression is a type of Mantel test that allows for a response matrix to be regressed against multiple explanatory matrices that represent dyadic attribute relationships. The response matrix contained observed association strengths (HWI) in the social network, while genetic relatedness, OCs and stable isotopes matrices served as explanatory matrices. OCs and stable isotopes matrices were created as similarity matrices between individuals by Euclidean distances in PROXY package (Meyer and Buchta, 2015) in R 3.1.2 (R Core Team, 2014).

Multiple regression quadratic assignment procedures (MRQAP) by the Double-Semi-Partialling or DSP (Dekker et al., 2007) was used with asnipe package (Farine, 2013) in R 3.1.2 (R Core Team, 2014) to test whether similarity in genetic relatedness, OCs and stable isotopes were significant predictors of association.

3. Results

3.1 Surveys

Between 1999-2011, we had 109 sightings in the SoG and 1 in 2009 in the CI (Fig. 1). In the SoG 47 individuals were identified, and 16 in the CI. No matches were made between the study areas. A total of 9 biopsy samples were taken in the CI during 2009, and 11 between 2006-2010 in the SoG. An additional sample from a female stranded in 2006, Vega, was obtained in the SoG.

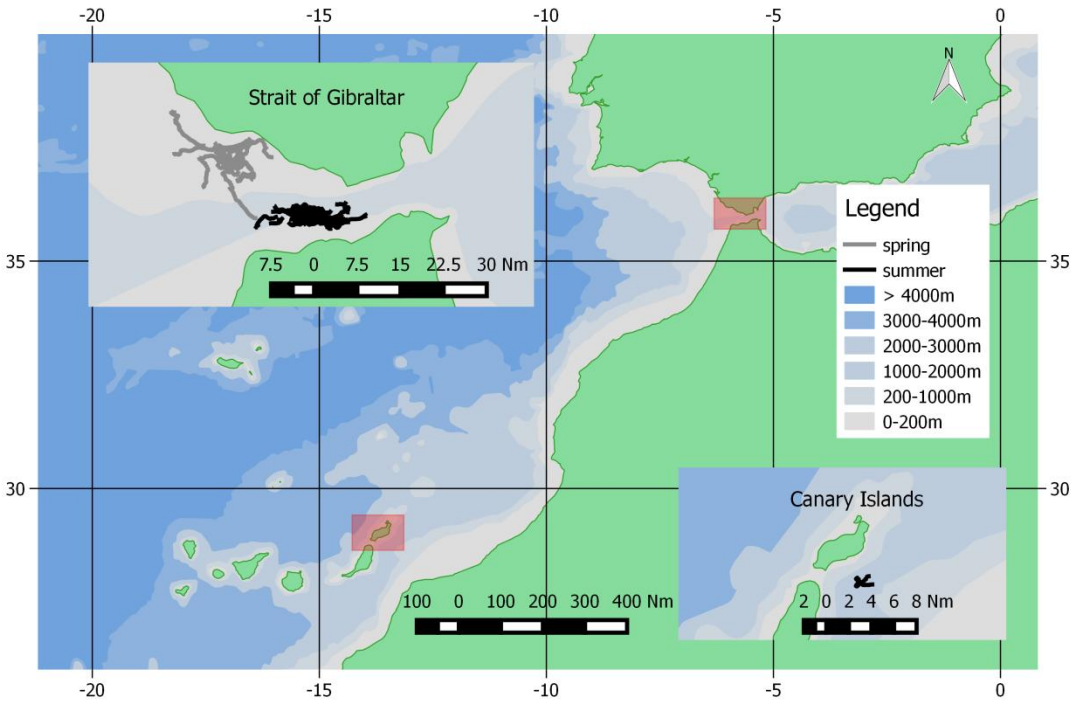

Figure 1: Map of killer whale tracks during sightings in the SoG and the CI.

3.2 Social structure

After restrictions, 28 individuals from the SoG and 16 from the CI were included in the social structure analyses. Modularity of 0.54 assigned individuals to six pods (Fig. 2), 5 pods in the SoG and one pod in the CI. There were social bonds between A1, A2, B and C pods from the SoG, but these pods were never observed in association with D pod.

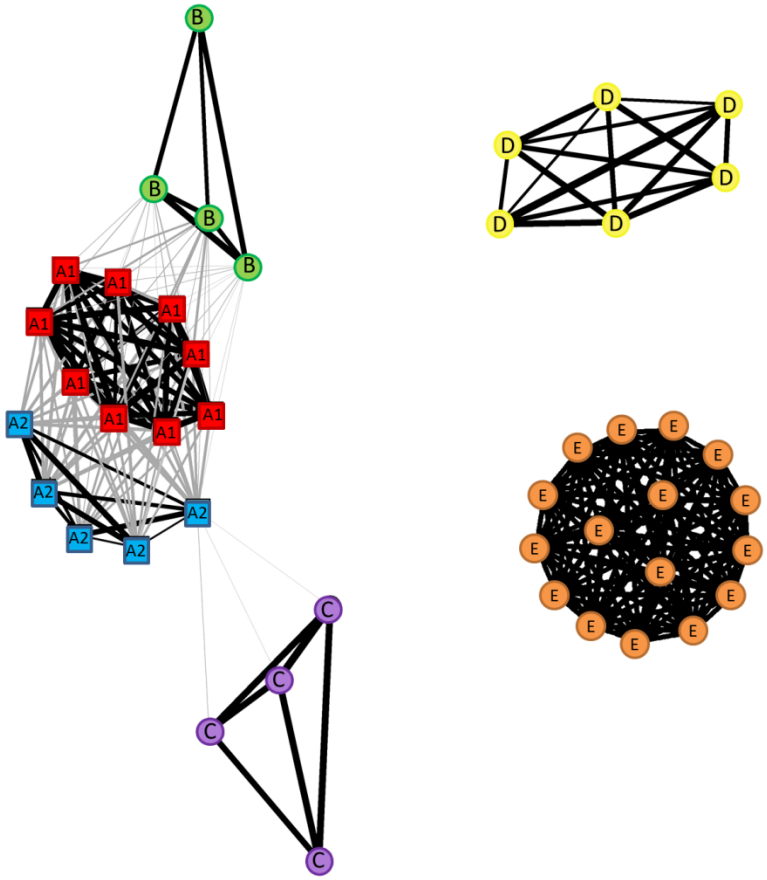

Figure 2: Network diagram of killer whales. Colors indicate different pods described by modularity: A1, A2, B, C and D (including Vega) are pods previously described in the SoG (Esteban *et al*. in review) and E are whales from the CI. Black lines indicate relation of individuals within the same pod, and grey lines between pods. Interacting individuals are indicated by squares, and non-interacting by circles.

3.3 Genetic structure

Genetic differentiation between the SoG pods and the CI individuals was relatively low (F_{ST} = 0.084, 95% CI: 0.004-0.155). Recent dispersal rates (m) estimated by BAYESASS+ suggest directional gene flow with a small proportion of SoG individuals inferred to be derived from the same population as CI individuals *m* = 0.05 (S.D. = 0.04), compared with the proportion of CI individuals inferred to be derived from SoG pods *m* = 0.21 (S.D. = 0.12). Using all 19 loci, the results from BOTTLENECK were marginal for determining if SoG pods had undergone a recent change in effective population size. Under infinite alleles model there was significant heterozygosity excess (*p* = 0.013), however, this was not the case under stepwise mutation model (*p* = 0.483) and the allele frequency distribution had a normal L-shape. One mitochondrial DNA control region haplotype (989-bp) was shared by all individuals sampled in the SoG with the exception of the stranded female from pod D (Vega) that shared a control region haplotype with some of the CI individuals (see Foote et al. 2011). A comparison of complete mitochondrial genome sequences (~16,390-bp) generated by Morin et al. (2015, 2010) and Foote et al. (2011) for a subset of these individuals and which has greater phylogenetic resolution than the control region (Duchene et al. 2011), indicated there were no shared mitochondrial haplotypes between SoG and CI individuals (Fig. 3).

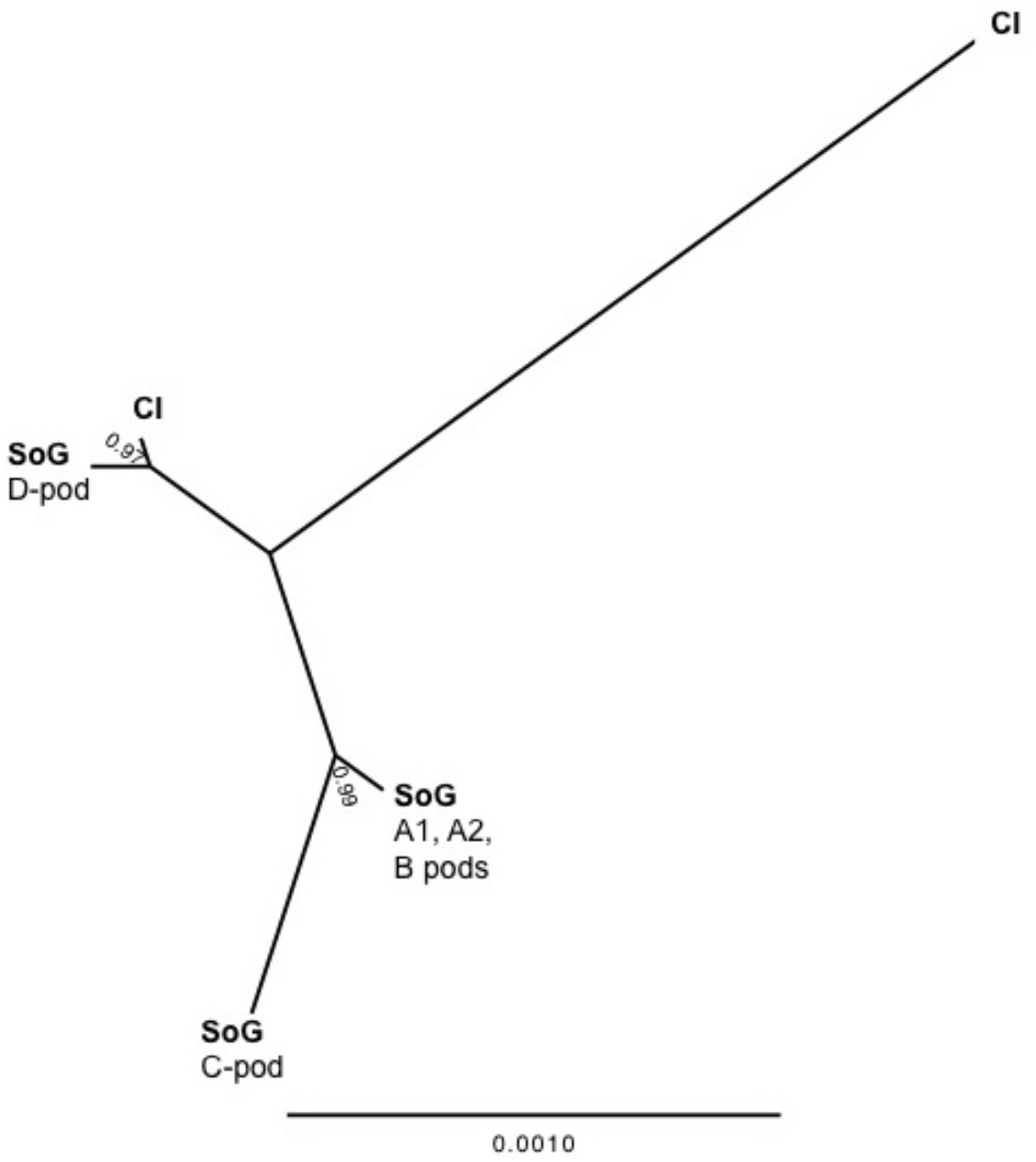

Figure 3: Maximum-likelihood reconstruction of the genetic relationships between pods based upon complete mitochondrial genome sequences. Tip labels indicate population (SoG or CI) and pod for SoG. Node labels indicate approximate likelihood ratio test (aLRT) support values.

3.4 Chemical tracers

Blubber samples were available for 8 killer whale individuals from CI (1 male and 7 females), and 8 from SoG (2 males and 6 females, including Vega) (Table 1). We did not find any sex-related differences in OCs compounds, nor in their variances. As a consequence, individuals of

both sexes were combined for subsequent comparisons. The highest concentrations observed were: HCB 1.25 mg kg-1 lipid weight in a CI male, and sum 25 CBs 138.32 mg kg-1 lipid weight and *pp*'-DDE 602.73 mg kg-1 lipid weight in the stranded SoG female, Vega. Consequently Vega's sample was excluded for average measures of OCs compounds from the SoG. Highest OCs values were found in the CI samples than in the SoG (Table 1). NMDS provided a 2-dimensional graphical configuration (stress = 0.007, Fig. 4). No overlap was found between study areas for the convex hulls and the ellipses.

Table 1: Stable isotopes ratios and OCs concentrations for killer whales SoG and CI killer whales. Comparisons between sex and study areas were made by ANOVA for OCs and Kruskall Wallis test for SI.

Parameter	Study areas	CI (pod E)			SoG (pods A1, A2, B and C)			Vega Pod D		Between sex	Between study areas
		Mean	S.D.	n	Mean	S.D.	n		n		
Stable isotopes	**δ^{15}N**	14.72	0.32	n=9 (1 male, 8 females)	12.72	0.30	n=10 (2 males, 8 females)	13.3	n=1 (1 female)	*p*=0.52	***p*<0.01**
	δ^{13}C	-15.47	0.29		-16.88	0.34		-15.5		*p*=0.59	***p*<0.01**
Ocs	**% lipid**	2.91	1.06	n=8 (1 male, 7 females)	11.09	7.79	n=7 (1 male, 6 females)	46.3	n=1 (1 female)		
	25 CBs	24.92	11.17		21.88	16.58		138.32		*p*=0.28	***p*=0.04**
	HCB	0.76	0.33		0.15	0.05		0.58		*p*=0.83	***p*<0.01**
	***pp'*-DDE**	128.72	108.63		117.75	119.01		602.73		*p*=0.17	*p*=0.29

Blubber OCs concentrations (mg.kg^{-1} lipid weight), and skin δ^{15}Nand δ^{13}C split by study area significant *p*-values in bold

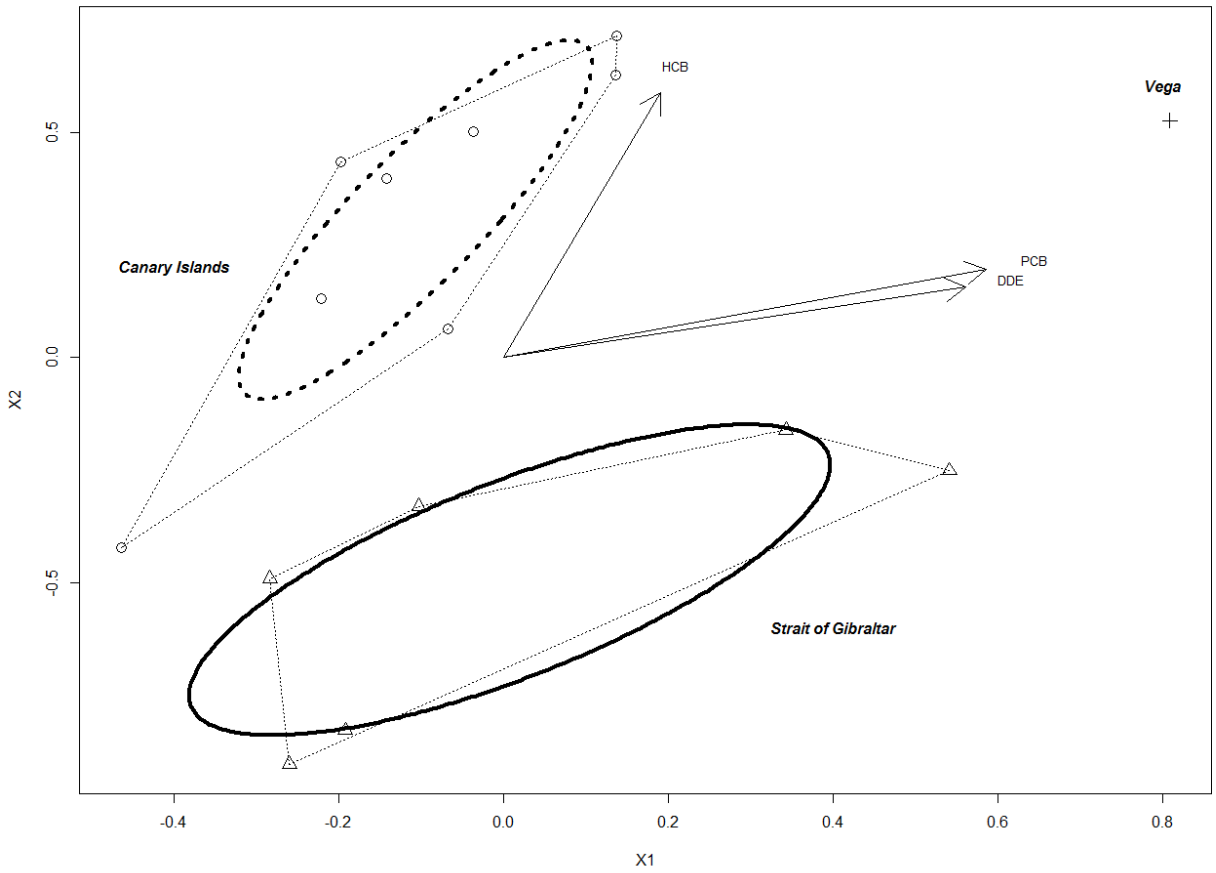

Figure 4: Two-dimensional NMDS scaling configuration of similarities among study areas (stress = 0.003). Circles: CI samples; triangles: SoG Samples and cross: stranded female in the SoG (Oo_GIB_021), Vega from pod D. Their correspondence convex hulls are delimited by dotted lines; and posterior estimates of the standard ellipses for CI (dotted black line) and SoG (solid black line). The arrows are vectors of pollutants concentrations that point towards where these pollutants increase strongest.

Skin samples were available for 9 individuals in the CI (1 male and 8 females), and 11 individuals in the SoG (2 males and 9 females, including Vega). Vega's sample also presented different values, and it was excluded from samples of the SoG (Table 1). No differences were found between sexes in stable isotopes ratios, so individuals of both sexes were combined for subsequent analyses. Great differences were found between study areas, with CI samples presenting higher values of both δ13C and δ15N (Table1). Bi-plot shows no overlap between study areas for the convex hull and the Bayesian ellipses (Fig. 5). No significant differences were found between laboratories both in δ^{15}N (p=0.371) or δ^{13}C (p=0.704) (Appendix A, Table A.1). The sizes of the ellipses do not vary significantly among communities (Appendix A, Fig. A.1)

with pairwise test indicating that CI is not larger than SoG (with probability= 0.457), showing a similar size of niche width between study areas.

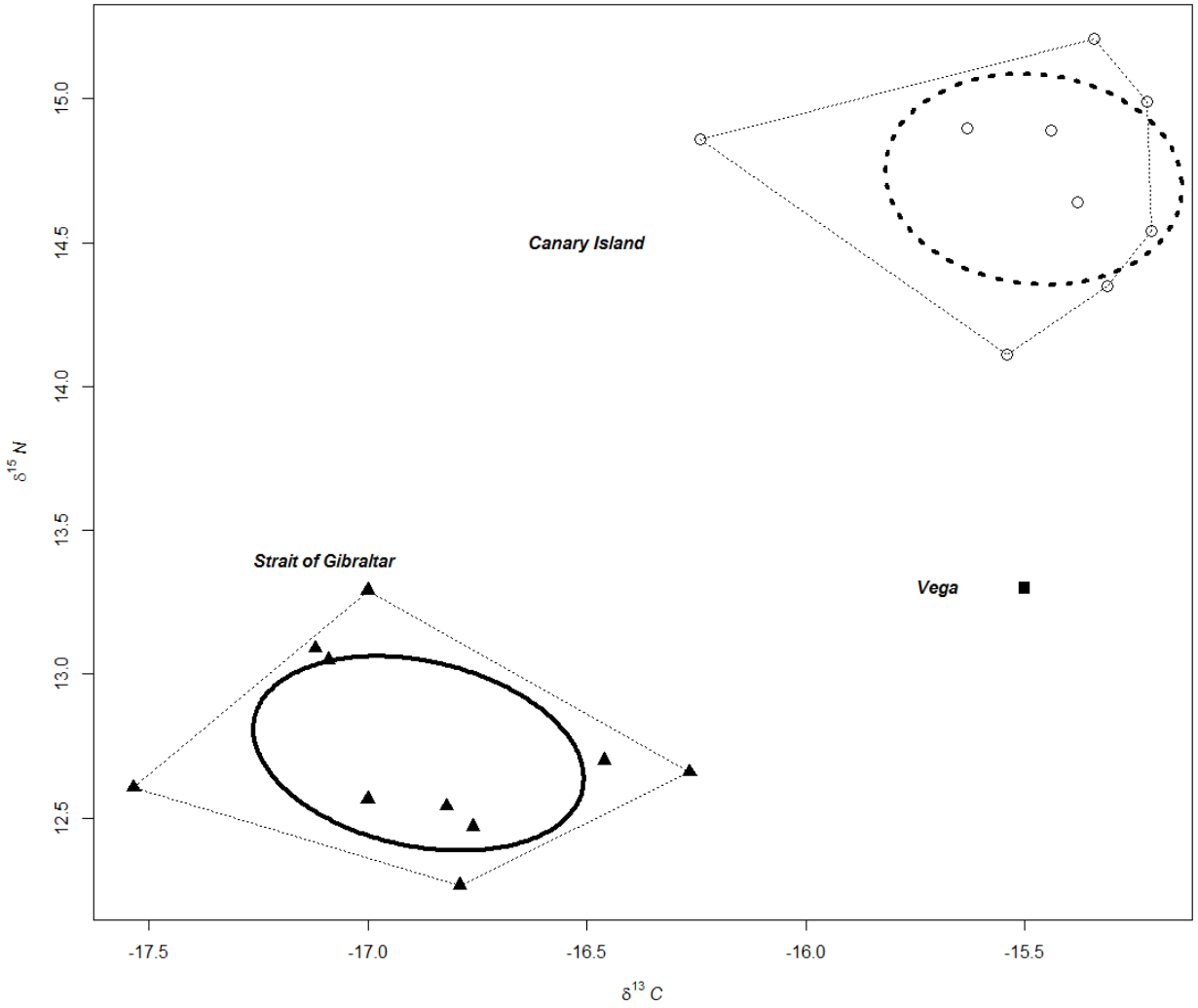

Figure 5: Stable isotopes bi-plot for killer whales in the Strait of Gibraltar, the CI and Vega. Convex hulls are delimited by dotted line; and standard ellipses for CI (dotted black line) and SoG (solid black line).

3.5 Management units

Only 13 individuals were used for this analysis, as they had complete records of all measures; 6 from the SoG and 7 from the CI. The MRQAP analysis showed a significant effect of genetic relatedness, OCs and stable isotope on the observed HWI of all connected dyads (Table 2).

Table 2: MRQAP regression model

	Regression coefficient	*p*
Genetic relatedness	0.50	**0.00**
Pollutant concentrations	1.56	**0.00**
Stable isotopes rates	0.70	**0.00**

N = 13, r^2=0.60. Significant p-values are indicated in bold

4. Discussion

By using a multi-disciplinary approach, here we have improved upon previous studies, and infer that pods of killer whales inhabiting the SoG appear to be reproductively, socially and ecologically differentiated from individuals sampled on the CI, , and therefore they are named as different subpopulation. In the SoG we identified 5 pods; 4 of them (A1, A2, B, C) were associated, but one (D, including Vega) was never seen in association with the others. In the CI we only had data from one sighting and all individuals were seen associated together (E pod). None of the individuals identified in the CI have ever been observed in the SoG. Consistently, genetic data inferred that approximately only 1-9% of the SoG subpopulation was derived from the same subpopulation as CI individuals. No complete mitogenome haplotypes were shared between the CI and SoG suggesting that there is no permanent female dispersal between these areas, and that any migration must be via male-mediated gene flow during rare short-term associations (Foote et al., 2011; Hoelzel et al., 2007; Pilot et al., 2010). Study areas are 1,100 km apart, but killer whales are known to be able to travel long distances (Matthews et al., 2011) up to 8,300 km (Rasmussen et al., 2007). Despite the low estimate of gene flow between the two putative subpopulations, genetic differentiation between them is relatively low (but comparable to that between the neighbouring Northern Resident and Southern Alaska Resident populations in the North-eastern Pacific, Barrett-Lennard 2000). This F_{ST} value is likely to be inflated due to sampling multiple individuals from within the same matrilineal pod (Foote et al., 2011) and this sampling bias may also explain the high uncertainty around the point estimate indicated by the wide 95% confidence intervals. The low differentiation in combination with low levels of migration and the lack of any recent bottleneck signal, for example due to a founder effect, are consistent with a very recent population split.

OCs and isotopic niche showed no overlap between study areas (Fig. 4 and 5). In general, CI individuals had higher pollutant loads than those in the SoG. One exception was Vega, which

had the highest pollutant load. It was found stranded and in poor body condition, and its high OCs concentrations could be due to food deprivation that promotes metabolism of lipid stores, releasing sequestered OCs into circulation. However, we also found differences in stable isotope signatures (Fig. 5). García-Tíscar (2009) previously suggested that isotopic signature of the SoG individuals was consistent with a diet of mainly ABFT with the exception of the Vega that presented a ^{13}C-enriched diet. Here, Vega's isotopic signatures fall in-between the values from SoG and CI individuals, with Nitrogen values similar to SoG while Carbon values are similar to CI (Fig. 5). Unfortunately, we do not have any information about their prey in the CI, but our results suggest that these whales could be feeding at a higher trophic level and on different species. An alternative explanation is that they are feeding on similar preys but the isotopic baseline between SoG and CI is different. Marine carbon and nitrogen isoscapes for the Atlantic Ocean based on a meta-analysis of published plankton δ^{13}C and δ^{15}N values (McMahon et al., 2013) show similarities in both areas, although the resolution of these isoscape maps is very low. Moreover, as PCBs and *pp*'-DDE are persistent and biomagnify through food webs, the higher pollutant load of CI individuals also support the first hypothesis. In any case, both hypotheses indicate that both subpopulations are ecologically different, by feeding either on different prey or on the same prey from different areas. HCB is the OC that best explains their separation (Fig. 4). HCB is not generally magnified by fish, but it is magnified in other marine animals, such as polar bears, seabirds and cetaceans (Borgå et al., 2007; Clark and Mackay, 1991; Norstrom et al., 1990), while pinnipeds are able to eliminate it (Goerke et al., 2004). It is relatively volatile and is more concentrated at higher latitudes (Wania and Mackay, 1996).

Whilst differences in the sex/age of the whales sampled in each study area could influence the result, no clear differentiation was found between sexes (Table 1). In resident killer whales of the Northeast Pacific, they found lower values in recently reproductive females compared to non-reproductive females and adult males (Endo et al., 2007; Krahn et al., 2009; Ross et al., 2000; Ylitalo et al., 2001). Our comparison between sexes may have lacked power as we did not sample many males, and had no data about reproductive status of female killer whales sampled in the CI. However, the complete lack of overlap in either contaminant or isotopic signature suggests that ecological factors, rather than demographic differences between the two sample-sets, were the main driver of differentiation between the two study areas.

We found a clear correlation between social structure and every other factor measured in this study (Table 3). Taken together, the results indicate marked patterns of population segregation (Fig. 2-5). All variables distinguished CI and SoG samples. We also found a clear relationship between the separation between A1, A2, B and C pods with D pod within the SoG pods (Table 1). Behavioural differences have also previously been found within killer whales of the SoG: all whales have been seen actively hunting in spring at the western part of the SoG (Esteban et al., 2013; Guinet et al., 2007) (see Fig. 1), but only A1, A2 and B pods, have been seen actively hunting in summer, and only A1 and A2 have been observed interacting with the drop-line fishery (Esteban et al. 2015). This interaction has been suggested to be advantageous to these interacting pods resulting in recruitment through increased fecundity, in contrast the other pods have suffered low-to-zero recruitment during the same time period (Esteban et al. in review a). Further biopsy samples need to be collected from D-pod to better understand the relationship this pod with the others, in particular to identify whether this pod is the source of migrant alleles between the CI and SoG.

This multidisciplinary analysis highlights the more nuanced insights from a multi-disciplinary approach, than a purely population genetics approach to determining MUs in wild animals. Here we have determined that there are at least two MUs of killer whales off southern Spain, the first one comprising killer whales sighted in the CI (pod E) and another in the SoG (A1, A2, B and C pods). Furthermore, a third MU should be considered and be the focus of future research in the SoG, as Vega of pod D presented large differences with the other four pods of the SoG. For example, pod D is only sighted in spring and based on the isotopic and contaminant data presented here may not be dependent upon tuna year-round. These variations within the pods of the SoG subpopulation could underlie the different demographic trajectories among pods reported in Esteban (In review a), which could be masked if the 5 pods are considered as a single MU. For example, the high recruitment in pods A1 and A2 could mask the decline in the other SoG pods if annual census of the overall subpopulation size is the only parameter taken into account. The SoG and CI killer whale population can perhaps best be viewed as a metapopulation, where subpopulations, or MUs, are connected through movements of at least a few individuals, even if most of the animals remain physically separated (Levins, 1970).

Different foraging groups have been observed within SoG whales (Esteban et al. 2015), this could lead to different cultures being transmitted through social learning (Laland et al., 2009), and these foraging groups have few social or reproductive connections between them, and so conservation and management should also consider the probable cultural division (Whitehead, 2010) .We argue that this fine-scale understanding of the interaction between these social pods of top predators and their ecosystem allows for a more nuanced monitoring of their demographic trajectory and a better understanding of any underlying threats to long-term survival. By doing so, the arguably greater effort and expense of identifying such fine-scale management units may allow for less costly and more focused and effective conservation measures. In the meantime, the existence of ecological differences within an already very small and genetically isolated population further stress the necessity of implementing urgently a conservation plan for killer whales in Southern Spain, as well as revising the conservation status of the different MUs. Key steps to conserve genetic, cultural and ecological diversity within this population of killer whales.

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Using a multi-disciplinary approach to identify a critically endangered killer whale management unit

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Appendix A Comparison between stable isotopes rates ellipses

Figure A.1: The posterior estimates of the standard ellipse areas for SoG and CI. The boxes represent the 95, 75 and 50% credible intervals in ascending order of size, with the mode indicated by the black circles.

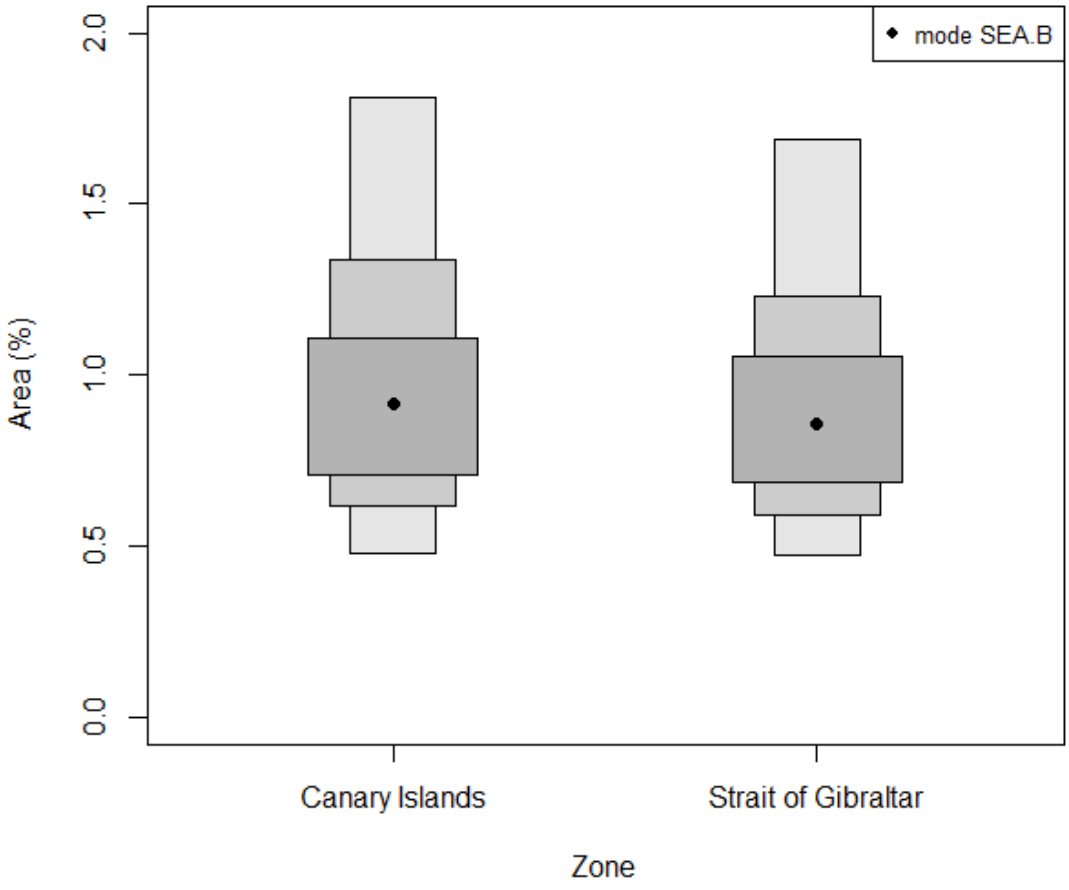

Table A.1: Paired-t test for laboratory comparison of stable isotopes ratios

	t	*p*
δ^{15}**N**	1.007	0.371
δ^{13}**C**	0.4086	0.704

N = 5