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1 **An holistic ecological analysis of the diet of Cory’s shearwaters using prey**
2 **morphological characters and DNA barcoding**

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Abstract

Knowledge of the dietary choices and trophic niches of organisms is the key to understanding their roles in ecosystems. In seabird diet studies, prey identification is a difficult challenge, often yielding results with technique-specific biases. Additionally, sampling efforts are often not extensive enough to reveal intra-population variation. Immature animals, which may constitute up to 50% of a population, may occupy a significantly different trophic niche to more-experienced birds, but this remains largely unexplored. We investigated the diet of Cory's shearwater (*Calonectris diomedea*) from Selvagem Grande, an island located off the northwest African coast, collecting a total of 698 regurgitate samples over three consecutive breeding seasons. The diet was assessed using two complementary approaches for prey identification: conventional morphological analysis (using fish vertebrae, otoliths and cephalopod beaks) and DNA barcoding of the 16S rRNA mitochondrial gene, in cases where a positive identification could not be retrieved. Species assignments employed BLAST and distance based methods, as well as direct optimization of the tree length based on unaligned sequences in POY. This method resulted in robust tree estimates and species assignments, showing its potential for DNA barcoding of stomach contents using hypervariable markers such as the 16S. The molecular approach increased taxonomic resolution and revealed an additional 17 taxa. Diet differed significantly according to breeding status, sex, breeding phase (pre-laying and chick-rearing) and year. Such direct evidence of trophic segregation within the same population has rarely been shown in seabirds and highlights the importance of including such variables in ecosystem-based management approaches.

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Introduction

Dietary studies are essential building blocks of the science of ecology. Only with the support of dietary studies can we properly assess the position of species in food webs, their role in energy flow within ecosystems, the importance of feeding resources for demographic regulation and the impact of predation on populations and communities. Despite this, the trophic niche of numerous ecologically important species, such as top marine predators, remains poorly understood (e.g., Naito *et al.* 2013). Two primary problems in previous research have been difficulties with prey identification and failure to sample relevant population segments (Barrett *et al.* 2007; Bowen *et al.* 2013) that may potentially display niche differentiations (Polis 1984). Amongst seabirds, which are major pelagic consumers, much effort has gone into sampling the diet of breeding birds (often only at the chick stage) while virtually nothing is known about non-breeders (Barrett *et al.* 2007). This gap is particularly relevant when one considers that non-breeders (mostly immature individuals) may represent >50% of the fully grown individuals in a population.

Trophic niche differentiation between immature and adult reproducing individuals is to be expected in species where growth is protracted and niche is strongly influenced by body size (e.g., Lucifora *et al.* 2009). However, in other taxa, particularly in birds, size varies little amongst fledged individuals. Nevertheless, even for a relatively invariable body size, we may expect differences linked to, for example, (a) age-related improvements in foraging competence (Kitowski 2003; Daunt *et al.* 2007), (b) the competitive exclusion of subdominant (generally younger) individuals by more dominant conspecifics (Goss-Custard *et al.* 1982), or (c) differential spatial distribution arising from the need of reproducing individuals to regularly attend breeding sites. Despite these expectations, we currently know very little about

69 whether there are ontogenetic changes of trophic niches in birds, or their possible causes and
70 consequences.

71 Molecular techniques, such as DNA barcoding (Kochzius *et al.* 2010; Zhang &
72 Hanner 2012), are revolutionising dietary studies and are now being extensively applied in
73 dietary analyses of vertebrate and invertebrate carnivores and herbivores (reviewed in
74 Symondson 2002; Pompanon *et al.* 2012). Prey species can be identified even from highly
75 degraded tissue (as found in faeces and regurgitates), using PCR. Most of these studies have
76 identified prey species from homogenised meta-samples (guts or faeces), with quantitative
77 estimates of species consumed derived from sequences obtained for each identified prey using
78 either a cloning and sequencing technique or Next Generation Sequencing. Nevertheless,
79 differences among prey species in the mitochondrial copy numbers per cell, as well as in the
80 binding efficiency of the primers (Symondson 2002; Pompanon *et al.* 2012), may lead to
81 substantial biases. One way to overcome this problem is to use a combined approach, using
82 morphological analyses for quantitative estimates of prey (hard parts recovered from guts or
83 faeces) plus augmentation of species identification using DNA barcoding of tissues (Barnett
84 *et al.* 2010; Dunn *et al.* 2010). Applying this approach to pelagic top-predators has the
85 potential to enhance understanding of trophic dynamics and, as such, marine conservation and
86 ecosystem-based management.

87 Birds are amongst the best studied animal classes, yet few studies have used molecular
88 techniques to improve our understanding of their trophic ecology (e.g., Deagle *et al.* 2007;
89 Jedlicka *et al.* 2013). Recently, molecular approaches has been used to investigate the dietary
90 habits of seabirds, but those few studies have analysed faeces only (Deagle *et al.* 2007;
91 Bowser *et al.* 2013; Jarman *et al.* 2013; but see Jarman *et al.* 2002), which implies that
92 quantification of identified prey remained relatively crude (Deagle *et al.* 2010). The first aim

93 of the present paper is to develop the technique and illustrate the tremendous potential of
 94 using DNA barcoding combined with morphological tools to provide an unusually refined
 95 picture of the diet of birds (in this case, of a pelagic seabird).

96 Our study model is the Cory's shearwater (*Calonectris diomedea borealis*), an oceanic
 97 predator of the Northeast Atlantic, which breeds on several islands and islets from the Azores
 98 and Berlengas archipelagos in the north to the Canary archipelago in the south (Thibault *et al.*
 99 1997). The feeding ecology of Cory's shearwaters has been studied at several colonies
 100 (Granadeiro *et al.* 1998; Paiva *et al.* 2010; Xavier *et al.* 2011; Neves *et al.* 2012), but little is
 101 known about their diet in the southern area of their breeding range (but see den Hartog &
 102 Clarke 1996; Paiva *et al.* 2010 for studies with limited sampling effort). More importantly,
 103 Cory's shearwaters are long-lived birds that only start reproducing at a mean age of 9 years
 104 and frequently skip breeding seasons, even after their first reproduction (Mougin *et al.* 1997).
 105 As such, a large proportion of Cory's shearwater populations is comprised of non-breeders,
 106 but their trophic ecology has never been investigated. We also have a poor understanding of
 107 male-female differences in the ecology of this dimorphic species (Navarro *et al.* 2009; Ramos
 108 *et al.* 2009) and most studies carried out found no evidence of spatial (Navarro *et al.* 2009) or
 109 foraging niche sexual segregation (Navarro *et al.* 2007; Ramos *et al.* 2009). Hence, the second
 110 broad objective of this paper is to characterise the diet of Cory's shearwaters in the southern
 111 part of its breeding range and assess within-population sources of variation, with a particular
 112 interest in differentiation between breeders and non-breeders, indicative of ontogenetic shifts
 113 in the trophic niche of this seabird.

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Methods

Study area and species

Fieldwork was conducted in the Selvagem Grande island (30° 09' N, 15° 52' W), where ca. 30,000 Cory's shearwater breeding pairs nest (Granadeiro *et al.* 2006). This subtropical oceanic island is located ca. 350 km from up-welling enriched shelf areas off the African coast. Cory's shearwaters are long-distance migrants returning in early March from their wintering sites in the south Atlantic (Thibault *et al.* 1997). During the extended pre-laying period, birds re-occupy their nest cavities, protecting them from prospecting birds and eventually finding a mate. Egg-laying takes place at the end of May with the chicks hatching at the end of July. The chick-rearing period lasts approximately 97 days, until early November, when chicks fledge (Thibault *et al.* 1997).

Diet sampling and analysis

Sampling was conducted in the pre-laying period of 2010 (11 to 20 April) and during the chick-rearing periods of 2008, 2009 and 2010 (28 July to 2 October). Shearwaters returning from the sea were captured by hand when entering the nest or preparing to feed their chick. Non-breeding birds were also sampled in the chick-rearing period of 2009. In the incubation period, when birds are more sensitive to disturbance and are more likely to have empty stomachs, only non-breeders were sampled (15 to 25 June of 2010). Non-breeding Cory's shearwaters tend to stay outside the nest cavities and to be very vocal and socially interactive, and are, therefore, easily selected for sampling.

Birds were sexed based on their distinctive vocalisations (Thibault *et al.* 1997) or using a discriminant function based on bill measurements, which has a 98.8% success rate (Granadeiro 1993).

Prey samples were collected from the birds using the water off-loading technique (Wilson 1984). By selecting different areas each day and marking the birds with wax markers, we guaranteed that birds and nests were only sampled once. Fresh prey items found in food samples were identified using specialised guides (Quéro *et al.* 2003) and stored in 70% ethanol. Digested fish were quantified and identified to the lowest possible taxon from vertebrae and other hard remains (otoliths, dentaries and scales), using our own reference collection and published guides (Tuset *et al.* 2008). Cephalopods were identified from their beaks and quantified based on the number of mantles, other fresh remains (tentacles, flesh) and fresh beaks (upper and lower beaks were counted).

A large number of fish from the genus *Scomber* were identified as *Scomber colias* (41.1%, N = 538). None was identified as *Scomber scombrus* and it was only possible to identify the remaining individuals to genus level (*Scomber*). Given this result, we pooled *Scomber colias* and *Scomber* sp. in all further analyses. We calculated frequencies of occurrence (FO): the number of samples with a given prey type, expressed as a percentage of the total number of samples and numerical frequencies (NF): the number of individuals of a given taxon, as a percentage of the total number of prey items.

Given their small size (less than 3 mm), most unidentified crustaceans and insects found in the samples were unlikely to be their direct prey, and were probably part of the diet of fish captured by shearwaters (secondary predation). Considering their parasitic habits, crustaceans from the family Isopoda were also probably captured along with fish prey. None of these prey were included when calculating the numerical importance of prey. The

exception were three larger crustaceans (more than 30 mm, Decapoda) that were considered to be part of the shearwater diet.

Genetic analysis

A total of 83 muscle samples (27 cephalopods and 56 fish), either unidentified through conventional diet analysis (45 samples) or only identified to higher taxonomic levels (*Trachurus* sp. and Exocoetidae) (38 samples), were examined using DNA barcoding (16S rRNA). Although the cytochrome c oxidase subunit I (COI) has gathered wide consensus as a genetic marker for species discrimination of unknown taxa (Hebert *et al.* 2003), the 16S barcode provided a higher sequence database coverage within the range of prey identified in Cory's shearwater diet. For example, in teleosts, all genera within the family Exocoetidae were covered for the 16S, but only three for the COI. A search on squid "Teuthida" in the GenBank database retrieved 359 matches against 305 (after excluding the family Loliginidae, which is by far the best represented in Genbank). Therefore, the 16S was more informative in the context of this study.

We collected pieces of tissue from prey associated with hard structures (e.g., vertebrae) that could not be identified morphologically and used these for DNA barcoding. To extract prey DNA, individual prey tissue was washed with ddH₂O to remove adherent ethanol. As in other barcoding studies, that identified prey remains in stomach contents (e.g., Barnett *et al.* 2010), we chose where possible the inner parts of the tissue, since tissue from complex meta-samples may be contaminated with DNA of other prey. The DNA was extracted using the DNeasy Blood and Tissue Extraction Kit (Qiagen) following the protocol for purification of total DNA from animal tissues. Individual prey DNA from regurgitates was amplified using the universal primers of Palumbi (1996): 16ar, 5'-

CGCCTGTTTATCAAAAACAT-3' and 16br, 5'-CCGGTCTGAACTCAGATCACGT-3',
with an expected amplicon length of ca 550-620bp.

Polymerase Chain Reactions (PCR) were performed with the Multiplex PCR Kit (Qiagen) using the following PCR reagent mixtures: 10µl of Multiplex PCR Master Mix (1X), 0.25µM of each primer, 0.1mM of BSA, 3.6 µl ddH₂O, 2.4µl (~50 – 100 ng/µl) of template DNA in a total volume of 20 µl. Thermal cycling conditions were as follow: 95°C for 15min; 35 cycles of 94°C for 30s, 52°C for 90s, 72°C for 90s, and a final extension at 72°C for 10min. PCR products were cleaned using ExonucleaseI and Antarctic Alkaline phosphatase enzymes (New England, Biolabs) and sequenced using the EZ-seq services of MacroGen, Inc (Amsterdam, Netherlands).

Molecular identification of prey using BLAST

Sequences were compared with those in GenBank using the BLAST algorithm (Altschul *et al.* 1990). Each taxonomic assignment was based on the percentage of similarity with the reference sequences in GenBank. Species were directly assigned when the query sequence produced an identical match to the reference sequence (100% of identity). For BLAST matches higher than 99.0%, species were assigned when the query sequence matched monotypic genera or when the distribution range of potential con-specifics was outside our study area, but only if no other species was retrieved with this value. Inter-specific divergences in teleosts are > 2% (i.e., Kochzius *et al.* 2010, Zhang & Hanner 2012) and in cephalopods 1.3-12.7% (Dai *et al.* 2012). Therefore, the above criteria were expected to produce robust species identifications.

Molecular identification of prey using phylogenetic analysis

Specimens were assigned using phylogenetic inferences in cases where the percentage of similarity between the query sequence and the reference sequence was lower than 99.0%. Two methods were used: (a) distance based Neighbour-Joining (NJ) trees and (b) direct optimisation (DO) of the tree length.

NJ trees were constructed in Mega 6 (Tamura *et al.* 2013) using the Kimura-2-parameter model of evolution (Kimura 1980). The nodal support was obtained using a 1000 bootstrap replicates. NJ trees were estimated from eight different data sets of aligned sequences, each corresponding to the families that produced the nearest match with the query sequences. Sequences for which no positive identifications were obtained in BLAST were included and aligned with all available representative genera of that family using Clustal W (Thompson *et al.* 1994) as implemented in BioEdit (Hall 1999).

DO analysis were performed in POY v 5.0.0 (Varón *et al.* 2010). This program infers the tree directly from unaligned sequences and overcomes, therefore, potential uncertainties in sequence alignment (e.g., the hypervariable 16S mtDNA, where different numbers of indels between sequences can significantly impact tree estimates). To generate the POY tree we used the reference sequence that produced the nearest match in BLAST, including all other congener reference sequences of the same family. The tree estimated in POY did not include cephalopods since only two taxa could not be identified using BLAST (only represented in NJ trees). Sequences were trimmed to produce the exact same sequence terminals (374-396 base pairs), since sequences that are absent in the terminals can account for erroneous indel event counts in POY (De Laet 2010).

Because POY uses empirical gap cost criteria to optimise the tree length, we first performed sensitivity estimates under five different affine gap costs regimes: (2,1,1), (2,1,2),

(2,1,3), (2,1,5), (2,1,7) (substitution cost, gap extension, gap opening). Trees based on parsimony were constructed using 100 initial trees generated by random addition sequences using Subtree Pruning and Regrafting (SPR) and Tree Bisection and Reconnection (TBR) branch swapping. The tree producing the most congruent topology with what is known of the evolutionary relationships of teleost fish was chosen as the “optimum” tree. Nodal support was calculated using a 1000 bootstrap replicates with alternate SPR and TBR swapping.

Assignments of families and genera were obtained using a strict criterion based on how query sequences clustered in the NJ and DO trees (Wilson *et al.* 2011). According to this criterion, a taxon (family or genera) is identified if the query sequence nests within a clade that comprises members of that taxon.

As some of our query sequences that produced 100% matches in BLAST showed high similarities with other congeners (>98%) (families Carangidae and Exocoetidae), those were also included for phylogenetic analysis to validate species assignments. Species were identified if the query sequences clustered monophyletically with the taxon that produced an identical match in BLAST and with no other congeners. Unidentified vertebrae based on morphological analysis but otherwise positively identified using DNA barcoding were later used to identify those species and quantify their occurrence in all samples.

Statistical analysis

We initially checked for overall differences in the diet between sexes and among years using permutational multivariate analysis of variance based on distance matrices, implemented using the package “vegan” (Oksanen *et al.* 2011) running in R software (R Development Core Team 2010). The method undertakes a partitioning of the sums of squares of a multivariate data set, using semi-metric and metric distance matrices to produce a

“pseudo-F value”. We tested for the effects of sex and year on the frequency of occurrence of all prey items (with frequencies larger than 5% in one breeding phase). Whenever these tests provided significant results, we further explored the effects of these factors (and their interaction) on the occurrence of each prey using binomial GLMs (Generalized Linear Models), with a logit link function. The statistical significance of each factor was tested through log-likelihood ratio tests of increasingly simpler nested models, based on chi-squared distributions.

Results

A total of 698 regurgitates were collected from adult Cory’s shearwaters. From these samples, a total of 2018 prey items were collected, 76.6% of which were successfully identified to the species or genus level.

Morphological inspection of the 2018 prey items retrieved 40 different prey types, but only 23 of those could be identified to species or genera. The use of DNA barcoding on morphologically unidentifiable specimens increased the prey list to 17 new taxa (12 species, 3 genera and 2 families).

Prey discrimination

DNA barcoding of the 16S ribosomal RNA gene produced longer fragments in teleost fish than in cephalopods varying approximately between 550-600 and 460-500 base pairs, respectively. DNA sequences were submitted to GenBank (Table S1).

BLAST comparisons in GenBank allowed for positive identification of 35% of the sequences to the species level, while phylogenetic inferences successfully discriminated

another 46% to the genus level. From these sequences, 14 (10 species, 2 genera and 2 families) matched taxa that have never been identified in the diet of Cory's shearwaters using morphological characters (Table 1). We also confirm the presence of the neon flying-squid (*Ommastrephes bartramii*) in the diet of these birds, where the beaks of small specimens were difficult to distinguish from those of the European flying-squid (*Todarodes sagittatus*).

It is noteworthy that the values of similarity between species and genera varied substantially, depending on the families and prey groups analysed. While most teleost families and cephalopods showed sequence homologies lower than 98% between conspecifics and congeners (within the reported divergences of vertebrate species), members of the families Exocoetidae and Carangidae presented very high homologies even between genera (ca. 99%). Therefore, identifications in both families were only obtained based on phylogenetic inferences. Regardless of the method employed for estimating trees (NJ or DO) the terminal topologies between the different trees were highly congruent (Fig. 1, Figs. S1, S2). Congeners clustered in highly supported monophyletic groups, with the exception of some members of the family Myctophidae and the genus *Cheilopogon*, that were paraphyletic and polyphyletic, respectively. Query sequences clustered, generally, with the reference sequences that produced the highest sequence homology in BLAST. Moreover, DO inferences resulted in a highly resolved tree at both internal and terminal nodes with an "optimal" tree obtained using the following settings: cost regime of substitutions = 2, indels = 1 and gap opening = 3. A total of seven major clades with high bootstrap support (85-100) were obtained, with each representing a different family of teleost fish.

Based on phylogenetic assignments using strict and liberal criteria we were also able to increase the taxonomic resolution of morphologically unidentified Exocoetidae and *Trachurus* specimens, identifying two species of *Cheilopogon* (*C. melanurus* and *C.*

pinnatibarbatus) and the species *Trachurus picturatus*. Furthermore, morphologically unidentified members of the family Exocoetidae presented seven distinct Molecular Operational Taxonomical Units (MOTUs), revealing a high diversity among these prey items. Sequences of morphologically unidentified *Trachurus* specimens presented two distinct MOTUs, where most sequences clustered separately from *T. picturatus* and the reference sequences. The congruence in tree topologies as well as the taxonomic resolution obtained suggests that genetic variability within the 16S rRNA gene is sufficient to discriminate between species.

Diet composition

During the chick-rearing period, the diet of Cory's shearwaters was essentially composed of fish (FO range = 88.7 to 91.1%) and cephalopods (FO range = 27.2 to 46.8%). Chub mackerel (*Scomber colias*/sp.) was the most common prey (FO range = 35.6 to 51.2%) (Table 2). Pilot-fish (*Naucrates ductor*; FO range = 13.3 to 16.1%), sardine (*Sardina pilchardus*; FO range = 7.8 to 20.2%) and flying-fish (Exocoetidae; FO range = 7.4 to 14.4%) also occurred frequently. Among flying-fish, two genera were found, namely *Exocoetus* (FO range = 1.2 to 10%) and *Cheilopogon* (FO range = 1.1 to 3.2%). Subsequently, four species were identified: the tropical two-wing flying-fish (*Exocoetus volitans*), the bandwing flying-fish (*Cheilopogon exsiliens*), the Atlantic flying-fish (*C. melanurus*) and Bennett's flying-fish (*C. pinnatibarbatus*). The diet of Cory's shearwaters was diverse, being composed of at least 33 fish species from 20 different families (Table 2). Unidentified fish were found in 13.3 to 19.7% of the samples mostly because they were too digested or lacked identifiable hard remains.

The most common cephalopods were the neon flying-squid (*Ommastrephes bartramii*; FO range = 6.4 to 13.7%) and *Histioteuthis arcturi* (FO range = 1.1 to 5.3%). In addition, ten other cephalopod species from nine different families were present in the diet of Cory's shearwaters (see Table 2).

Crustaceans (FO range = 4.4 to 14.4%), mostly from the order Isopoda (FO range = 1.1 to 11.1%) and insects, from the family Halobatidae (FO range = 0 to 0.8%), were also present in the diet samples (Table 2). Fishery hooks were also found in three diet samples (FO = 0.5%).

Diet of non-breeders

During the chick-rearing period of 2009, the diet of non-breeders and breeders differed significantly (pseudo- $F_{1,240} = 3.63$, $p = 0.04$; Fig. 3). Non-breeders preyed heavily on cephalopods (FO = 63.2%), compared to breeders of the same year (FO = 35.1%), and consumed less chub mackerel (Fig. 3). During this period, neon flying-squid (FO = 36.8%), chub mackerel (FO = 31.6%), pilot-fish (FO = 21.1%) and horse/blue mackerel (FO = 10.5%) were the most frequent prey item of non-breeders (Fig. 3). In the incubation period, non-breeders also consumed less fish (FO = 12.1%) while cephalopods were much more frequent in their diet (FO = 93.9%), particularly neon flying-squid (FO = 45.5%).

Sex and inter-annual variations in diet

During the chick-rearing period, we found significant dietary differences between sexes (pseudo- $F_{1,513} = 10.63$ $p < 0.001$; Table 3). Females delivered significantly more chub mackerel to their chicks than males. In contrast, males provided the chicks with more sardines (Table 3). We also found significant inter-annual variations in the diet of shearwaters

(pseudo- $F_{2,513} = 11.52$, $p < 0.001$), which were particularly noticeable in 2010, when the consumption of sardine and cephalopods was higher (Table 2), in comparison to previous years.

Seasonal variations in diet

The diet of shearwaters was substantially different among periods of the same year (pseudo- $F_{1,200} = 17.55$, $p < 0.001$). In the pre-laying period of 2010, trumpet fish (*Macroramphosus scolopax*; FO = 36.7%, NF = 83.3%) and horse/blue jack mackerel (*Trachurus* sp.; FO = 40.0%, NF = 9.7%) dominated the diet of Cory's shearwaters (Fig. 2, Table 2). In this period, cephalopods were found to occur less frequently (FO = 13.3%, NF = 1.3%), than during the chick-rearing period of the same year (Fig. 2, Table 2). Other fish, particularly sardine and pilot-fish were frequent during the chick-rearing period, but completely absent from the diet during the pre-laying stage (Fig. 2, Table 2).

Discussion

This study of the diet of Cory's shearwaters from Selvagem Grande provides detailed information on the feeding ecology of this species. We used morphological and DNA barcoding methods in a complementary way to characterise and quantify the dietary composition of a pelagic seabird, showing the advantages of combining both techniques in diet studies of marine predators. The large number of samples collected during the provisioning period revealed dietary differences between birds of different breeding status and between sexes, something rarely shown to occur in seabirds.

The use of DNA barcoding in prey identification

DNA barcoding greatly improved our knowledge of Cory's shearwater prey range, identifying species that would be overlooked in an analysis based solely on morphological traits. This was the case for small mesopelagic prey (such as myctophids), but also of some epipelagic and bathypelagic species that tend to be underestimated in morphological analyses due to a lack of representation in reference collections. Moreover, DNA barcoding proved to be effective in the identification of juvenile cephalopods, such as the neon flying-squid, a dominant prey in the diet of Cory's shearwater. Indeed, the identification of cephalopods from their beaks is challenging, particularly for small individuals, as many diagnostic characters only develop later in life.

In generalist predators such as seabirds, prey species can only be identified if a comprehensive database of reference sequences across different prey groups exists (Hebert *et al.* 2003). Mitochondrial 16S rRNA sequences are the most widely used marker in marine systems and provided the most extensive database of sequences relevant to the potential prey of Cory's shearwaters. We found that inter- and intra-specific variability of the 16S barcode was effective for accurate prey species discriminations in teleosts and squid, with GenBank reference sequences showing high percentage matches in BLAST and congeners clustering monophyletically in the NJ and DO trees. Depending on the studied species and on the potential prey, the 16S mtDNA gene seems to be a reliable marker for dietary analysis of marine predators.

The incompleteness of reference databases has been widely acknowledged as the main factor limiting accurate taxonomic assignments using DNA barcodes (Valdez-Moreno *et al.* 2012), but is also a limiting factor in morphological analyses. In the case of the families Trichiuridae and Synphobranchidae only a few species and genera are represented in the

GenBank database and, therefore, only family level assignments were obtainable. The expansion of the taxonomic and geographic scope of fish and cephalopod reference material in GenBank, particularly for oceanic species, is needed to disentangle the identification of closely related species.

Prey identified in the families Exocoetidae and Carangidae produced very similar BLAST matches to different genera and species in the GenBank database. In the case of Exocoetidae, query sequences produced matches with percentage of similarity > 98% even between different genera. Although most of our sequences could be reliably assigned to genus level based on the trees, many species were polyphyletic suggesting that evolutionary relationships in these genera are unclear (especially *Cheilopogon*). These findings may be an artefact inherent to the use of single markers, which represent only a small snapshot of the evolutionary history of species. However, studies using mitochondrial and nuclear markers (cytb and RAG2) have also reported polyphyly of the genus *Cheilopogon* (Lewallen *et al.* 2011). Therefore, species assignments based on divergence thresholds should be interpreted with caution in these groups. Regardless of the success in species identification, DNA barcoding also allowed identification of a high number of MOTUs, reflecting the diversity of flying-fishes around the Selvagens islands.

The complementary use of DNA and conventional methods allowed us to identify fish vertebrae of particular species (e.g., *Ranzania laevis*, *Katsuwonus pelamis*) and to use those in subsequent identification and quantification, bridging some of the gaps in our morphological reference collection. We should note that it is not always possible to collect viable tissue samples from digested prey remains (frequent in Procellariiform diet samples) in order to perform genetic analysis. Therefore, relying on a combined approach, we were able to maximise the identification and quantification of different types of prey.

Diet of non-breeders

The diet of non-breeding/immature seabirds has been seldom studied, mainly due to the difficulty of obtaining a sufficient number of samples at the breeding colony (Barrett *et al.* 2007; Granadeiro *et al.* 2009). Most researchers have relied on the isotopic analysis of tissues, and suggested that immatures feed at a lower trophic level (Forero *et al.* 2002; Votier *et al.* 2011), but the lack of taxonomic resolution of this approach prevented a better understanding of those differences.

At Selvagem Grande, a high number of non-breeding individuals, mostly composed by immature individuals, attend the colony during the breeding period (Granadeiro *et al.* 2009). We found that, during the chick-rearing period (August/September), the diet of non-breeders was substantially different from that of breeders, with a higher incidence of cephalopods (FO = 63.2% versus 35.1%) in the former group. Furthermore, in June of the same year, the incidence of cephalopods (mostly neon-flying squid) in the diet of non-breeders was even higher (FO = 93.9%). These results strongly point towards an ontogenetic shift in the trophic niche, the causes of which need to be evaluated by further studies. Non-breeders are less constrained by the need to attend the nesting colony and as such we would have expected them to feed more on distant (coastal) prey. However, the opposite pattern was revealed by our data, as squid in our system is more often captured in offshore waters (unpublished data). Does this differentiation reflect a difference in foraging abilities of breeders and non-breeders? Or could non-breeders be forced, by the competitively superior breeders, out of the rich feeding areas of the coastal upwelling (Ramos *et al.* 2013)? Our results urge more research in this area. Given the potential susceptibility of pelagic seabirds, such as the Cory's shearwater, to mortality linked to fishing vessels (Belda & Sanchez 2001) and to changes in

the availability of their prey (Paiva *et al.* 2013), these results have clear implications. They suggest that different segments of seabird populations are likely to respond differently to ecosystem changes, or to the impacts of human activities, and those need to be taken into account, for example, in demographic modelling (Oro *et al.* 2010).

The influence of sex on diet

Direct evidence of sex-related dietary differences in pelagic seabirds is scarce (e.g., Xavier & Croxall 2005, Castillo-Guerrero *et al.* 2011) and most studies that investigated this issue were based on a small number of samples (e.g., Zavalaga *et al.* 2007; Xavier *et al.* 2011). Despite that, many studies (mostly based on stable isotopes or tracking) clearly showed the existence of a sex-related spatial or isotopic segregation in several seabird populations, often linked to sexual dimorphism (Phillips *et al.* 2011). We found clear dietary differences between sexes in Cory's shearwaters during the chick-rearing period, with males feeding more on sardines and less on chub mackerel than females. Despite the marked morphologic differences between sexes (Navarro *et al.* 2009; Ramos *et al.* 2009), sex-related differences in the diet or in foraging ranges of Cory's shearwaters were not found in previous studies (Navarro *et al.* 2007; Navarro *et al.* 2009; Xavier *et al.* 2011).

Male Cory's shearwaters are heavier, with larger bills and longer wings than females (Navarro *et al.* 2009; Ramos *et al.* 2009). It is possible that the higher wing load of males could provide them with greater mobility (Ramos *et al.* 2009) and enable them to increase their foraging range, in relation to females. Indeed, Cory's shearwaters from Selvagem Grande are known to prey on sardines mostly during long-distance foraging trips along the African coast (unpublished data). Weimerskirch *et al.* (2006) also described a greater foraging range of females in relation to males in red footed boobies *Sula sula*, presumably due the

larger size of females. However, sexual divergence in provisioning or foraging specialisation could also explain diet differences (Phillips *et al.* 2004) and this issue requires further investigation.

Inter-annual and seasonal variations in diet

There were inter-annual differences in the occurrence of some prey species in the diet of the shearwaters, namely sardine and cephalopods, which were more frequent in 2010. Cory's shearwaters are generalist predators (Thibault *et al.* 1997) and it is likely that these temporal variations may reflect a change in the abundance or availability of their main prey. However, inter-annual differences were smaller than variations linked to season and to foraging domain, found in this and in other studies (Paiva *et al.* 2010; Neves *et al.* 2012). Our results also contrast with previous studies at the Azores, where much more marked inter-annual variations in the consumption of fish and cephalopods were detected (Granadeiro *et al.* 1998; Paiva *et al.* 2010; Xavier *et al.* 2011; Neves *et al.* 2012). This suggests that the marine environment in the vicinity of the Selvagens Islands presented limited inter-annual changes in summer, which may be a general feature of these pelagic subtropical waters.

The diet of Cory's shearwaters was substantially different between the pre-laying and chick-rearing periods. During the pre-laying period, shearwaters fed mainly on trumpet fish and horse/blue jack mackerel. These prey species were of low importance during the chick-rearing period, when shearwaters increased the consumption of chub mackerel, sardine and pilot-fish. This variation in diet could be related to increased selectivity in prey choice, since parents are expected to select larger or higher-quality prey for their chicks (Wilson *et al.* 2004). Moreover, foraging areas explored by Cory's shearwaters are known to vary through

the breeding season (e.g., Navarro *et al.* 2007), possibly contributing to these striking seasonal changes in diet.

Conclusions

Our study highlights the importance of combining different techniques to accurately describe the diet of a pelagic seabird. The use of DNA barcoding and morphological analysis proved to be very efficient to study the diet of Cory's shearwaters, by improving both the taxonomical resolution and the quantification of prey species. This approach is likely to be useful in future seabird dietary studies. We also show the occurrence of trophic segregation between birds of different breeding status and sex, highlighting the need to further investigate the dietary choices of different population segments. Understanding the sources of dietary variation within a seabird population will be important for instituting appropriate conservation or population management measures.

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Data accessibility

Sequence data have been deposited to GenBank (accession numbers KC603479–KC603537). Input files for POY and Mega 6 have been deposited in Dryad.

Authors contributions

JPG, PC, HA and SW designed the study; PC, JPG, JAR and WOCS provided guidance during the study; HA, JPG and PC collected the data in the field; HA and JX identified and quantified prey through conventional methods; HA and JPG analysed the data; SW performed the molecular part of the study; HA, SW, PC, JPG, and WOCS wrote the manuscript.

Supplementary information

Additional supporting information may be found in the online version of this article.

Figure S1 NJ tree based method for assignment of morphologically unidentified specimens in Cory's shearwater diet.

Figure S2 NJ tree based method for assignment of morphologically unidentified specimens in Cory's shearwater diet.

Table S1 Genbank accession numbers of prey identified using DNA barcoding.

Figures and tables

Fig. 1 Tree estimated in POY for identification of teleosts using direct optimisation (DO) method. Query sequences and Genbank accession numbers of morphologically unidentified specimens for which no reliable identification could be obtained in BLAST are shown (unidentified specimens code). Representative genera of the families that produced the nearest match are included in the tree. Query sequences clustering with *Cheilopogon pinnatibarbatus*, *C. melanurus* and *Trachurus picturatus* corroborate previous BLAST results (100% of similarity). The tree shows the number of different MOTUs (different prey) obtained in each family. Nodal support is presented for bootstrap values ≥ 70 .

Fig. 2 Diet of Cory's shearwaters *Calonectris diomedea* in the pre-laying (30 diet samples with 318 prey) and chick-rearing (188 diet samples with 553 prey) periods of 2010: a) Frequency of occurrence of each prey type (%), b) Numerical frequency of each prey type (%).

Fig. 3 Diets of breeders (N = 248 diet samples) and non-breeders (N = 19) among Cory's shearwaters *Calonectris diomedea* during the chick-rearing period of 2009 (Frequency of occurrence, %).

Table 1 Cory's shearwater (*Calonectris diomedea*) prey identified using DNA barcoding of 16S mtDNA.

	Family	Genus/Species	Specimens	Percentage of Similarity	Phylogenetic analysis
Teleostei	Carangidae	<i>Trachurus</i> sp.	5		**
	Carangidae	<i>Trachurus picturatus</i>	2	100	***
	Coryphaenidae	<i>Coryphaena equiselis</i> *	1	100	
	Diretmidae	<i>Diretmus argenteus</i> *	1	99.8 ^a	
	Exocoetidae	<i>Cheilopogon melanurus</i> *	1	100	***
	Exocoetidae	<i>Cheilopogon pinnatibarbatus</i> *	2	100	***
	Exocoetidae	<i>Cheilopogon</i> sp.	1		**
	Exocoetidae	<i>Exocoetus</i> sp.	16		**
	Exocoetidae	Unidentified	2		
	Halosauridae	<i>Halosaurus</i> sp.*	1		**
	Molidae	<i>Ranzania laevis</i> *	2	100	
	Myctophidae	<i>Diaphus</i> sp.	1		**
	Myctophidae	<i>Lampadena atlantica</i> *	1	100	
	Neoscopelidae	<i>Neoscopelus macrolepidotus</i> *	1	100	
	Scombridae	<i>Katsuwonus pelamis</i> *	2	100	
	Sparidae	<i>Boops boops</i> *	2	99.1 ^b	
	Sternoptychidae	<i>Argyropelecus</i> sp.*	1		**
	Synphobranchidae*	Unidentified	2		**
	Trichiuridae*	Unidentified	2		**
Cephalopods	Chiroteuthidae	<i>Chiroteuthis mega</i> *	1	99.8 ^b	
	Cranchiidae	<i>Taonis pavo</i>	1	100	
	Histioteuthidae	<i>Histioteuthis</i> sp.	1		**
	Ommastrephidae	<i>Ommastrephes</i>	8		**

Single (*) asterisk correspond to taxa not recorded previously in the diet of Cory's shearwater (den Hartog & Clarke 1996, Granadeiro *et al.* 1998, Paiva *et al.* 2010, Xavier *et al.* 2011, Neves *et al.* 2012). Double (**) and triple (***) asterisks represent positive genus and species assignments based on the Neighbour joining (NJ) and DO trees. Similarity percentages with the GenBank reference sequences for species identifications using BLAST are shown (a) Monotypic species, (b) assignment based on the geographical distribution of the taxa

Table 2 Frequency of occurrence (FO %) and numerical frequency (NF %) of prey, identified by a combined use of morphologic analysis and DNA barcoding, in the diet of Cory's shearwaters (*Calonectris diomedea*). Diet samples were collected in two different periods, pre-laying (only in 2010) and chick-rearing (in 2008, 2009 and 2010), at Selvagem Grande. Number of samples and prey is presented in brackets.

	Pre-laying		Chick-rearing					
	2010 FO (30)	NF (318)	2008 FO (180)	NF (416)	2009 FO (248)	NF (631)	2010 FO (188)	NF (553)
CEPHALOPODA	13.3	1.3	27.2	13.9	35.1	24.9	46.8	22.8
Chiroteuthidae								
<i>Chiroteuthis mega</i> *	3.3	0.3						
<i>Chiroteuthis</i> sp.					1.2	0.5		
Cranchiidae								
<i>Taonius pavo</i>	3.3	0.3	0.6	0.2			1.1	0.5
<i>Cranchia</i> sp.								
Grimalditeuthidae								
<i>Grimalditeuthis bonplandi</i>			0.6	0.2				
Histioteuthidae								
<i>Histioteuthis arcturi</i>			1.1	0.5	1.2	0.5	5.3	2.0
<i>Histioteuthis meleagroteuthis</i>							0.5	0.2
<i>Histioteuthis</i> sp.			1.1	0.5	0.8	0.3		
Mastigoteuthidae								
<i>Mastigoteuthis</i> sp.					0.4	0.2		
Unidentified							2.1	0.7
Neoteuthidae								
<i>Neoteuthis</i> sp.							0.5	0.2
Ommastrephidae								
<i>Ommastrephes bartramii</i> *	3.3	0.3	7.7	3.8	13.7	9.2	6.4	2.7
Octopoteuthidae								
<i>Taningia danae</i>			0.6	0.2	0.4	0.2		
Onychoteuthidae								
<i>Ancistroteuthis lichtensteinii</i>					0.4	0.2		
Sepiidae								
Unidentified							0.5	0.2

Unidentified cephalopods	3.3	0.3	17.2	8.4	25.8	13.9	36.2	16.3
FISH	86.7	98.7	91.1	86.1	88.7	75.1	89.9	76.9
Belonidae								
<i>Belone belone</i>			2.2	1.0	0.4	0.2	3.7	1.6
Caproidae								
<i>Capros aper</i>					0.4	0.2	0.5	0.2
Carangidae								
<i>Naucrates ductor</i>			15.6	15.9	16.1	14.9	13.3	10.7
<i>Trachurus picturatus*</i>			0.6	0.2	0.4	0.2		
<i>Trachurus</i> sp.	40.0	9.7	1.7	0.7	2.8	1.4	4.8	4.0
Clupeidae								
<i>Sardina pilchardus</i>			7.8	4.1	9.3	4.3	20.2	13.2
<i>Sardinella</i> sp.					0.4	0.2		
Congridae								
<i>Conger conger</i>					0.8	0.3	0.5	0.2
Coryphaenidae								
<i>Coryphaena equiselis*</i>					0.4	0.2		
<i>Coryphaena</i> sp.			0.6	0.2			1.1	0.5
Diretmidae								
<i>Diretmus argenteus*</i>							2.7	0.9
Engraulidae								
<i>Engraulis encrasicolus</i>			1.7	1.0	3.6	4.0	1.1	0.5
Exocoetidae								
<i>Exocoetus volitans</i>			1.7	0.7				
<i>Exocoetus</i> sp.			8.3	4.1	1.2	0.5	4.8	1.6
<i>Cheilopogon exsiliens</i>					1.2	0.5		
<i>Cheilopogon melanurus*</i>					0.4	0.2		
<i>Cheilopogon pinnatibarbatus*</i>					0.4	0.2		
<i>Cheilopogon</i> sp.			1.1	0.5	1.2	0.5	1.1	0.4
Unidentified	3.3	0.3	6.7	2.9	4.0	2.5	2.7	0.9
Halosauridae								
<i>Halosaurus</i> sp.*			0.6	0.2	0.4	0.2		
Unidentified			0.6	0.5			0.5	0.2
Macroramphosidae								

<i>Macroramphosus scolopax</i>	36.7	83.3					1.1	0.4
Molidae								
<i>Ranzania laevis</i> *							3.7	1.3
Myctophidae								
<i>Diaphus</i> sp.*					0.4	0.2		
<i>Lampadena atlantica</i> *							0.5	0.2
Unidentified			1.7	1.0			1.6	0.9
Neoscopelidae								
<i>Neoscopelus macrolepidotus</i> *			0.6	0.2				
Scomberesocidae								
<i>Scomberesox</i> sp.	3.3	0.3	2.2	1.7	1.2	0.5	4.3	2.9
Scombridae								
<i>Scomber colias</i> /sp.	23.3	3.8	46.7	39.2	51.2	36.7	35.6	23.3
<i>Katsuwonus pelamis</i> *			0.6	0.2	3.2	1.3	2.7	0.9
Sparidae								
<i>Boops boops</i> *					0.4	0.2	1.1	0.5
Sternoptychidae								
<i>Argyropelecus</i> sp.*			0.6	0.5				
Synphobranchidae*								
Unidentified			1.1	0.5	1.2	0.5	3.7	1.4
Trichiuridae*								
Unidentified			4.4	1.9	1.2	0.5	4.8	1.8
Unidentified fish	13.3	0.9	19.4	8.9	13.3	5.5	22.3	8.3
CRUSTACEA			4.4		6.5		14.4	
Decapoda	3.3	0.3					0.5	0.4
Isopoda			1.1		3.6		11.1	
Unidentified crustacean	13.3		3.3		2.8		2.7	
INSECTA					0.8			
Halobatidae					0.8			

Single (*) asterisk represent taxa first identified through DNA barcoding of the 16S mtDNA.

Table 3 Frequency of occurrence (FO %) of prey in the diet of male and female Cory’s shearwaters (*Calonectris diomedea*), during the chick-rearing period of 2008, 2009 and 2010, at Selvagem Grande island. Sample size is presented in brackets. Differences among sexes were tested using a binomial GLM with a logit link function, controlling for the effect of year.

	Males (316)	Females (191)	Sex		Year	
	FO (%)	FO (%)	F	p	F	p
Fish	88.0 (278)	94.2 (180)	5.3	< 0.05	0.08	0.92
<i>Scomber colias</i> /sp.	38.0 (120)	58.6 (112)	21.7	< 0.001	5.9	< 0.01
<i>Naucrates ductor</i>	15.8 (50)	13.6 (26)	0.5	0.50	0.6	0.55
<i>Sardina pilchardus</i>	16.1 (51)	8.9 (17)	5.5	< 0.05	6.0	< 0.01
<i>Trachurus</i> sp.	4.7 (15)	2.1 (4)	2.3	0.13	0.4	0.69
Exocoetidae	10.1 (32)	5.8 (11)	2.9	0.09	0.9	0.41
Cephalopods	35.4 (112)	34.0 (65)	0.10	0.74	8.9	< 0.001

Breeders

Non-Breeders

Chub mackerel

Pilot-fish

Sardine

Flying-fish

Cephalopods

Other prey

100 75 50 25 0 25 50 75 100

Frequency of Occurrence %

Figure S1. NJ tree based method for assignment of morphologically unidentified specimens in Cory's shearwater diet. Positive family level identifications were obtained for the families (a) Synaphobranchidae and (b) Trichiuridae. Specimens were positively assigned when monophyletic clusters with con-genera of the family were obtained. (*) Represent positive assignments. Bootstrap values are above nodes and are presented for a cut-off value > 50.

Figure S1. Continued

2a Carangidae: *Trachurus*, *T. picturatus*

Figure S2. NJ tree based method for assignment of morphologically unidentified specimens in Cory's shearwater diet. Positive genus and species level identifications were obtained within the teleost families a) Carangidae b) Exocoetidae c) Halosauridae (d) Myctophidae (e) Sternoptychidae and cephalopods: f) Histioteuthidae g) Ommastrephidae. Specimens were positively assigned according to a strict criterion. (**) and (***) represent positive genus and species level assignments, respectively. Bootstrap values are above nodes and are presented for a cut-off value > 50

2b Exocoetidae: *Exocoetus* sp, *Cheilopogon* sp, *C. pinnatibarbatus*, *C. melanurus*

Figure S2. Continued

2c Halosauridae: *Halosaurus* sp

Figure S2. Continued

2d Myctophidae: *Diaphus* sp

Figure S2. Continued

2e Sternoptychidae: *Argyropelecus* sp

Figure S2. Continued

2f Histoteuthidae: *Histoteuthis* sp

Figure S2. Continued

2g Ommastrephidae: *Ommastrephes bartramii*

Figure S2. Continued

Table S1 Genbank accession numbers of prey identified using DNA barcoding. NI corresponds to unidentified prey taxon

Family	Genus	Species	Number of individuals	Genbank accession number
Histioteuthidae	<i>Histioteuthis</i>	NI	1	KC603485
Ommastrephidae	<i>Ommastrephes</i>	<i>Ommastrephes bartrammi</i>	9	KC603479, KC603480, KC603482-KC603484, KC603486-KC603489
Chroteuthidae	<i>Chroteuthis</i>	<i>Chroteuthis mega</i>	1	KC603490
Cranchiidae	<i>Taonius</i>	<i>Taonius pavo</i>	1	KC603481
Carangidae	<i>Trachurus</i>	<i>Trachurus picturatus</i>	2	KC603529, KC603532
Carangidae	<i>Trachurus</i>	NI	5	KC603530, KC603533, KC603535-KC603537
Coryphaenidae	<i>Coryphaena</i>	<i>Coryphaena equiselis</i>	1	KC603517
Dirymidae	<i>Dirymus</i>	<i>Dirymus argenteus</i>	1	KC603521
Exocoetidae	<i>Cheilopogon</i>	NI	1	KC603494
Exocoetidae	<i>Cheilopogon</i>	<i>Cheilopogon melanurus</i>	1	KC603504
Exocoetidae	<i>Cheilopogon</i>	<i>Cheilopogon pinnatibarbus</i>	1	KC603499
Exocoetidae	<i>Exocoetus</i>	NI	16	KC603491-KC603493, KC603495-KC603497, KC603500, KC603501, KC603505-KC603510, KC603512, KC603527
Exocoetidae	NI	NI	3	KC603498, KC603502, KC603503
Halosauridae	<i>Halosaurus</i>	NI	1	KC603516
Moridae	<i>Ranzania</i>	<i>Ranzania laevis</i>	2	KC603525, KC603526
Myctophidae	<i>Lampadena</i>	<i>Lampadena atlantica</i>	1	KC603522
Myctophidae	<i>Diaphus</i>	NI	1	KC603519
Neoscopelidae	<i>Neoscopelus</i>	<i>Neoscopelus macrolepidotus</i>	1	KC603513
Scombridae	<i>Katsuwonus</i>	<i>Katsuwonus pelamis</i>	2	KC603511, KC603528
Sparidae	<i>Boops</i>	<i>Boops boops</i>	2	KC603531, KC603534
Sternoptychidae	<i>Argyropelecus</i>	NI	1	KC603514
Synphobranchidae	NI	NI	2	KC603520, KC603524
Trichiuridae	NI	NI	2	KC603518, KC603523