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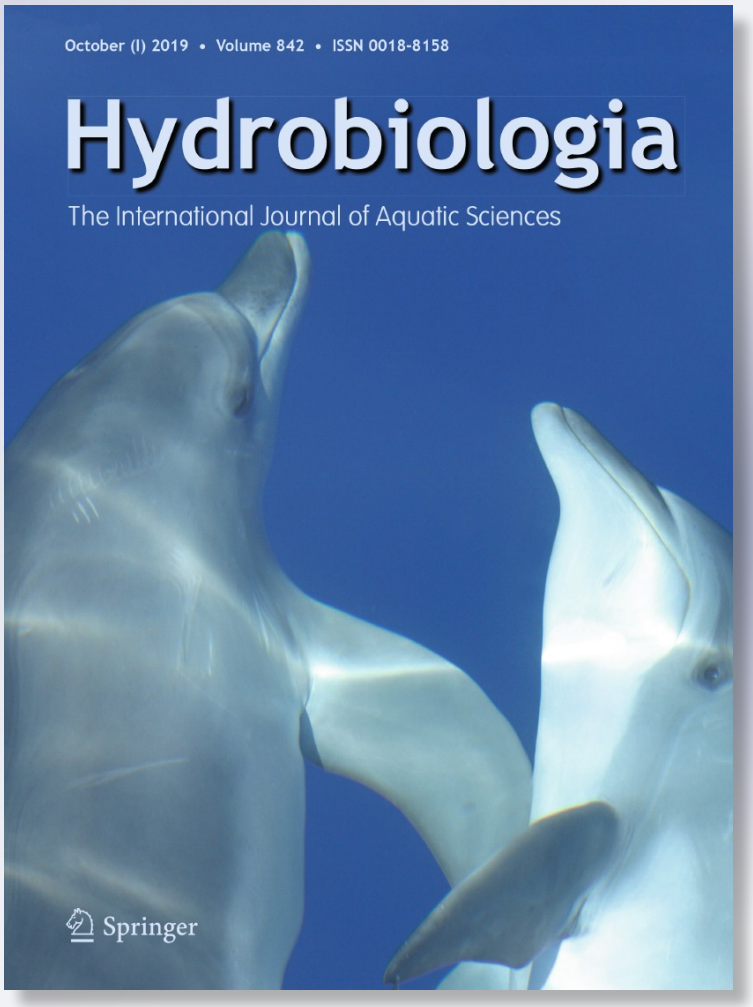

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PRIMARY RESEARCH PAPER

Genetics and stable isotopes reveal non-obvious population structure of bottlenose dolphins (*Tursiops truncatus*) around the Balearic Islands

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Abstract The effective management of wildlife requires that populations are defined in a biological sensible manner. We investigated the population structure of bottlenose dolphins (*Tursiops truncatus*) in waters around the Balearic archipelago using two complementary techniques; DNA markers (i.e. microsatellites and a portion of the mitochondrial control region) and stable isotopes (δ^{13}C, δ^{15}N). We used tissue samples from biopsies (n = 50) and fresh carcasses (n = 7) obtained around the islands of Gimnèsies and Pitiüses, and Comunitat Valenciana (Western Mediterranean Sea). Genetic differentiation between individuals from Gimnésies and Pitiüses and between individuals from across these two areas and individuals from Comunitat Valenciana was significant when assessing F_{ST}, but no substructure was found using clustering methods (i.e. DAPC and Bayesian clustering). δ^{13}C and δ^{15}N profiles were not significantly different between dolphins from Gimnésies and Pitiüses. Dolphins from both areas showed coastal carbon isotopic values and similar trophic niche levels. However, the trophic niche of dolphins from Gimnésies was broader than the trophic niche of Pitiüses' dolphins. These results indicate non-obvious population structure between the mainland and the archipelago, or between islands within the archipelago. The use of combined techniques, which integrate information over different time scales, is applicable to other species and areas.

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Introduction

One of the challenges in the effective management of wildlife is to define populations in a biological sensible manner. This is especially challenging for mobile marine species, inhabiting an environment without obvious physical barriers which could shape population structure. Stock or population identification is an essential instrument for the management of highly mobile marine species, such as fish (Begg et al., 1999; Cadrin et al., 2013) as well as for the conservation of vulnerable and threatened marine species such as small cetaceans (ASCOBANS, 2009).

The use of genetic markers has been the main approach to understand the structure of wildlife populations. However, a wide variety of other methods are available. Recent studies have shown the strengths of combining several methodologies to unravel population substructure and define ecological management units (Foote et al., 2009; Louis et al., 2014a, b; Reis-Santos et al., 2015; Ozerov et al., 2016a, b; Giménez et al., 2018). Importantly, genetic differentiation originates after several generations and for management purposes, stock and population identification methods that reflect more recent events, such as current population demography or differentiated ecological niches, are likely to complement genetic approaches (Giménez et al., 2018).

In addition to genetics, ecological tracers such as stable isotopes in animal tissues have aided in determining population structure (e.g. Fernández et al., 2011; Louis et al., 2014a; Reis-Santos et al., 2015; Giménez et al., 2018). Carbon and nitrogen stable isotopes (δ^{13}C and δ^{15}N) have also been used as tracers to examine the origin of marine food webs (i.e. coastal vs. offshore food webs) and trophic relationships between taxa (Peterson & Fry, 1987; Rau et al., 1992; Hobson et al., 1995; Smith et al., 1996). In addition, the C:N ratio has been previously used as an indicator of nutritional status in marine organisms (e.g. Box et al., 2010). As nitrogen is mostly present as part of proteins, diets with a low C:N ratio contain a greater proportion of protein and are of higher quality to a consumer relative to diets with a high C:N ratio (Fantle et al., 1999).

Previous genetic studies concluded that Mediterranean bottlenose dolphins (*Tursiops truncatus*, Montagu 1821) are differentiated from the neighbouring Northeast Atlantic populations (Natoli et al., 2005). Furthermore, within the Mediterranean, two distinct populations were identified; the Eastern and Western Mediterranean populations, separated by the Italian Peninsula. The boundaries between these two populations were related to hydrographic characteristics (Natoli et al., 2005). More recent research suggests that North Atlantic and Mediterranean bottlenose dolphins may be part of the same metapopulation, composed of pelagic and coastal populations, where pelagic populations act as a genetic source for coastal ones (Gaspari et al., 2015).

In the Mediterranean and adjacent areas, the species forages mainly on demersal and bentho-pelagic prey (Miokovic et al., 1999; Blanco et al., 2001; Giménez et al., 2017a). In other parts of the world the advanced learning ability of this species has been found to increase foraging specialization (Pryor et al., 1990; Chilvers & Corkeron, 2001; Krützen et al., 2005; Donaldson et al., 2012; Kopps et al., 2014) that could result in incipient population structure. For example Chilvers & Corkeron (2001) identified two overlapping communities of bottlenose dolphins in Moreton Bay (Australia) differentiated based on the presence or absence of dolphin association with trawlers. Also, Krützen et al. (2005) demonstrated that sponging (i.e. use of marine sponges as foraging tools) resulted mainly from vertical social transmission within a single matriline. Louis et al. (2014a) suggest that environmental opportunity to specialize may be the major factor driving ecological, genetic and morphological divergence for this species. Indeed, the trophic niches of pelagic versus coastal ecotypes of bottlenose dolphins have been found to be highly segregated (Louis et al., 2014a). Thus, combining genetic markers (reflecting several generations to evolutionary times) with more dynamic ecological tracers, such as stable isotopes, should be encouraged in order to investigate fine-scale population structure for such a species.

The bottlenose dolphin is classified as "vulnerable" by the Spanish Catalogue of Endangered Species. The same threat category applies to Mediterranean bottlenose dolphins according to IUCN criteria based on

the suspected population decline of at least 30% over the previous 60 years (Bearzi & Fortuna, 2006; Reeves & Notarbartolo di Sciara, 2006). This suspected decline was based on concerns about habitat loss and degradation in addition to mortality due to direct killing (mainly historical) and incidental bycatch in fishing gear. Although the Balearic Islands are considered a hotspot for the species within the Mediterranean, the abundance estimate of bottlenose dolphins is relatively low (517, CV = 12.4%; Gonzalvo et al., 2014).

No significant differences in stable isotope signatures have been found between bottlenose dolphins from the Balearic Islands and from Catalonia/Valencia, although differences found in organochlorine compounds suggest that deep waters between the archipelago and the peninsula could represent an effective barrier for the movement of individuals (Borrell et al., 2006). The present study aims to evaluate whether movements of bottlenose dolphins between the Balearic archipelago and the Iberian Peninsula, and between the islands that form the archipelago, are restricted. We predict that if these restricted movements have resulted in population differentiation, it will be reflected in their genetic structure and/or isotopic profiles. Thus, in the present study two complementary techniques, namely DNA markers (i.e. microsatellites and mitochondrial DNA) and stable isotope profiles (δ^{13}C and δ^{15}N), have been used to explore fine-scale population structure. The identification of fine-scale population structure will have implications for the management and conservation of the species.

Methods

Study site

The study site covered the waters around the Balearic Islands (Western Mediterranean, Spain) and off Comunitat Valenciana (East coast of the Iberian Peninsula, Spain). The study site was divided into three areas (separated by deep water channels of similar dimensions): Gimnèsies (islands of Mallorca and Menorca) and Pitiüses (islands of Ibiza and Formentera) in the Balearic Islands, and Comunitat Valenciana (Fig. 1).

Sample collection

Skin and blubber samples from the region posterior to the dorsal fin were taken from 50 bottlenose dolphins (n = 26 from Gimnèsies, n = 22 from Pitiüses, n = 2 from Comunitat Valenciana), from March 2009 to May 2011. Biopsies were collected from small boats (< 10 m) powered by outboard engines, using a 150 lb pull crossbow (manufactured by Barnett International). The crossbow was fitted with 25 mm cutting heads mounted on carbon fibre arrow shafts with moulded flotation (designed by Ceta-Dart, F. Larsen, Copenhagen, Denmark).

To increase the number of samples from Comunitat Valenciana, seven samples from strandings occurring between July 2008 and March 2009 were added to this study. Only very fresh animals (estimated < 3 days post-mortem) were sampled to avoid carcasses resulting from long-range drift.

Biopsy samples were divided into two subsamples: the first was preserved at − 18°C for stable isotope analysis and the second was preserved in 90% ethanol at − 20°C for DNA extraction.

DNA extraction and population genetic analysis

DNA extraction was performed employing Nucleospin reagents (Macherey–Nagel GmbH & Co. KG) according to the manufacturer's' instructions. DNA concentration was quantified with a nanodrop 1000 spectrophotometer from Thermo Scientific and stored at − 20°C.

Sex determination

To determine the sex of each sampled individual a PCR co-amplification of ZFX and SRY genes was carried out based on Rosel (2003). PCR were carried out with a Multiplex PCR kit from (QIAGEN). PCR included 5 µl of multiplex mix, 3 µl of primer mix and 2 µl of DNA (10 ng). Primer concentration was 10 pM of ZFX0582F and ZFX0923R and 3 pM of TtSRYR and PMSRYF. PCR started with 15 min of denaturation at 95°C to activate the HotStart Taq polymerase of the Multiplex kit, followed by 30 cycles of 30 s at 94°C, 90 s at 51°C and 45 s at 72°C, final extension was carried out for 2 min at 72°C. The PCR products were visualized with UV light in a 2% agarose gel dyed with ethidium bromide.

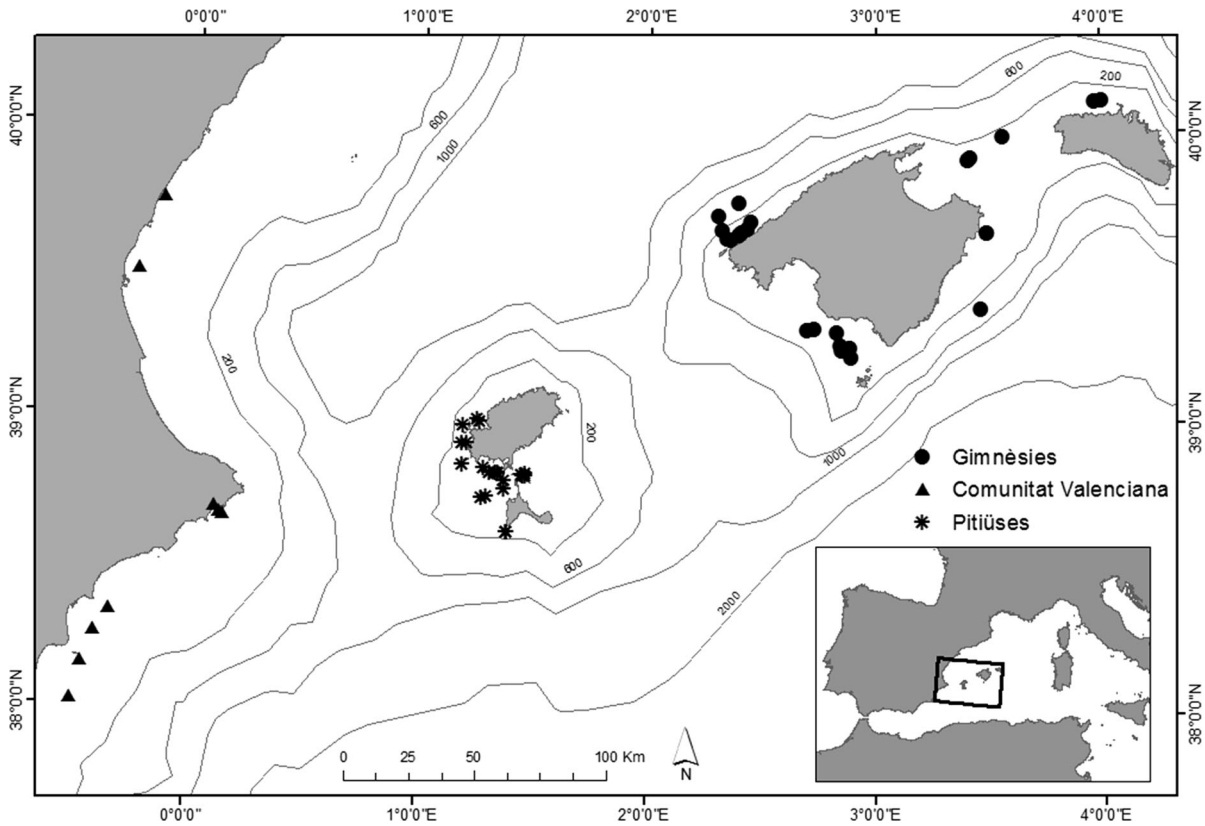

Fig. 1 Study area with indication of sites for bottlenose dolphin biopsies and stranding locations

Microsatellites

Eleven previously reported polymorphic nuclear microsatellite loci (see Supplementary Table 1) were analysed for all 57 samples. After elimination of two individuals with more than three genotypes missing the sample size was 55 (Gimnesies 24, Pitiüses 23, Comunitat Valenciana 9). Microsatellites were amplified with fluorescent dye using a Multiplex PCR kit from QIAGEN. PCR consisted of 10–20 ng of genomic DNA, 5 µl of Multiplex Mix and 3 µl of primer mix in a 10 µl reaction. The PCR profile was as follows: 95°C for 15 min followed by 30 cycles of 94°C for 30 s, 60°C for 90 s and 71°C for 45 s, with a final extension of 72°C for 2 min. Microsatellites were amplified and automatically sequenced in a Beckman Coulterer system. Genotyping error was calculated by reamplifying 15 random individuals (27%). All loci were tested for null alleles, misgenotyping and stutter bands, in the program Microchecker (Van Oosterhout et al. 2004).

For each putative population (Gimnésies, Pitiüses and Comunitat Valenciana) expected and observed heterozygosity (H_E and H_O) along with Hardy–Weinberg equilibrium probability tests were obtained with Arlequin (Excoffier & Lischer, 2010) and allelic richness was calculated with FSTAT 2.9.3.2 (Goudet 1995). Pairwise comparisons of genetic differentiation (F_{ST}) were conducted with the program GENEPOP (Raymond & Rousset, 1995) and FSTAT 2.9.3.2 was used to test for significance. Linkage disequilibrium for each locus was estimated with GENEPOP. A sequential Bonferroni correction (Rice, 1989) was applied to assess significance values. Relatedness within each sampling location was tested with RERAT (http://people.musc.edu/~schwaclh/). In order to test whether the inclusion of related individuals could influence population structure, we estimated the Queller & Goodnight (1989) relatedness index using a jackknife procedure with 100 repetitions.

The genetic structure of the sample set was analysed through three different methods as each

performs differently, depending on the level of population structure. Structure 2.3.1 (Pritchard et al., 2000) was used with a burn-in period set to 150,000 iterations and the probability estimates were determined using 3,000,000 Markov chain Monte Carlo (MCMC) iterations. Runs were conducted with K (the potential number of populations within our sample set) set from 1 to 10 with 10 runs for each value of K. The admixture model assumes that individuals are allowed to have a mixed ancestry and it is good at dealing with hybrid zones. The no-admixture model assumes that all the individuals are drawn purely from one of K populations and this model is good at detecting subtle population structure. Both models were tested with correlated and uncorrelated frequencies. The uppermost hierarchical level of structure was obtained in the program *STRUCTURE HARVESTER* (Earl & vonHoldt, 2012) using the Evanno et al. (2005) method to determine the value of K. As this method is not able to identify $K = 1$, LnP(D) and membership coefficients for each individual were examined.

In addition, a CAR (conditional autoregressive) admixture model was run in TESS (Durand et al., 2009) because it is recommended to avoid overestimation of the values of K (Guillot 2009) using 120,000 MCMC steps with a burn-in of 20,000 steps and 10 replicate runs for each K values from 2 to 10. TESS was run several times, removing two individuals from each pair of highly related individuals (relatedness coefficient $r \geq 0.5$) in order to test for the influence of related individuals in the population structure. DIC (Deviance Information Coefficient) for each value of K and individual assignment probabilities were examined.

The discriminant analysis of principal components (DAPC), which is a multivariate method to determine population structure, was also employed. This method has been shown to work well with complex population structure and does not rely on population genetic models (Jombart, 2008). Prior to running the DAPC the optimal number of clusters was identified using k-means and the adegenet package in R v2.1.1 (Jombart, 2008). The values of each K were compared using Bayesian Information Criterion (BIC). For the DAPC analysis the optimization α-score was obtained to determine the number of retained PCs, several runs were performed, using a different number of clusters in each run, until the one that could explain most of the variance was found.

Mitochondrial DNA

To determine the relationship of the Balearic dolphins lineages with the rest of the Mediterranean and North Atlantic populations, seven individuals in Gimnésies, five in Pitiüses and six in Comunitat Valenciana were chosen at random and a 660 bp section of the mtDNA control region was amplified using the primers: Rev (5′GTGACGGGGCCTTTCTAA 3′) (this study) and F2 (5′CTC ACC ACC AAC ACC CAA AG 3′) (this study). PCR conditions were as follows: 150 µM dNTPs, 1.5 mM $MgCl_2$, 20 mM Tris–HCl pH 8.0, 50 mM KCl, 0.3 µM of each primer, 1.25 U/µl of Taq (Bioline) and 20 ng of DNA for a 25 µl total PCR. The PCR cycling profile consisted of 4 min at 95°C, 30 cycles of 45 s at 94°C, 1 min at 55.8°C and 1 min at 72°C, followed by a final extension of 5 min at 72°C. PCR products were purified with a QIAGEN-QIA quick gel extraction kit and were sequenced on an automated sequencer. In those cases ($n = 8$) where the sequences were not of high quality, individuals were sequenced in both directions (forward and reverse) to verify the identity of each nucleotide. Sequences were edited and checked by eye and aligned with the Clustal W application in BIOEDIT 7.0.5.3BIOEDIT 7.0.5.3.

A haplotype network was constructed with statistical parsimony in TCS 1.18 (Clement et al., 2000) with Popart (*Population Analysis with Reticulate Trees*; http://popart.otago.ac.nz), including 112 haplotypes from Quérouil et al. 2007 (haplotypes from pelagic known populations of Azores and Madeira) and from the most recent comprehensive analysis of population structure in the North East Atlantic (NEA) (Louis et al. 2014b). From the latter study, haplotypes that had frequencies of > 0.1 were characterized as Pelagic Atlantic, Coastal North, and Coastal South and were highlighted in the network (see Supplementary Tables 2 and 3 for GenBank Accession Numbers).

Stable isotope analysis

Only biopsy samples were used for stable isotope analyses (SIA). For each biopsy, the skin was separated from the blubber. Once at the laboratory, the portion of skin for SIA was rinsed with distilled water and was dried at 60°C for 48 h and then ground to a fine powder using a mortar and pestle. Skin and blubber samples contain large amounts of lipids, which can interfere with carbon ($^{12}C/^{13}C$) isotope

analysis results (Giménez et al. 2017b). Therefore, lipids must be extracted and eliminated from the sample before analysis. For lipid extraction a modification of Folch's method (Folch et al., 1957), suggested by Morin & Lesage (2003), was carried out. Lipids were removed by rinsing the ground sample several times with a 2:1 chloroform:methanol mixture using an automated agitator for 10 min. The samples were kept for at least 12 h at 4°C; then, they were centrifuged at 750 rpm for 10 min and the supernatant was eliminated. The extraction was repeated twice more, at room temperature with 1 h periods between extractions.

The samples were left to dry and solidify at 60°C for at least 12 h. The solid residue was dissolved in 10 ml of distilled water and was agitated during 5 min with an automated agitator, centrifuged at 1200 rpm for 10 min, and the supernatant was eliminated. This process was repeated two more times and then the solid residue was left to dry at 60°C for at least 12 additional hours. Finally, 2 ± 0.1 mg of this dried sample was placed into a tin capsule and combusted for δ^{15}N and δ^{13}C isotope analyses with a continuous flow mass spectrometer (Thermo Finnegan Delta x-plus). Analyses were conducted at the Scientific-Technical Services Institute (SCTI) of the University of the Balearic Islands. Reference standards used were Vienne Pee Dee Belemnite (VPDB) for C and atmospheric nitrogen for N. Every eight samples of bottlenose dolphin one sample of an internal reference material (Bovine Liver standard (BLS); 1577b; U.S Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD 20899, USA) was analysed in order to calibrate the system and to compensate for drift over time. The analytical precision of the stable isotope analysis was based on the standard deviation of replicates of the BLS reference and was of 0.08‰ for δ^{13}C and 0.09‰ for δ^{15}N. Stable isotopes ratios were expressed in δ notation (Peterson & Fry, 1987) with units of parts per thousands (‰) according to the following equation, where R is the corresponding $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio (Abelson & Hoering, 1961):

$$^{13}C/^{12}C \text{ or } ^{13}N/^{12}N = [(\text{R sample}/\text{R reference} - 1)] \times 100$$

To analyse differences in the isotopic values of δ^{13}C and δ^{15}N and the C:N ratio of *Tursiops truncatus* between geographical localities and sex a permutational multivariate ANOVA was performed (PERMANOVA; Anderson et al., 2008). The design incorporated two factors: 'Macrosite' (fixed) with two levels: Gimnèsies and Pitiüses and 'Sex' (random and nested within the factor 'Macrosite') with two levels: males and females. The resemblance matrix was built on the Euclidean distance. Samples from Comunitat Valenciana (n = 2) were not included in this analysis given the small sample size.

Carbon and nitrogen stable isotopes values from dolphins from both geographical areas were used to describe the isotopic niche. The Total Area (TA) was calculated from the convex hull areas as a measure of total niche (Layman et al., 2007). Along with this metric, five more Layman metrics based on carbon and nitrogen stable isotopes were calculated. These metrics corresponded to the δ^{15}N range (NR) providing information on the trophic length of the community; the δ^{13}C range (CR) which gives an estimate of the diversity of basal resources; the mean distance to centroid (CD) which provides additional information on niche width but also species spacing; the mean nearest neighbour distance (MNND) which provides a measure of density and clustering of species within the community; and the standard deviation of the nearest neighbour distance (SDNND) which gives a measure of evenness of spatial density and packing (Jackson et al., 2011). Additionally, bootstrapping (n = 10,000 replicates) based on the minimum sample size (n = 16) was performed allowing comparison among areas with different sampling effort (Jackson et al., 2011). Following this approach, the mean bootstrapped TA value was also obtained for comparison. In addition, the Standard Ellipse Area (SEA) and the small sample size corrected Standard Ellipse Area (SEAc) were calculated using a p. Interval value of 0.95 (encompassing 95% of the core isotopic data). The Bayesian estimate of the Standard Ellipse Area (SEAb) which is unbiased with respect to sample size (Jackson et al., 2011) was also reported. Stable isotopic niches of dolphins from both areas were calculated using ellipse-based metrics with SIBER (Stable Isotope Bayesian Ellipses in R package; Jackson et al., 2011) implemented in the SIAR package version 4.2 (Parnell & Jackson, 2013) of the R Programming Environment (v.3.3.1).

Results

Microsatellites

Genotyping error rate was 0.055 (10 incorrect genotypes out of 180). Microchecker found no evidence of null alleles or allele dropout among the 11 loci employed. Allele distributions did not deviate from expected Hardy–Weinberg equilibrium levels for most loci, except for D22 and Dde70 in Gimnésies and TV5 in Pitiüses (Table 1). No loci were found to be linked. Allelic richness varied from 1.87 to 7.44 with the lowest number of alleles per locus being two and the highest 11. Heterozygosity values from individual loci varied from 0.206 to 0.952, but overall values for each sampling location were very similar, ranging from 0.566 to 0.668. After Bonferroni correction ($\alpha = 0.016$) significant differentiation (F_{ST}) was found between all the pairwise comparisons (Table 2). After molecular sexing five females and 19 males were identified from Gimnésies, six females, 15 males and one undetermined from Pitiüses, and one female and eight males from Comunitat Valenciana. Overall

Table 2 F_{ST} based on microsatellites below diagonal with P values obtained after: 3000 permutations. Indicative adjusted nominal level (5%) for multiple comparisons is: 0.0167

Area	Gimnèsies $N = 24$	Pitiüses $N = 22$	Comunitat Valenciana $N = 9$
Gimnèsies			
Pitiüses	0.0103 $P = 0.012$		
Comunitat Valenciana	0.0475 $P = 0.007$	0.0451 $P = 0.006$	

Table 1 Microsatellite genetic diversity per population

Locus	Gimnèsies $N = 24$				Pitiüses $N = 22$				Comunitat Valenciana $N = 9$			
	Ho	*He*	*n*	*AR*	*Ho*	*He*	*n*	*AR*	*Ho*	*He*	*n*	*AR*
DO8	0.833 $P = 0.897$	0.787	7	5.27	0.773 $P = 0.175$	0.791	6	5.28	1.000 $P = 1.000$	0.838	6	5.69
D22	**0.273** $P = \mathbf{0}$	**0.765**	8	5.57	0.545 $P = 0.252$	0.538	6	3.91	0.778 $P = 1.000$	0.660	5	4.52
TV7	0.364 $P = 1$	0.359	2	1.99	0.227 $P = 1.000$	0.206	2	1.87	0.222 $P = 0.168$	0.523	2	2.00
TV5	0.364 $P = 0.678$	0.438	5	2.93	**0.455** $P = \mathbf{0.000}$	**0.689**	7	4.87	0.444 $P = 0.065$	0.778	5	4.73
MK6	0.619 $P = 0.221$	0.840	7	5.89	0.636 $P = 0.024$	0.846	7	6.07	0.556 $P = 0.226$	0.706	5	4.55
MK8	0.739 $P = 0.332$	0.720	7	5.15	0.682 $P = 0.976$	0.629	5	3.81	0.667 $P = 0.212$	0.810	6	5.51
Tur117	0.636 $P = 0.068$	0.697	5	3.97	0.636 $P = 0.901$	0.693	5	4.1	0.556 $P = 0.255$	0.778	4	3.99
Dde61	0.696 $P = 0.989$	0.709	7	4.86	0.727 $P = 0.851$	0.682	6	4.6	0.778 $P = 0.730$	0.680	6	5.3
Dde70	**0.625** $P = \mathbf{0.008}$	**0.879**	11	7.44	0.952 $P = 0.747$	0.836	9	6.41	0.667 $P = 0.841$	0.621	6	5.12
Tur138	0.833 $P = 0.961$	0.793	5	4.74	0.773 $P = 0.752$	0.728	4	3.85	0.778 $P = 1.000$	0.660	3	3.00
Dde84	0.458 $P = 0.255$	0.484	6	3.78	0.476 $P = 1.000$	0.405	4	3.04	0.333 $P = 1.000$	0.314	4	3.33
Mean	0.566	0.681			0.596	0.635			0.636	0.668		
SD	0.197	0.165			0.212	0.185			0.225	0.143		

Sample size per population (N), number of alleles (n), allelic richness (AR), observed (Ho) and expected (He) heterozygosity. Hardy–Weinberg equilibrium test P value is shown and significant values after Bonferroni correction $\alpha = 0.0015$ are shown in bold

relatedness within the sampling locations was: Gimnésies $R = 0.008$ with a standard error (SE) of 0.023, Pitiüses $R = 0.094$ with a SE of 0.044, and Comunitat Valenciana $R = 0.114$ with a SE of 0.065.

Population structure was analysed with three different clustering methods. The program *STRUCTURE HARVESTER*, performed the Evanno method (Evanno et al., 2005) for both the admixture and no-admixture models with correlated and uncorrelated frequencies. The Evanno method is unable to identify $\Delta K = 1$. Thus, it chose the most likely number of populations as $\Delta K = 2$ for the admixture and no-admixture models. However, the value of the MeanLnP(K) was highest for $\Delta K = 1$ for the admixture model with correlated and uncorrelated frequencies (− 1733.38 and − 1733.32, respectively) as well as for the no-admixture model with correlated frequencies (− 1733.42). In the case of the no-admixture model with uncorrelated frequencies the value of the MeanLnP(K) was highest for $\Delta K = 3$ (− 1680.34). The case for $K = 1$ being the best value is strengthened by the fact that for $K = 2$ all the individuals show mixed ancestry in equal proportions (Fig. 2).

The Bayesian analysis performed with TESS cannot test for $K = 1$. For each of the runs, DIC values suggested different optimal values of K (Online Appendix, Figs. 13 to 17 indicating instability given the lack of $K = 1$. However, when examining the assignment proportion of each individual, they mostly belong to one grouping except for two individuals that show admixture (individual 2 and 13 in the barplot) (Online Appendix Fig. 19). This pattern of high assignment of individuals to one main cluster was shown across all runs, including those runs when individuals that were highly related to other individuals were removed from the analysis.

In DAPC the *Find.clusters* function obtained the lowest value of BIC at $K = 2$ followed by the value of BIC at $K = 1$ that was relatively low as well. Several runs of the analysis were performed retaining different number of Principal Components (PCs). Finally, a total of 20 PCs were retained, this was the combination that showed higher proportion of conserved variance (0.849) without overfitting the data. This analysis indicated no population differentiation, as the structure found did not correspond to the geographical structure, indicating that the clusters found were likely to be spurious (Appendix Fig. 18); this strengthens the case that the real value of K is one.

Mitochondrial DNA

From the 18 individual mtDNA sequences obtained in this study, there were 11 unique haplotypes (four from Gimnésies, three from Pitiüses, and four from Comunitat Valenciana). The haplotype network showed the majority of the haplotypes from the three sampling locations spread across the network along with the published haplotypes from other geographical areas. One haplotype from Comunitat Valenciana was two mutational steps from those of Gimnésies/Pitiüses, while all other haplotypes from Comunitat Valenciana were situated at least nine mutational steps from those of Balearic individuals (Supplementary Fig. 2).

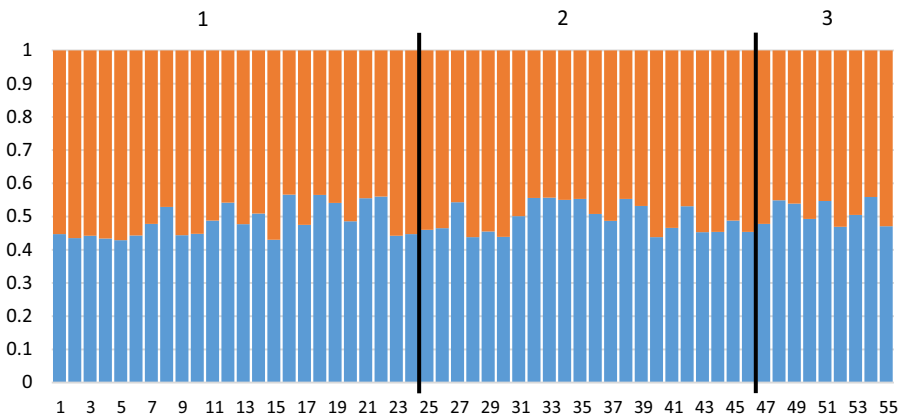

Fig. 2 Structure barplot showing individual inferred ancestry for $K = 2$. Each column represents an individual in each of the three geographical areas analysed: (1) Gimnésies, (2) Pitiüses and (3) ComunitatValenciana. The membership proportion to each of two genetic clusters is shown in the y-axis represented with a different colour

Stable isotopes analyses

Isotopic $\delta^{13}C$ and $\delta^{15}N$ values of dolphins are summarized in Table 3 and Fig. 3. No significant differences were found between males and females (PERMANOVA $P > 0.05$). Dolphins from Gimnèsies had slightly higher average $\delta^{13}C$ and $\delta^{15}N$ values than those from Pitiüses but these differences were not statistically significant (PERMANOVA $P > 0.05$; Table 4). However, Pitiüses dolphins had significantly higher C:N ratios than the ones from Gimnèsies (PERMANOVA $P < 0.001$; Table 4).

In relation to niche area, carbon and nitrogen ranges were narrower for dolphins from Pitiüses than those from Gimnèsies (Table 5). Consequently, the convex hull area TA values were lower in Pitiüses (TA = 1.62) than in Gimnèsies (TA = 3.34). The mean bootstrapped TA values were only slightly lower in Pitiüses ($TA_b = 0.002$) than in Gimnèsies ($TA_b = 0.003$). In addition, the corrected Standard Ellipse Area (SEAc) values were also lower in Pitiüses ($SEA_c = 0.63$) than in Gimnèsies ($SEA_c = 1.51$) (Table 5), which means that niche width in Pitiüses was 2.39 times smaller than in Gimnèsies. Similarly, the full sample TA values were 2.07 times lower in Pitiüses than in Gimnèsies (Fig. 4). The SEAb was computed (see Fig. 4) as well as a density plot for the mode and the 50, 95 and 99% credible confidence intervals for the Bayesian standard ellipse area (Fig. 5).

Discussion

The degree of genetic differentiation found between individuals from Gimnésies/Pitiüses and Comunitat Valenciana (Table 2) based on F_{ST} suggests a limited amount of movement of dolphins between the archipelago and the peninsula. Distances and water depths between the islands of Gimnèsies and Pitiüses are comparable to those encountered between Comunitat Valenciana and the whole archipelago. This is reflected in the fact the genetic differentiation based on F_{ST} between bottlenose dolphins from Gimnèsies and Pitiüses was lower but also statistically significant (Table 2). These results are not consistent with

Table 3 Mean ± SE isotopic values for $\delta^{13}C$, $\delta^{13}N$ and C:N for bottlenose dolphins sampled at Gimnèsies and Pitiüses

	n	$\delta^{13}C$ Mean	$\delta^{15}N$ Mean	C:N Mean
Sites				
Gimnèsies	18	− 14.94 ± 0.10	13.69 ± 0.25	1.88 ± 0.01
Pitiüses	16	− 15.14 ± 0.10	13.43 ± 0.15	1.93 ± 0.01

n number of individuals used to calculate the mean values

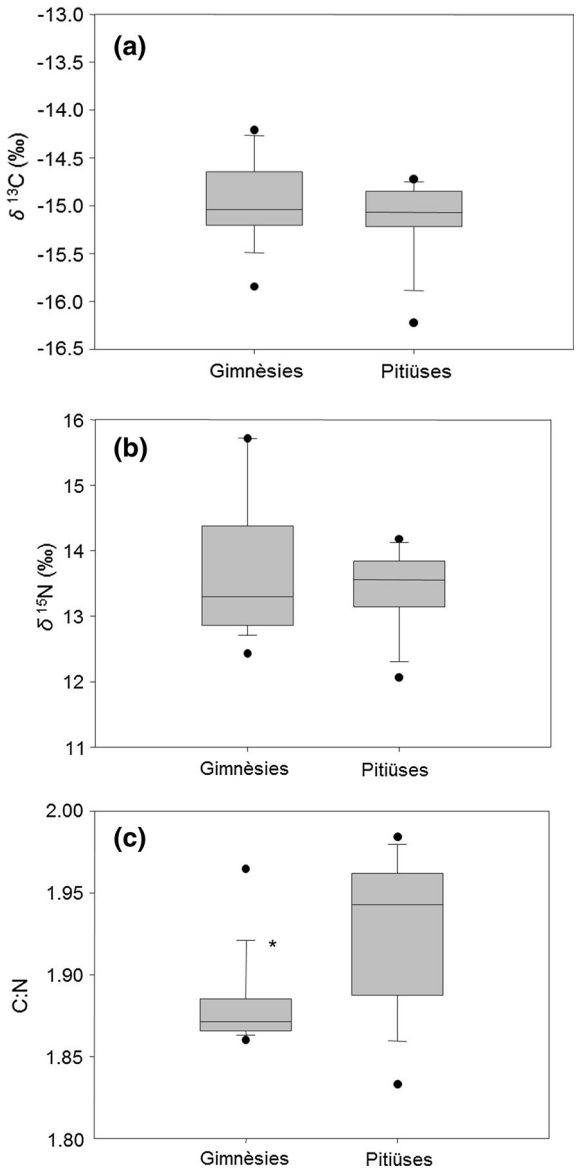

Fig. 3 Box plots with isotopic values for **b** nitrogen (‰), **a** carbon (‰) and **c** carbon:nitrogen ratio for the sampling areas, Gimnèsies and Pitiüses. The significant differences between the locations are shown with an asterisk (*) based on a PERMANOVA pairwise test ($P < 0.001$). Horizontal lines inside the box represent the mean value

Table 4 Summary of the results of the PERMANOVA to test $\delta^{13}C$, $\delta^{15}N$ and C:N ratio at Gimnèsies and Pitiüses and between sex

Source of variation	$\delta^{13}C$				$\delta^{15}N$				C:N			
	Df	SS	MS	*F*	Df	SS	MS	*F*	Df	SS	MS	*F*
Macrosite	1	0.13	0.13	$1.95^{n.s}$	1	0.02	0.02	$0.03^{n.s}$	1	0.01	0.01	19.88*
Sex	2	0.01	0.006	$0.03^{n.s}$	2	0.70	0.35	$0.45^{n.s}$	2	0.0002	0.0001	$0.09^{n.s}$
Residual	29	5.28	0.18		29	22.72	0.78		29	0.04	0.001	

Df degrees of freedom, *SS* sum of squares, *MS* mean sum of squares, *F* value by permutation, asterisk indicates statistical significance at $P < 0.001$, and n.s indicates no significant differences *P* values based on 999 permutations

Table 5 Results for the layman metrics in Gimnèsies and Pitiüses

	TA	TA_b	SEA	SEA_c	CD	MNND	SDMND	NR	CR
Gimnésies	3.34	0.003	1.42	1.51	0.98	0.26	0.29	3.29	1.64
Pitiüses	1.62	0.002	0.59	0.63	0.55	0.30	0.28	2.11	1.50

TA total area, *TAb* bootstrapped total area, *SEA* standard ellipse area, *SEAc* corrected standard ellipse area, *CD* mean distance to centroid, *MNND* mean nearest neighbour distance, *SDNND* standard deviation of the nearest neighbour distance, *NR* nitrogen range, *CR* carbon range values

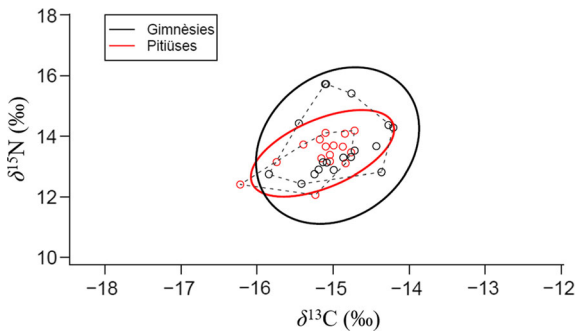

Fig. 4 Isotopic niche according to the convex hull area (solid lines) and the Bayesian estimate of the standard Ellipse Area (dashed lines) for populations of *Tursiops truncatus* at Gimnèsies (black lines/circles) and Pitiüses (red lines/circles) based on carbon and nitrogen isotopic values from dolphins' skin

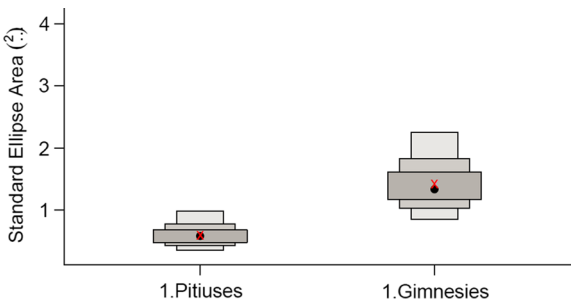

Fig. 5 Density plot showing the confidence intervals of the standard ellipse areas for Pitiüses and Gimnèsies. The black point corresponds to the mode for each area while the grey and white boxes reflect the 50, 95 and 99% confidence intervals

Bayesian cluster analyses or the multivariate approach in DAPC. *STRUCTURE HARVESTER* and *TESS* failed to show any differentiation between the three geographical areas analysed and mainly indicated a shared cluster membership of each individual in scenarios of different values of K, consistent to previous results obtained by Natoli et al. (2005) for the Western Mediterranean. However, it is known that when genetic differentiation is very subtle the analyses carried out using these software packages have limitations in detecting this substructure (Latch et al., 2006). The relatedness value for individuals from Comunitat Valenciana was higher than for individuals from Gimnésies and Pitiüses, which could be influencing the genetic structure observed using F_{ST} values, especially given that the number of samples and markers was relatively small. Of the nine individuals sampled in Comunitat Valenciana, seven came from strandings and the true origin of these individuals could not be determined. Genetic diversity values including observed and expected heterozygosity, along with the allelic richness found in this study are similar to ones previously reported for this species

in the Western Mediterranean (e.g. Natoli et al., 2005). However, the low number of targeted individuals and markers may have limited the amount of genetic differentiation identified in the present study.

The haplotype network showed all the new haplotypes obtained in this study were spread throughout the network and were close to the pelagic haplotypes from the North East Atlantic. In the present study, the number of individuals sequenced from each sampling location is small and cannot be considered representative of the whole community or assigned to the two ecotypes (pelagic and coastal bottlenose dolphins) described in the North East Atlantic and Mediterranean Sea (Louis et al., 2014b; Gaspari et al., 2015).

The ability to resolve population structure and niche separation through stable isotope analyses depends on the presence of sufficiently large and distinct isotopic differences between prey types or foraging locations (Newsome et al., 2010). Results obtained here came from fresh samples, while most results from the literature and the research area come from stranded individuals (Borrell et al., 2006; Cardona et al. 2007; Capelli et al., 2008; García-Tiscar 2010). However, tissue decomposition was not found to change the isotopic ratios of striped dolphin, *Stenella coeruleoalba* (Meyen 1833), muscle or skin (Payo-Payo et al., 2013). Therefore, a valid comparison can be made between results in the literature, obtained from stranded and biopsied individuals, and results from the present study. Nitrogen isotopic values (δ^{15}N) are generally used to quantitatively assess the trophic level of a given taxa (Hobson et al., 1995), although Chouvelon et al. (2012) suggest that in contrasted ecosystems nitrogen isotopic ratios may also be indicator of the feeding area. Carbon isotopic values (δ^{13}C) are applied to indicate the relative contributions to the diet of different potential primary sources in a food web, giving evidence of inshore versus offshore food intake (higher δ^{13}C values are more related to an inshore/benthic origin of food sources (Hobson et al. 1995)). The present study showed generally higher isotope ratios, especially those of δ^{13}C (-14.94 ± 0.10‰ and -15.14 ± 0.10‰), compared to values obtained from the literature [e.g. -18.94 ± 0.83‰ (Balearic Islands), -18.17 ± 1.16‰ (Catalonia), and -18.88 ± 1.04‰ (Valencia) (Borrell et al., 2006), -17.30 ± 0.90‰ (Balearic Islands; Cardona et al., 2007), -18.6‰ and -17.1‰ (Ligurian Sea; Capelli et al., 2008)]. This could suggest a more coastal habitat or a higher consumption of coastal prey by the bottlenose dolphins included in the present study. In addition, dolphins from both study areas were feeding at the same trophic level (similar δ^{15}N values) and the overlap of isotopic niches in both areas suggested similar diets and foraging habitats (Cherel & Hobson, 2007). In support of this, no statistical significant difference was found between the δ^{15}N and δ^{13}C signatures of dolphins from Gimnèsies and Pitiüses. However, the trophic niche for Gimnèsies' individuals was found to be larger than that of Pitiüses' individuals, shown by a higher mean distance to centroid value as well as larger δ^{13}C and δ^{15}N ranges, which give an estimate of the diversity of basal resources and provide information on the trophic length of the community, respectively (Jackson et al., 2011). This could indicate that dolphins from Gimnèsies have a broader range of prey, specialize in different prey, or that the habitat around Gimnèsies is more diverse. Additionally, this boarder trophic niche could also reflect more diverse oceanic conditions or a larger mobility range of dolphins from shallower to deeper areas in Gimnèsies (Gibbs et al., 2011).

Louis et al. (2014a) demonstrated that the ecological niches of pelagic and coastal bottlenose dolphins were highly segregated in the North East Atlantic, applying population genetics and ecological approaches. The authors found higher δ^{13}C and δ^{15}N values in coastal areas and also a consistency of coastal and pelagic prey types in stomachs of dolphins from coastal and pelagic ecotypes, respectively. Given that isotopes from our study come from biopsies, no stomach content analysis could be performed to provide complementary information. However, our results do highlight significant differences between the areas based on C:N ratios, which could indicate different quality in bottlenose dolphins' diets and a different nutritional status of dolphins from each sampling area. This highlights the utility of C:N as biological tracer. We found that Gimnèsies' dolphins had both lower C:N ratios and a boarder trophic niche, suggesting that a higher quality diet may be related to a more diverse range of preys. As isotopic profiles are measured in skin, reported information represents the diet of the animals over approximately the previous 15–70 days (Giménez et al., 2016), indicating that these individuals had been feeding in areas with high carbon isotopic basal values during the last one to two

months. Regardless of the absence of significant differences in $\delta^{13}C$ or $\delta^{15}N$ values between dolphins from Gimnèsies and Pitiüses, photo-id data has shown a certain degree of site fidelity at smaller scales within the archipelago (Gonzalvo et al., 2014). Dolphins from both sampled areas showed $\delta^{13}C$ signatures typical from coastal environments which suggests limited feeding in pelagic waters between islands, and in turn supports site-fidelity to coastal areas as reported by Gonzalvo et al. (2014). Such site fidelity could facilitate the gradual isolation of local groups.

Bottlenose dolphins form fission–fusion societies (Bearzi et al. 2005) and ecology and cultural transmission of tool use have been reported to contribute to genetic and social structuring for this species (Krützen et al., 2005; Kopps et al., 2014). Thus, the use of combined techniques to discriminate populations, as applied in the present and previous studies (Louis et al., 2014a; Giménez et al., 2018), is advisable, and is relevant to studies of other species and areas (Foote et al., 2009). Particularly when population structure over both long and short time scales are of interest, a combination of genetic and ecological markers may be preferred.

Our study was limited by the relatively small number of both individuals sampled, DNA markers and ecological tracers used. An increase of coverage both in terms of sample size of individuals, genetic markers, and ecological tracers as well as complementary studies addressing social structure may contribute to more definite conclusions. However, our study showed that the deep waters that separate the Iberian Peninsula and the Balearic archipelago, and Gimnèsies and the Pitiüses within the archipelago do not constitute strong barriers to gene flow in bottlenose dolphins, although subtle population structure was observed and should be further investigated. This has implications for the local management of the species because (i) they belong to a wider Mediterranean population considered to be in decline (Blanco & González, 1992; Borrell et al., 2000; Bearzi et al., 2004; Bearzi & Fortuna, 2006), (ii) Balearic individuals are subject to mortality risk resulting from interactions with local artisanal fisheries (Brotons et al., 2008; Gazo et al., 2008), (iii) the estimated population size in the archipelago is relatively small (Gonzalvo et al., 2014), and (iv) their coastal habitat is subject to increasing anthropogenic pressures, as it is a mass tourism destination (Gonzalvo et al., 2014). Even with subtle population substructure, direct management actions can be undertaken in the area, including fishery-specific reductions in fishing effort, modifications to fishing gear and stricter regulations regarding marine pollutants, recreational boat traffic and anchoring.

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Compliance with ethical standards

Conflicts of interest Author JMB declares that he has no conflict of interest. Author VI declares that she has no conflict of interest. Author CA declares that she has no conflict of interest. Author AT declares that she has no conflict of interest. Author RF declares that she has no conflict of interest. Author SD declares that she has no conflict of interest.

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