Diet of adult and immature imperial cormorants, Leucocarbo atriceps, from southern Patagonia. A combined dietary approach and an exploratory analysis of stable isotopes of pellet membrane

Annick Morgenthaler (amorgenthaler@uaco.unpa.edu.ar)
Universidad Nacional de la Patagonia Austral
Ana Millones
Universidad Nacional de la Patagonia Austral
Patricia Gandini
Universidad Nacional de la Patagonia Austral
Esteban Frere
Universidad Nacional de la Patagonia Austral

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Abstract

Age-related trophic segregation between breeding adults and immature individuals has been reported in seabirds. The use of combined conventional and Stable Isotope Analyses (SIA) to study the diet of seabirds has become very frequent. Unfortunately, information on the trophic ecology of immature seabirds remains scarce because the sampling of tissues to perform SIA is often very limited due to the difficulty to capture these birds. The koilin membrane, which covers the regurgitated pellet casts of many seabirds, could offer an interesting non-intrusive alternative tissue to perform SIA. In this two-year study of the diet of imperial cormorants in southern Patagonia, we 1) compare the diets of breeding and non-breeding birds through conventional pellet analysis; 2) describe the diet of breeding adults and chicks through a combined method of pellet analysis and whole blood SIA; and finally, 3) compare SIA values of breeding adults obtained from blood with those obtained from koilin membranes. We found that immature individuals incorporated abundant invertebrate taxons, compared with breeding adults which relied mainly on fish. Younger and inexperienced individuals, which are not under the constraint to feed chicks, are feeding on more predictable, but lower trophic and less energetic prey. By comparing the stable isotope values of koilin and blood in breeding adults, a correlated interannual difference between the two seasons was found. Under the light of our results, the koilin offers an encouraging alternative to blood in the study of trophic ecology, particularly for ages or stages in which capture is not possible.

Introduction

Seabirds as mid-top predators respond to the accelerating ecological changes observed in oceans. Dietary studies, in addition to providing information on the trophic ecology of seabirds, can also help interpreting possible changes in their populations. The dietary composition of seabirds responds to intrinsic and extrinsic factors. Within a specific seabird species, diet can differ among colonies, likely due to the effect of the community structure and habitat characteristics (Karnovsky et al. 2008; Chiaradia et al. 2012; Fernandez et al. 2019). Diet composition can vary among years in response to changes in food availability and, also, to the individuals' stages of their life cycles, age or sex (Carscadden et al. 2002; Jaeger et al. 2014; Barrionuevo et al. 2018; Ibarra et al. 2018). Trophic segregation can be observed between breeding adults and non-breeding immature individuals in seabirds (Forero et al 2002; Michalik et al 2013). This could be explained not only by the different energetic requirements and constraints of breeding and non-breeding individuals (Votier et al 2011; Campioni et al. 2018), but also possibly by the improvement of individual foraging abilities gained with age (Wunderle 1991; Morrison et al. 1978; Daunt et al. 2007). Unfortunately, due to the difficulty to study immature seabirds, information on their trophic ecology remains scarce (see Campioni et al. 2018 for a review).

The analysis of pellet casts (regurgitated indigestible remains) and Stable Isotope Analysis (SIA) of carbon and nitrogen are both widely used techniques to study the diet of seabirds (Barrett et al. 2007; Bond and Jones 2009). When used in combination, these methods potentiate each other, thanks to the possibility to integrate prior information obtained from pellet analysis into stable isotope mixing models (Moore et al. 2008; Layman et al. 2012; Morgenthaler et al. 2020). The choice of the tissue for SIA

responds to the possibilities of sampling these tissues, which may be more or less intrusive, and to the temporal scale needed to represent. Each tissue stands for a different time scale of assimilated diet depending on the metabolic routing of stable isotopes and tissue turn-over rate (Bond and Jones 2009). Feathers can - in some occasions - be obtained in a non-intrusive manner, but since the information it provides corresponds to the period of time when the feather is growing, information on molting chronology is required for the correct temporal interpretation of its SIA values (Barrett et al. 2007). Blood (whole blood, or blood-red cell and plasma analyzed separately) is the most widespread tissue used for breeding individuals, but it implies capturing and manipulating the birds for sampling, and this is therefore more invasive and not always applicable (Bond and Jones 2009). Other tissues, like muscles or organs, are sometimes also used by sampling randomly dead animals (Barrett et al. 2007). Egg shells or membranes can be used non-intrusively, but these reflect a very specific period of time of females' diet (Polito et al. 2009; Quillfeldt et al. 2009). Finally, the seabird prey remains regurgitated as pellet casts are often wrapped by a thin membrane called koilin membrane (Marshall 2013). This functions as a protective layer in the gizzard of birds and is formed both by secretion from glands (carbohydrate/protein complex) and by entrapped sloughed epithelial cells and cellular debris (Marshall 2013). This membrane has received little overall attention by ecologists (Kim et al. 2016). To the researchers' knowledge, no SIA of this tissue has been undertaken in seabirds (or any bird species), even though the koilin membrane could potentially offer an easy and non-intrusive tissue to sample for SIA, which could be performed alongside the conventional analysis of the prey remains found in the pellet. Therefore, this could allow for the study of immature or non-breeding individuals which are usually hard to study through the analysis of other tissues.

Like all shags and cormorants, the imperial cormorant, *Leucocarbo atriceps*, is a coastal foot-propelled pursuit-diver. Among the four cormorant species found in southern South America, the imperial is the largest, the most abundant and the one that can dive deeper and travel farther offshore (Frere et al 2005; Quintana et al. 2011; Gomez Laich et al. 2012). The composition of its diet has been studied by conventional techniques (pellets, stomachs or regurgitates analysis) at several locations along the Atlantic Patagonian coast and at Malvinas/Falkland Islands, showing that it was feeding on both benthic and pelagic prey, primarily on fish but also on crustaceans, cephalopods and polychaetes (Punta et al. 2003; Ferrari et al. 2004; Bulgarella et al. 2008; Michalik et al. 2010; Yorio et al. 2017). The prey composition and the proportions of pelagic-benthic prey differed among locations (Frere et al. 2005, Ibarra et al. 2018). Within locations, differences were found among reproductive seasons and also among the stages of the same year. The main distinction was the presence of a higher proportion of small pelagic forage fish during the chick rearing stage compared with the rest of the reproductive season. The same feeding pattern has been found with respect to the non-reproductive period (Punta et al. 1993; Malacalza et al. 1994; Michalik et al. 2010; Ibarra et al. 2018). SIA were performed in a few ecological studies on imperial cormorant and were mainly focused on intra-specific trophic segregation more than on diet description (Quillfeldt et al. 2011; Michalik et al 2013; Harris et al. 2016). At Malvinas/Falkland Islands, Michalik et al. (2013) found that the isotopic signatures of immature birds

indicated feeding at a lower trophic level than adults during the winter. This was explained by possible poorer hunting abilities of young, inexperienced birds, when compared with adults.

In this work, we studied the diet of imperial cormorants in southern Patagonia, Argentina, using a combined technique of conventional diet assessment (pellet analysis) and stable isotope mixing models. Our objectives are to 1) compare the diets of breeding and non-breeding imperial cormorants through conventional pellet analysis; 2) describe the diet of breeding adults and chicks through a combined method of pellet analysis and whole blood SIA; and finally, 3) compare SIA values obtained from blood with those from koilin membranes in order to explore the possibility to use pellet membrane isotopic values for diet characterization.

Methods

Study area

The study area is found in southern Patagonia, near the town of Puerto Deseado, on the northern coast of Santa Cruz Province in Argentina (47°45'S, 65°53'W). Part of the fieldwork was carried out at the imperial cormorant breeding colony of Isla Chata, situated in the *Parque Interjurisdiccional Marino Isla Pingüino* (47°55'S, 65°44'W), one of the largest breeding sites for the species, hosting 6613 ± 1541 breeding pairs (Morgenthaler 2019). The other location is the non-breeding site of Peninsula Foca, situated at the entrance of the Ría Deseado; a long (> 40 km), narrow inlet formed by the partial submergence of a river valley by the sea, belonging to the protected area *Reserva Provincial Ría Deseado* (47°44'S, 65°50'W). Peninsula Foca is a small rocky island connected to the mainland during low tide, which is the roosting ground of around 200-300 non-breeding imperial cormorants (pers. obs.). The two sampling sites, Isla Chata and Peninsula Foca, are situated 20 km apart.

Conventional diet sampling

At Isla Chata, pellets were collected in December 2012 and 2013, during chick-rearing stage, around nests situated at the periphery of the colony. At Peninsula Foca, they were collected between late November and early-February of the same seasons, by walking throughout the roosting area. The pellets were analysed with a binocular microscope and hard prey remains were used to quantify and identify prey at the lowest taxonomic level possible. Identification was carried out by using our own collections and available literature and catalogues (Lombarte et al. 1991; Boschi et al. 1992; Gosztonyi and Kuba 1996; Pineda et al. 1996; Piacentino 1999; Volpedo and Echeverría 2000; Tombari et al. 2010). The frequency of occurrence (%FO) and the number of occurrences (%N) were calculated for all prey items, and expressed as percentages. Published or our own allometric regressions were used to estimate the average total length (TL) and wet weight (W) of different prey types (Pineda et al. 1996; Koen-Alonso et al. 2000; Torroglosa et al 2012). The length of not worn fish otoliths and cephalopod mandibles were used for TL and W calculations. Finally, the Shannon-Weaver diversity index was calculated for each species (Tramer 1969).

SIA sample collection and processing

Whole blood samples of adult and chick imperial cormorants were collected during two consecutive breeding seasons (2012 to 2013) at Isla Chata by late-December for SIA (overall N = 30). Adults and two-to-three week old chicks were captured directly from nests by hand or with the help of a pole. The manipulation of each animal lasted less than five minutes. Chicks were then put back promptly to their nest. At release, adults flew directly to the water, and returned to their nests shortly afterwards. Approximately 0.3-0.5 mL of blood was extracted from the brachial vein of adults and chicks and preserved in 70% ethanol before processing in the laboratory (Hobson et al. 1997).

The pellets used for conventional diet analysis at Isla Chata were collected on the same day and at the same place as the blood sample. The membranes of these pellets were cleaned, all prey items or parasites were removed, and rinsed with distilled water in order to be used for SIA.

The samples were dried at 60°C for >24 h for whole blood, and for >48 h for pellet membrane, and ground to a fine, homogenized powder. Carbon and nitrogen isotope ratios were measured in the Center for Stable Isotopes at the University of New Mexico, USA, by Elemental Analyser Continuous Flow Isotope Ratio Mass Spectrometry using a Costech ECS 4010 Elemental Analyser coupled to a Thermo Fisher Scientific Delta V Advantage mass spectrometer via a CONFLO IV interface. Isotope ratios were reported using the standard delta (δ) notation relative to AIR and Vienna Pee Dee Belemnite (V-PDB), respectively, and expressed in units per thousand (‰) as follows: δ = (Rsample/Rstandard - 1), where Rsample and Rstandard are the molar ratios of the heavy to light isotopes ($^{13}C/^{12}C$ or $^{15}N/^{14}N$) of the sample and standard, respectively. Average analytic precision based on routine analysis of a laboratory protein standard was < than 0.1‰ (1σ). The laboratory standard was calibrated against IAEA-N-1, IAEA-N-2, USGS 42 and USGS 43 for nitrogen and NBS 21, NBS 22 and USGS 24, USGS 42 and USGS 43 for carbon.

Five potential prey sources were chosen for SIA according to our preliminary dietary results and information of the same area (Gandini and Frere, unpublished data). These were the three demersal-benthic fish groups: the channel bull blenny (*Cottoperca gobio),* the Patagonian rock cod (*Patagonotothen cornucola)* and the eelpouts (zoarcidae fish), and two different cephalopods: the demersal-pelagic squid (*Dorytheutis gahi,* known as Patagonian squid) and the benthic octopus (*Enteroctopus megalocyathus,* known asred octopus). Specimens of four of these species were collected in the study area and period, and their SIA values have been previously published in Morgenthaler et al. (2016, 2020) (Table 1). Since no specimens of *Cottoperca gobio* were collected at the study site, SIAvalues from samples collected in 2014 at the central Patagonian Shelf break between 45-47°S and 60-61°W were used for the mixing models (Zhu et al. 2018) (Table 1).

Table 1 Stable isotope values of main prey of imperial cormorant used in the mixing models. Data are presented as means with standard deviation (in parentheses). All data are corresponding to the study site and period (originally published in Morgenthaler et al. 2016, 2020), but *Cottoperca gobio* which samples were collected at the central Patagonian Shelf break in 2014 (Zhu et al. 2018)

PREY	*n*	δ^{13}C (s.d.)	δ^{15}N (s.d.)
Cottoperca gobio (channel bull blenny)	5	-17.9 (0.3)	14.4 (0.3)
*Patagonotothen cornucola (*rock cod)	10	-14.9 (1.2)	17.4 (0.6)
Zoarcidae (eelpout)	8	-14.3 (0.6)	17.8 (0.9)
Dorytheutis gahi (Patagonian squid)	6	-18.1 (1.0)	14.2 (1.3)
Enteroctopus megalocyathus (red octopus)	4	-16.6 (1.0)	17.0 (0.3)

Trophic discrimination factors (TDF) selection and mixing polygons inspection

Since no diet to whole blood trophic discrimination factors (TDF) has been experimentally determined for the imperial cormorant, choice was made to use SIDER package in R (Healy et al. 2018), which has been successfully used for Neotropic cormorant at the same study site (Morgenthaler et al. 2021). SIDER package, run in R 3.6.3 (R Core Team 2020), uses a phylogenetic regression model using Bayesian inference-based on a compiled dataset to estimate the TDFs of a consumer considering its tissue type and feeding ecology. This package uses a standard database with numerous worldwide published TDF values, however since *P. auritus* and *S. magellanicus* TDF values (Craig et al. 2015, Ciancio et al. 2016) were not included in this database, they were added manually before running the models (see Morgenthaler et al. 2021). TDF values obtained with SIDER werechannel bull blenny: Δ^{13}C: 0.70 (±1.40); Δ^{15}N; 2.20 (±0.93), Patagonian rock cod: Δ^{13}C: -0.24 (±1.45); Δ^{15}N:1.59 (±1.01), eelpouts: Δ^{13}C: -0.40 (±1.47); Δ^{15}N: 1.51 (±1.03), Patagonian squid: Δ^{13}C: 0.75 (±1.40); Δ^{15}N: 2.24 (±0.91) and red octopus: Δ^{13}C: 0.32 (±1.41); Δ^{15}N: 1.67 (±1.01).

To the authors' knowledge, no experimental TDF values for pellet membrane have been published for any bird species. By comparing the isotopic values of imperial cormorant pellet membranes with those of the different prey in our study, it appeared that their ranges were similar (Fig. 2, Table 3). Applying any positive Δ^{15}N values to perform the mixing models would have increased the probabilities of consumers falling outside the mixing space. So the choice was made to maintain null TDF values for both isotopes for all prey to perform the mixing models of pellet membrane as an experimental approach, and to discuss the results obtained with these values and the implication of such choice (see discussion).

For each model (year-tissue) we applied the simulation method of Smith et al. (2013), using the aforementioned TDF values, to ensure that the consumer data were situated within 95% of the source isotopic mixing polygon. The assumption was made that no important source was missing. In the case of whole blood, the 2012 average of probabilities of consumer falling in the mixing polygon was 0.57 (min: 0.46; max: 0.73), and the one for 2013 was 0.58 (0.43- 0.77). In the case of pellet membrane, the 2012 average of probabilities of consumer falling in the mixing polygon was 0.36 (0.11-0.52), and in 2013, it was 0.46 (0.20-0.68).

Stable isotope mixing models

The relative contribution of the potential prey to the diet of the imperial cormorant from Isla Chata based on their isotopic values (whole blood and pellet membrane) was estimated with Bayesian mixing models from the 'simmr' package (Parnell and Inger 2016) in R 3.6.3 (R Core Team 2020). Models were run for each year and tissue separately due to inter-annual differences of cormorant isotopic data. Two different models were run for each year: 1) an initial model, with no prior information, and 2) an informed model with the following priors based on conventional diet estimates from Isla Chata (%W): channel bull blenny: 0.6 (±0.2); rock cod: 0.2 (±0.1); eelpout: 0.1(±0.05); Patagonian squid: 0.05 (±0.02) and red octopus: 0.05 (±0.02). A standard deviation was associated with each of these priors to account for uncertainty in the prior information (Parnell and Inger 2016).

Statistical analyses

For conventional diet, multivariate similarity analyses (ANOSIM) using the R 'vegan' package were used to test for differences in the biomass estimates of the main prey types among years and among breeding stages (Oksanen et al. 2016). The isotopic centroid positions were examined using nested linear models and residual permutation procedures (Turner et al., 2010). Centroid locations were compared between years for each tissue, and between tissues within each year, and were considered different if the Euclidean Distance (ED) between centroid locations was significantly greater than zero (Turner et al. 2010).

Results

Conventional diet

Out of the 80 pellets analysed, 28 different prey items belonging to six different taxa (teleost fishes, cephalopods, crustaceans, polychaetes, gasteropods and algae) were identified (Table 2). Overall prey diversity was higher at the breeding colony of Isla Chata (Shannon-Weaver Index: 1.62) than at the non-breeding site of Peninsula Foca (Shannon-Weaver Index: 1.01). The most frequent (highest frequency of occurrence) and abundant (highest numerical percentage) prey at Isla Chata were the channel bull blenny (*Cottoperca gobio*) and the rock cod *(Patagonotothen* spp.) (Table 2). At Peninsula Foca, the most frequent and abundant prey were the polychaete worms from family Polynoidae and the rock cods; the Patagonian squid was also frequent but not so important numerically (Table 2). The dietary composition, considering the biomass estimates of the main prey types (Fig.1), presented a moderate overlap of diet (70%) with significant differences between sites (R: 0.303, $p<0.001$) and no interannual differences within each site (Chata: R: 0.001, p: 0.402; Foca: R: 0.012, $p=0.297$). At Isla Chata, the most important prey, based on the biomass estimates, were the channel bull blenny followed by the rock cod, while at Peninsula Foca, the Patagonian squid *(Dorytheutis gahi)* was the most important prey, followed by the channel bull blenny and the rock cod (Fig. 1). The species which most contributed (43.6 %) to the inter-site difference was the channel bull blenny ($p<0.001$), whose biomass estimates were much higher at the breeding colony compared with the non-breeding site. All the main prey of the diet of the imperial cormorant are considered demersal-benthic or benthic, except for the demersal-pelagic Patagonian squid.

Table 2 Percentages of prey in the diet of imperial cormorant at the breeding colony of Isla Chata and at the non-breeding site of Peninsula Foca. Number of individual prey (n), percent number (%N), percent frequency of occurrence (%FO) of prey obtained from a total of 80 pellets collected in 2012 and 2013. The ecological group of each prey is shown in parentheses (P=pelagic, B=benthic, DP=demersal pelagic, DB=demersal benthic). Values of specific taxon with %FO>60 and %N>10 were highlighted in bold

	ISLA CHATA				PENINSULA FOCA			
	BREEDING COLONY				NON-BREEDING SITE			
	2012		2013		2012		2013	
	%N	%FO	%N	%FO	%N	%FO	%N	%FO
N prey items - N pellets	*735*	*20*	*693*	*20*	*1372*	*20*	*3216*	*20*
TELEOST FISHES	*81.8*	*95.0*	*84.0*	*100.0*	*33.2*	*85.0*	*15.6*	*90.0*
Patagonotothen spp. (DB)	**30.3**	**80.0**	**33.2**	**90.0**	**10.3**	**70.0**	3.6	**70.0**
Paranotothenia magellanica (DB-DP)	0.1	5.0	0.0	0.0	0.0	0.0	0.0	0.0
Eleginops maclovinus (DB)	0.0	0.0	0.1	5.0	0.0	0.0	0.0	0.0
Zorcidae (DB)	4.1	50.0	5.9	50.0	3.9	45.0	1.7	25.0
Austrolycus laticinctus (DB)	0.3	5.0	0.0	0.0	0.5	15.0	0.1	5.0
Iluocoetes elongatus (DB)	1.8	15.0	0.0	0.0	0.4	15.0	0.0	0.0
Phucocoetes latitans (DB)	0.7	5.0	1.9	15.0	2.3	15.0	0.1	5.0
Dadyanos insignis (DB)	0.5	10.0	0.0	0.0	0.0	0.0	1.3	5.0
Unidentified zoarcidae	0.8	15.0	4.0	35.0	0.8	25.0	0.3	15.0
Agonopsis chiloensis (DB)	6.1	55.0	7.5	40.0	6.4	**65.0**	2.8	55.0
Cottoperca gobio (DB)	**18.5**	**90.0**	**16.3**	**95.0**	2.3	50.0	0.5	40.0
Salilota australis (DP)	3.5	25.0	1.3	35.0	0.1	10.0	0.2	25.0
Bovichthys argentinus (DB)	0.0	0.0	0.0	0.0	0.1	5.0	0.0	0.0
Merluccius hubbsi (DP)	0.4	15.0	0.3	5.0	0.0	0.0	0.0	0.0
Engraulis anchoita (P)	0.0	0.0	0.1	5.0	0.0	0.0	0.0	0.0
Sprattus fuegensis (P)	0.0	0.0	0.1	5.0	0.0	0.0	0.0	0.0
Odontesthes spp. (DP)	0.0	0.0	0.0	0.0	0.0	0.0	0.1	5.0
Unidentified fish	18.6	95.0	19.2	95.0	10.1	50.0	6.7	70.0
CEPHALOPODS	*8.0*	*90.0*	*6.2*	*55.0*	*6.9*	*85.0*	*2.5*	*85.0*
Dorytheutis gahi (DP)	5.4	**80.0**	2.5	40.0	4.8	**65.0**	1.4	**65.0**
Enteroctopus megalocyathus (DB)	2.6	40.0	3.8	50.0	2.0	60.0	1.1	55.0

CRUSTACEANS	*3.5*	*80.0*	*2.6*	*55.0*	*1.1*	*60.0*	*1.4*	*85.0*
Decapoda	2.3	55.0	1.7	40.0	0.6	25.0	1.3	65.0
Eurypodius latreilli (B)	0.0	0.0	0.0	0.0	0.1	5.0	0.0	5.0
Peltarion spinosulum(B)	0.0	0.0	0.0	0.0	0.0	0.0	0.1	10.0
Betaeus truncatus (DB)	0.0	0.0	0.0	0.0	0.1	5.0	0.1	10.0
Caridae (DB)	0.0	0.0	0.0	0.0	0.0	0.0	0.1	15.0
Munida sp. (DP-DB)	0.4	5.0	0.4	10.0	0.2	5.0	0.1	10.0
Unidentified decapoda	1.9	55.0	1.3	30.0	0.4	20.0	1.0	40.0
Isopoda	0.8	25.0	0.1	5.0	0.3	15.0	0.0	5.0
Unidentified crustaceans	0.4	15.0	0.7	20.0	0.2	15.0	0.1	10.0
POLYCHAETES	*6.4*	*55.0*	*7.1*	*60.0*	*58.3*	*85.0*	*80.5*	*75.0*
Polynoidae (B)	1.9	35.0	1.3	45.0	**49.3**	**80.0**	**79.0**	**65.0**
Nereididae (B)	3.8	55.0	3.3	40.0	5.8	60.0	1.1	30.0
Eunice (B)	0.5	15.0	0.6	20.0	1.2	20.0	0.1	15.0
Unidentified polychaetes	0.1	5.0	1.9	15.0	2.0	30.0	0.3	10.0
GASTEROPODS (B)	*0.3*	*10.0*	*0.0*	*0.0*	*0.5*	*35.0*	*0.0*	*0.0*
ALGAE (B)	*0.0*	*45.0*	*0.0*	*35.0*	*0.0*	*5.0*	*0.0*	*0.0*
STONES	*13.7*	*65.0*	*4.0*	*55.0*	*0.0*	*15.0*	*0.0*	*5.0*

Whole blood stable isotope values and diet estimated from the mixing models

The stable isotope values of imperial cormorant whole blood ranged between -16,7 and -14,8‰ for $\delta^{13}C$, and between +16,6 and +18,0‰ for $\delta^{15}N$ (Table 3). No difference was found between adults and chicks in 2013 (ED = 0.12‰, p=0.735), and only a small but significant difference was found in 2012 (ED = 1.43‰, p<0.001; see Table 3). The position of the centroids (adults and chicks together) differed significantly between the two years (ED = 1,0‰, p<0,001), with higher $\delta^{13}C$ and $\delta^{15}N$ values in 2012 (Fig.2).

Table 3 Stable isotope values of whole blood and pellet membrane of imperial cormorants from Isla Chata**.** Data are presented as means with standard deviation (in parentheses)

Tissue	Year	Age	*n*	δ^{13}C (s.d.)	δ^{15}N (s.d.)
whole blood	2012	adult	6	-15.2 (0.3)	17.7 (0.2)
whole blood	2013	adult	6	-16.1 (0.3)	17.1 (0.3)
whole blood	2012	chick	8	-15,4 (0,3)	17,6 (0,2)
whole blood	2013	chick	10	-16,0 (0,4)	17,2 (0,4)
pellet membrane	2012	adult	10	-16.0 (0.5)	14.3 (0.6)
pellet membrane	2013	adult	10	-16.7 (0.3)	14.4 (0.6)

The results of the 2012 initial mixing models of imperial cormorant based on whole blood stable isotope values (adults and chicks together) showed a higher proportion (33.1%) of the channel bull blenny, followed by lower and similar proportions of the four other potential prey (<20% each) (Fig.3). In 2013, results were similar but with slightly higher proportions of the channel bull blenny (39.9%), followed by the Patagonian squid (21.4%), and low proportions (<15% each) of the other three prey (Fig.3). All proportion estimates of the initial models presented high credibility intervals (Fig. 3). The results of the informed mixing models (model 2) reduced the credibility intervals in all proportions of prey estimates, restricting the results to a range of proportions more similar to those obtained with conventional diet analysis, with the channel bull blenny found in higher proportions than with initial models (48.2% in 2012 and 58.3% in 2013), followed by the rock cod (27.6% and 21.9%), and low proportions (<13% each) of the other three prey (Fig.3).

Pellet membrane stable isotope values and mixing models prey proportion estimates

The stable isotope values of imperial cormorant pellet membrane ranged between -16.7 and -15.3‰ for δ^{13}C, and between +12.7 and +15.1‰ for δ^{15}N (Table 2). The position of the centroids differed significantly between the two years (ED = 0.7‰, p<0,003), due to higher δ^{13}C values in 2012 (Fig.2). Both years, δ^{13}C and δ^{15}N values of the membrane were much lower than whole blood values (Fig.2), but interestingly, their respective interannual differences changed accordingly, as both tissues presented higher δ^{13}C values in 2012. By comparing both tissues within each year, we found that in 2012, the Euclidean distance between their centroid position was 3.5 (p<0,001), with $\Delta\delta^{13}$C=0.8 and $\Delta\delta^{15}$N=3.4. In 2013, the Euclidean distance between both tissues was 2.8 (p<0,001), with $\Delta\delta^{13}$C=0.6 and $\Delta\delta^{15}$N=2.8.

The overall prey proportion estimates based on pellet membrane mixing models were quite similar to those obtained from blood. The initial mixing models showed slightly higher proportions of the channel bull blenny (27.9% in 2012 and 37.6% in 2013) and the Patagonian squid (25.4 and 28.5) than the other three prey types (<20% each), although with high credibility intervals (Fig.3). The results of the informed mixing models reduced the credibility intervals in all proportions of prey estimates, restricting the results to a range of proportions more similar to those obtained with conventional diet analysis, and also very similar to the blood mixing models results, with the channel bull blenny found in higher proportions than

with initial models (63.0% in 2012 and 68.0% in 2013), followed by the rock cod (19.5% and 16.1%), and low proportions (<10% each) of the other three prey (Fig.3).

Discussion

We have described for the first time the dietary composition of immature imperial cormorants during the spring-summer period and compared it with simultaneously breeding adults from the nearest breeding colony (20 km). Immatures' diet was different from the breeding adults as they fed primarily on invertebrates, mainly polychaete worms, squids and octopus, while secondarily they also fed on benthic fish. The main prey of the breeding adults, the channel bull blenny, was much less frequent and abundant in immatures. These differences are in accordance with the expected diet for younger and inexperienced individuals (Morrison et al. 1978; Wunderle 1991; Michalik et al. 2013) feeding on prey that are more predictable and less mobile (worms and cephalopods are much less mobile than fish), while less nutritive and energetic (Ciancio et al. 2007), and situated at a lower trophic level. Furthermore, immatures are not under the constraint of foraging in close range of a central place like breeding individuals, and their requirements are also different since they do not need to feed on particularly energetic food to raise chicks (Campioni et al. 2016). The mechanisms underlying these differences are not completely understood and can be hard to disentangle. Intraspecific competition near the breeding colony could play a role and lead the immatures to get away from breeding adults, leading to the segregation (or partial segregation) of their foraging areas towards less optimum areas.

Most of the important prey of breeding imperial cormorants from Isla Chata are benthic, but the squid. No pelagic foraging fish, like Fuegian sprat *Sprattus fuegensis*, which is found in the area and used by several seabird species extensively (Frere et al. 1996; Millones et al. 2005; Morgenthaler et al. 2016; Barrionuevo et al. 2018), nor gregarious crustaceans, like lobster-krill (*Munida* spp.),were found in important numbers. This is in contrast to what was found in the diet of breeding imperial cormorants from most of the other locations from the Atlantic Patagonian coast (Punta et al. 2003; Ferrari et al. 2004; Bulgarella et al. 2008; Yorio et al. 2010; Ibarra et al. 2018). Nevertheless, according to early-2000 unpublished observations (Frere & Gandini, unpubl.), lobster-krill was found to be abundant in some imperial cormorant pellets recollected at that time at Isla Chata . Since lobster-krill have a very patchily and unpredictable distribution, it is not unlikely that this prey could be used only momentarily when available, and could therefore go unnoticed in a spot pellet sampling. The Patagonian squid was much more abundant in immatures from Peninsula Foca than in breeding adults from Isla Chata. It was not found in such important numbers, however, in most of the other imperial cormorant studies. Its presence may respond to an increased availability of this particular prey in the study area, since it was found in the diet of two of the other three species of cormorants and two species of penguins (Frere et al. 1996; Millones et al. 2005; Morgenthaler et al. 2016, 2020; Barrionuevo et al. 2018). Finally, the most abundant prey in terms of biomass, at this breeding colony, was the benthic channel bull blenny. Surprisingly, it was neither found in important numbers in the diet of any other cormorant or penguin species from the study area, nor in the diet of imperial cormorants from other locations along the coast, unlike rock cods. The channel bull blenny, along with the rock cod species, must be very abundant and relatively predictable at

Parque Interjurisdiccional Marino Isla Pingüino to sustain this large colony of more than six thousand breeding pairs.

Considering the stable isotope values of pellet membrane (koilin), and particularly their correlation with interannual differences observed in the blood values, it appears that this tissue could offer an interesting alternative to blood in the study of cormorants. This is because its sampling is completely non-invasive and pellets can be collected throughout the year from roosting sites. Moreover, koilin membranes could be used to study some aspects of seabirds' trophic ecology, as for example in comparative studies, like trophic segregation or trophic structuring within communities. Although the differences between the blood and the membrane stable isotope values obtained in this study could serve as a proxy to calibrate membrane values, further studies and research should be carried out in order to fully interpret membrane SIA values. The turnover of this tissue is not known, but it is suspected to be relatively short, since cormorants produce around one pellet a day (Barrett et al. 2007). The metabolic routing of carbon and nitrogen from the koilin membrane needs to be clearly understood. Furthermore, the TDF of koilin membrane from cormorants is also unknown, and should ideally be determined experimentally through feeding trials of captive or semi-captive cormorants. Nevertheless, the similarities of SIA values between prey and pellet membranes indicate that $\delta^{13}C$ and $\delta^{15}N$ TDF values of pellet membranes from cormorants are probably very low (if not null). This is also supported by the results from the exploratory mixing models carried out with null TDF, which resulted very similar to those obtained with blood mixing models. . Our findings suggest that the koilin membrane values could reflect the prey values transferred or absorbed by direct contact in the gizzard more than assimilation through metabolic processes. Finally, the results of these pellet membrane exploratory analyses are encouraging and strengthen the need to undertake further studies to enhance the possibility to employ koilin membrane in seabird trophic ecology studies.

Declarations

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Author information

Affiliation

Centro de Investigaciones de Puerto Deseado. Universidad Nacional de la Patagonia Austral. Av. Prefectura s/n, 9050 Puerto Deseado, Santa Cruz, Argentina

Annick Morgenthaler, Ana Millones, Patricia Gandini, Esteban Frere

Centro de Investigaciones y Transferencia (CIT) Golfo San Jorge, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Ruta 1 - Km 4 s/n, Comodoro Rivadavia, Chubut Argentina.

Annick Morgenthaler

Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Prefectura s/n, 9050 Puerto Deseado, Santa Cruz, Argentina.

Patricia Gandini, Esteban Frere

Corresponding author: Annick Morgenthaler, Email: amorgenthaler@uaco.unpa.edu.ar

ORCID numbers

Annick Morgenthaler: 0000-0002-7346-3140; Ana Millones: 0000-0002-6725-7279; Patricia Gandini: 0000-0003-1171-0086; Esteban Frere: 0000-0001-9435-5440

Authors contributions

All authors contributed to the study conception and design. AMo and AMi conducted field work. AMo and several assistants conducted laboratory analyses of pellets, with the help of AMi. AMo prepared the samples for stable isotope analysis. AMo analysed and interpreted the data under the supervision of AMi, EF and PG. EF and PG obtained the external funding. The first draft of the manuscript was written by AMo with substantial input from EF. All authors contributed to the article revision and final approval.

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Figures

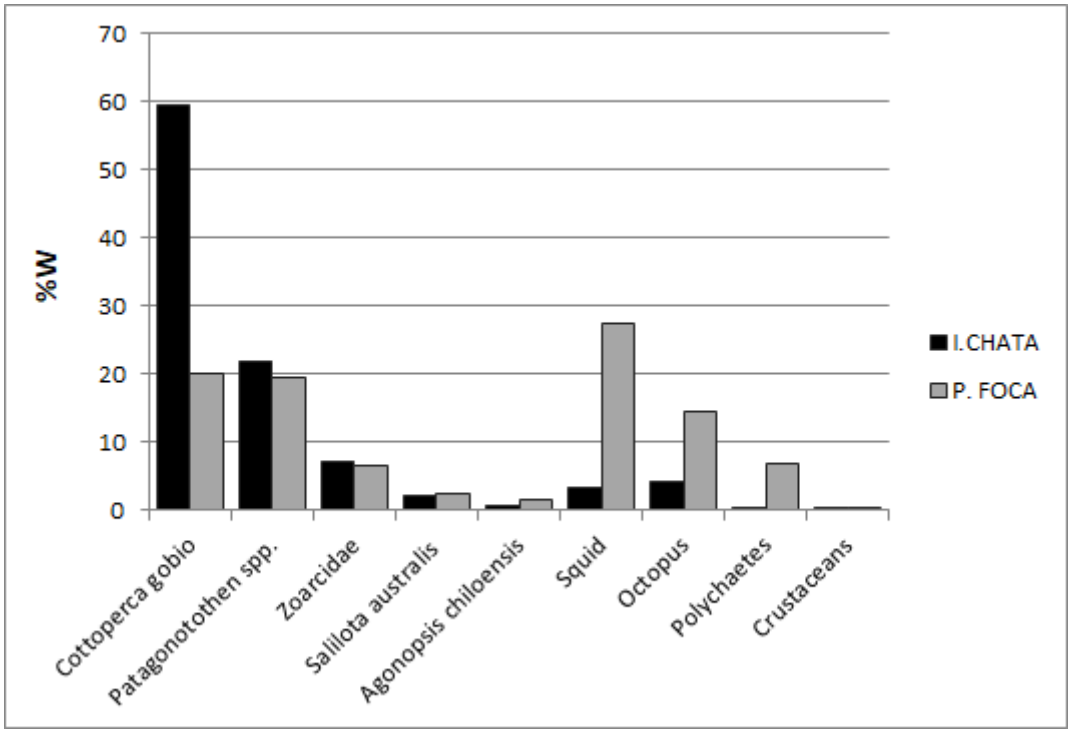

Figure 1

Percent estimated wet weight (%W) of the main prey in the diet of Imperial Cormorant based on pellet analysis (N=80) at the breeding colony of Isla Chata (black) and at the non-breeding site of Peninsula Foca (grey). Values are shown for each site, both years (2012 and 2013) together

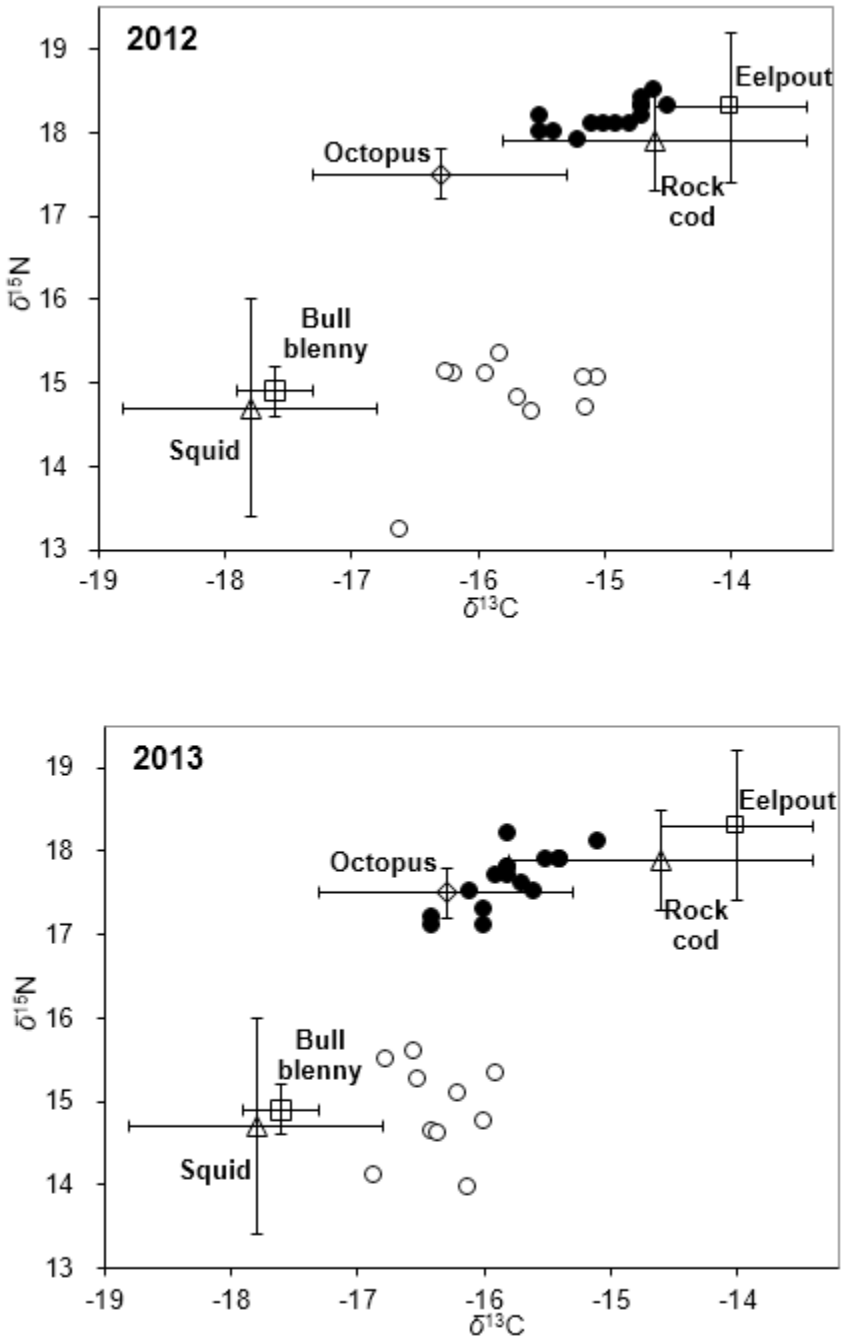

Figure 2

Stable isotope values of whole blood (black dots) and pellet membrane (white circles) of imperial cormorant from Isla Chata and their potential prey, presented by year. Values of prey are mean with standard deviation. Prey items: channel bull blenny, *Cottoperca gobio*; rock cod, *Patagonotothen cornucola*, eelprout (zoarcidae fishes); patagonian squid, *Dorytheutis gahi*, and red octopus, *Enteroctopus megalocyathus*

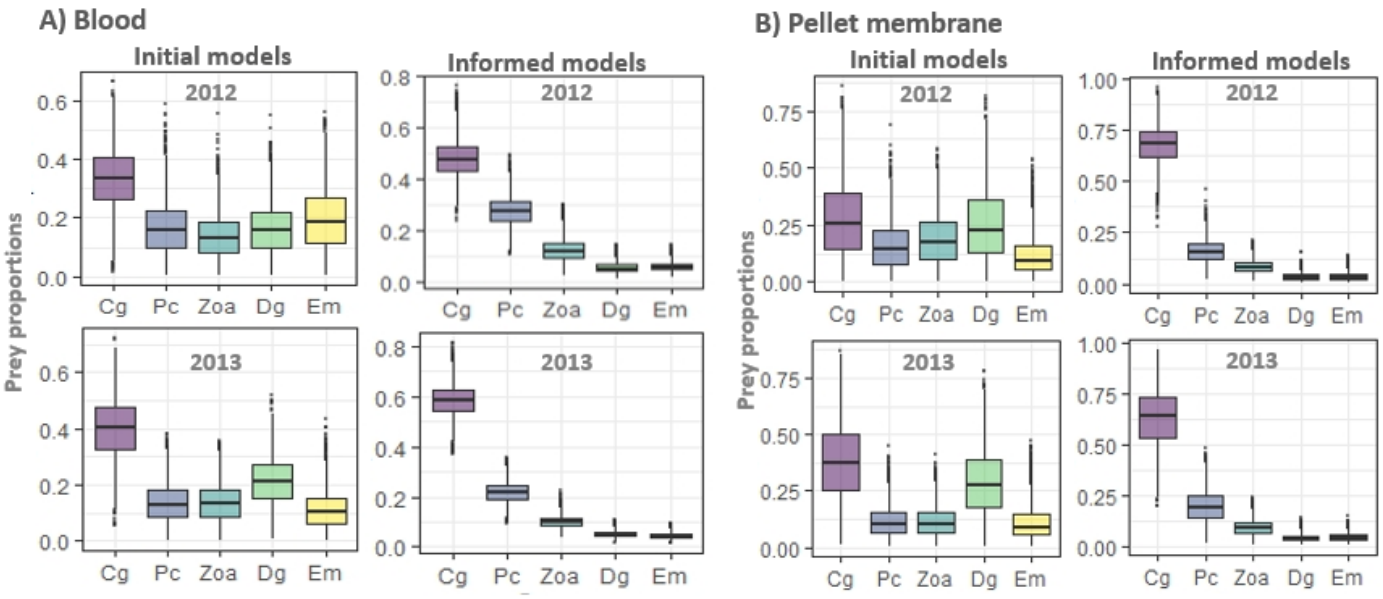

Figure 3

Estimated prey contributions to the diet of imperial cormorant obtained from initial (left) and informed (right) stable isotope mixing models, based on A) blood, B) pellet membrane. Box plots display the range between 25% and 75% credibility quantiles, with error bars extending to the maximum and minimal values (97.5% and 2.5%, respectively), and the median represented by the bold line. Prior data of informed models: channel bull blenny, *Cottoperca gobio:* 0.6 (±0.2); rock cod, *Patagonotothen cornula*: 0.2 (±0.1); eelpout, Zoarcidae fishes: 0.1(±0.05); Patagonian squid, *Dorytheutis gahi*: 0.05 (±0.02) and red octopus, *Enteroctopus megalocyathus:* 0.05 (±0.02)