

Invisible trophic links? Quantifying the importance of non-standard food sources for key intertidal avian predators in the Eastern Atlantic

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ABSTRACT: Coastal wetlands are heterogeneous systems with multiple inputs and complex interactions within local food webs. Interpreting such complexity is limited by incomplete knowledge of trophic interactions among organisms. Although widely recognized as secondary consumers and predators of intertidal macroinvertebrates, shorebirds can also consume lower-trophic-level food sources, and frequently forage in adjacent supratidal habitats. To ascertain potential trophic links between overwintering shorebirds and alternative non-standard food sources, we collected carbon and nitrogen stable isotope data of shorebirds and benthic organisms from 4 coastal wetlands along the Eastern Atlantic: Tejo Estuary, Portugal; Sidi-Moussa, Morocco; Banc d'Arguin, Mauritania; and Bijagós Archipelago, Guinea-Bissau. Using dual-isotope Bayesian mixing models, we evaluated the relative importance of intertidal benthic macroinvertebrates and 3 other potential food sources (biofilm and seagrass rhizomes from intertidal areas, and saltpan macroinvertebrates) in the diet of wintering shorebirds. Although intertidal macroinvertebrates form the main part of most shorebird species' diet, our data revealed that supratidal saltpans can contribute to >30% of the biomass ingested by several shorebird species. Seagrass rhizomes represented >10% of the diet of several species in Banc d'Arguin and in Sidi Moussa. Little stint *Calidris minuta* appears to consume biofilm on all 3 wetlands where they were sampled, which is the first time biofilm consumption by shorebirds has been detected along the East Atlantic Flyway. Empirical evidence for generalized consumption of alternative food sources by intertidal avian predators show the greater complexity and food web connectivity in and of intertidal habitats, and also with the surrounding habitats.

KEY WORDS: Shorebird · Diet · Macroinvertebrate · Biofilm · Saltpan · Seagrass · Wetland

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INTRODUCTION

A food web is a construct that describes which kinds of organisms in a community eat which other kinds (Pimm et al. 1991). It does not give the com-

plete picture, but the structure of a food web, including all the links and interactions among organisms positioned at various trophic levels (Olf et al. 2009), is certainly a good starting point to show species interactions, the possible dynamics of ecosystems as

a whole, and the population dynamics of individual species (Thébault & Loreau 2003, Bascompte 2010). A full understanding of food-web structure requires complete knowledge of the many links and nodes composing the networks and the strengths of the interactions. Such knowledge is often limited by difficulties in identifying missing links among species (Clauset et al. 2008).

Estuaries and other coastal wetlands form an intermediate transition zone, better described as an ecocline (Attrill & Rundel 2002), linking terrestrial and marine ecosystems. Understanding food-web structure in coastal systems is particularly challenging as they are affected by a mixture of autochthonous and allochthonous sources of nutrients and energy originating from adjacent terrestrial and marine areas (Bouillon et al. 2011, Careddu et al. 2015), which can promote contrasting patterns of interactions and organization within the local food web (Bouillon et al. 2011, Olin et al. 2013). Coastal tidal areas are also among the most productive ecosystems on Earth, supporting abundant and diverse macroinvertebrate populations (e.g. Compton et al. 2013) that attract large numbers of both resident and migratory vertebrates such as fishes and waterbirds (Wolff 1983, van de Kam et al. 2004).

Shorebirds (Aves: Charadrii) have long been known as key predators in intertidal areas, where they feed on the available macroinvertebrate fauna and have a significant impact on its abundance and population dynamics (e.g. Quammen 1984, Piersma 1987, van der Meer et al. 2001, Rosa et al. 2008). However, intertidal-feeding shorebirds are known to also use supratidal habitats (saltpans, marshes, rice fields), where they consume macroinvertebrates and seeds (e.g. Perez-Hurtado et al. 1997, Masero et al. 2000, Lourenço & Piersma 2008). In fact, rice fields are the main foraging habitat for the *Limosa l. limosa* population of black-tailed godwits during most of their non-breeding season (Lourenço & Piersma 2008, Santiago-Quesada et al. 2009), and during the fuelling periods, saltpans can also form the main foraging habitat for several shorebird species (Masero 2003). More recently, some studies have shown that in intertidal areas, shorebirds may feed at a trophic level lower than the macrobenthos (molluscs, worms, crustaceans), such as biofilm grazing by sandpipers in wetlands of the northern Pacific (Elnor et al. 2005, Kuwae et al. 2008, Jardine et al. 2015), and seagrass *Zostera noltii* rhizome consumption by black-tailed godwits *Limosa limosa* in France (Robin et al. 2013). Although these 'alternative' food sources can represent an important part of the diet of some species in

some areas (e.g. Kuwae et al. 2012, Robin et al. 2013), and therefore form important links in the local food webs, little is known of how widespread their use is. This is in part due to difficulties in identifying food items that leave no trace in droppings and are hard or impossible to detect through focal observations.

The response of an ecosystem to perturbations is mediated by both antagonistic and facilitative interactions among species. Therefore, the resilience of a community will depend on the network of trophic interactions (Guimerà et al. 2010). The widespread use of lower-trophic-level food sources by secondary consumers would indicate greater food-web interconnection and complexity in intertidal habitats (Collwell 2010, Kuwae et al. 2012), which may increase the stability and resilience of these food webs to species removal (Dunne et al. 2002). In particular, processes affecting intertidal macroinvertebrate communities, such as eutrophication (Lopes et al. 2000, Cardoso et al. 2004), climate change (e.g. Cheung et al. 2009), or direct human disturbance through dredging or sediment alterations (Ferns et al. 2000, Leewis et al. 2012), can have indirect effects on predator dynamics and distribution (e.g. Peterson et al. 2006, van Gils et al. 2006). That the consumption of lower-trophic-level foods could buffer such effects may be illustrated by the use of seagrass rhizomes, which allows shorebirds to use intertidal areas with lower abundances of their traditional macroinvertebrate prey (Robin et al. 2013). On the other hand, consumption of lower-trophic-level food is not necessarily a 'last resort' strategy, but can also be driven by preference (e.g. Beninger et al. 2011, Jiménez et al. 2015), potentially reducing predation pressure on benthic organisms and therefore lessening the strength of top-down regulation effects that secondary consumers have on benthic communities (e.g. Menge 2000, Rosa et al. 2008).

Stable isotope analysis is increasingly used as an analytical approach to determine diets and trophic relationships among organisms (e.g. Hobson & Clark 1992, Hobson et al. 1994, Kelly 2000), complementing traditional techniques for analyzing diets (Winemiller et al. 2007, Catry et al. 2016a) and even showing previously unknown trophic patterns (e.g. MacNeil et al. 2005, Cherel et al. 2008). The stable isotope ratios of food are incorporated into newly synthesized tissues, therefore ratios in tissues can integrate diet information over long periods, depending on the turnover rate of each body tissue (Bearhop et al. 2002, Caut et al. 2009). The use of tissues with slow turnover rates avoids biases caused by short-term variations in food abundance

or foraging site selection, thus providing a better picture (than when using tissues with fast turnover rates) of the general feeding patterns of a species over the course of a season. In particular, shorebird toenails can be easily sampled with minimum harm to birds and have half-lives of approximately 1 mo for both carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotope ratios (Lourenço et al. 2015a), thus nails provide a valuable source of isotopic information to infer avian diets (Bearhop et al. 2003). However, since toenails grow conically and therefore combine old and new diets (Hahn et al. 2014), they must be collected after the bird has spent sufficient time at a given site to fully incorporate the local isotopic signature.

Here we used stable isotope data collected in 4 Atlantic coastal wetlands in South Europe and West Africa to evaluate the relative importance of intertidal benthic macroinvertebrates and 3 other potential food sources—biofilm and seagrass rhizomes that are found within the intertidal zone, and macroinvertebrates found in nearby salt pans—in the diet of wintering shorebirds. We thus attempt to detect potential trophic links between shorebirds and alternative food sources, as well as investigate consistency in foraging choices of shorebirds in several key wintering areas along the East Atlantic Flyway.

MATERIALS AND METHODS

Study areas

Sampling took place in 4 key wintering areas for shorebirds along the East Atlantic Flyway (Delany et al. 2009), one in southern Europe and the other 3 in West Africa. The Tejo Estuary, on the central coast of Portugal ($38^{\circ}45' \text{N}$, $09^{\circ}01' \text{W}$), is a large estuarine wetland with an intertidal area of 97 km^2 . Most of this area is dominated by mudflats, with smaller areas of sandy sediments and virtually no seagrass-covered areas. The estuary is bordered by salt pans and salt-marshes that are also used by shorebirds for both feeding and roosting.

Sidi Moussa is a coastal lagoon located on the Moroccan Atlantic coast ($32^{\circ}58' \text{N}$, $8^{\circ}45' \text{W}$). The lagoon covers an area of 4.2 km^2 , separated from the ocean by a chain of consolidated dunes and a few rocky outcrops. It has a permanent connection to the ocean that exposes the lagoon to a tidal regime. The intertidal areas are a mixture of mud and sandflats, partly covered by the seagrass *Zostera noltii*, and there are several large salt pan areas surrounding the

lagoon that are heavily used by shorebirds throughout the tidal cycle.

The Banc d'Arguin, in Mauritania ($19^{\circ}52' \text{N}$, $16^{\circ}17' \text{W}$), is an area of tidal flats and shallow inshore waters off the tropical Saharan coast, covering over 500 km^2 . The intertidal area includes both sandy and muddy substrates, which are widely covered by extensive beds of seagrass *Z. noltii* (replaced at lower levels by the partially submerged *Cymodocea* sp.). There are no relevant supratidal feeding areas for shorebirds, even though they use supratidal beaches and shebkas (i.e. barren sandy flats situated above the mean high water mark that contain large quantities of sodium chloride and harbor no benthic organisms; Wolff et al. 1993) as roosting sites. Our main sampling sites were located near the village of Iwik, specifically at Baie d'Aouatif and Ebelk Aiznay.

The Bijagós Archipelago, in Guinea-Bissau, lies off the West African coast ($11^{\circ}12' \text{N}$, $15^{\circ}53' \text{W}$) and includes 88 islands and islets. The intertidal area covers roughly 760 km^2 of mud and sandflats, of which more than 350 km^2 are covered by mangroves that are only used by birds as roosting sites. There are no relevant seagrass beds or supratidal feeding areas for shorebirds in the Bijagós. All sampling took place at the islands of Bubaque, Canhabaque and João Vieira.

Field sampling took place in the winters of 2012–2013 in Sidi Moussa and Banc d'Arguin, and 2013–2014 in the Bijagós Archipelago and Tejo Estuary.

Sample collection and processing

In each area, intertidal benthic macroinvertebrates (bivalves, gastropods, crustaceans and polychaetes) were collected by hand or by using sampling cores and sieving the sediment ($0.5\text{--}1.0 \text{ mm}$ mesh-size). At Tejo Estuary and Sidi Moussa, benthic sampling was less extensive and focused on species that are known to be part of shorebird diets (e.g. Kersten et al. 1981, Piersma 1987, Moreira 1994, 1996, Lourenço et al. 2008, Martins et al. 2013). At Banc d'Arguin and Bijagós Archipelago, where much less was known about shorebird diets, we sampled all common macroinvertebrates and later selected for analysis the ones found in bird faeces collected in parallel to this study (Lourenço et al. 2016, authors' unpubl data). To avoid any biases caused by variation in isotopic signatures of macroinvertebrates with different sizes and ages, we preferentially sampled macroinvertebrates in the range of sizes known to be consumed by shorebirds (Goss-Custard et al. 2006).

We used ca. 1 mm mesh nets to sample Chironomidae larvae (Insecta) on the salt pans of Tejo Estuary and Sidi Moussa. These larvae are the most common macroinvertebrates in these areas and are known to be one of the main prey for shorebirds in salt pan habitats (Sánchez et al. 2006, Pedro & Ramos 2009). Salt pan and intertidal macroinvertebrates were kept alive for 24 h after collection in estuarine or marine water to clear their guts and were afterwards dried and stored in containers before further analysis.

At Banc d'Arguin and Sidi Moussa, we also collected rhizomes of *Z. noltii*. To avoid any contamination, prior to drying for storage, the rhizomes were carefully scraped to remove any epiphytes or mud attached to their surface. For macroinvertebrates and rhizomes, each replicate used for stable isotope analysis consisted of a pool of a variable number of individuals (see Table S1 in the Supplement at www.int-res.com/articles/suppl/m563p219_supp.pdf).

Microbial biofilms are composed of bacterial and microalgal cells and a matrix of extracellular polymeric secretions (Decho 2000). To determine the isotopic composition of this potential food source, we collected samples of sediment organic matter (SOM) and microalgae in the intertidal zone of all study areas. SOM was sampled by collecting the upper layer (~1 cm) of sediment, using an open vial over an area of roughly 5 cm² from which visible detrital particles were removed before drying the samples for storage. To collect microalgae, we placed textile panels (6–8 panels approximately 10 × 10 cm; Whatman, grade 105) in the sediment surface and waited about 2 h for the microalgae to migrate to the surface and cover the panels. Panels were then carefully placed in plastic containers, rinsed with estuarine or marine water and decanted to separate the microalgae from the sediment attached to the panels. The supernatant was then filtered onto pre-combusted Whatman GF/F filters and the filters were then dried. The content of the filters was later scraped into tubes and stored until further analysis. We collected 1 or 2 replicates of both SOM and microalgae at each study site (Table S1 in the Supplement).

Shorebirds were mainly captured in roosting areas using mist-nets during the night and crossbow (Martins et al. 2014), cannon or whoosh-netting techniques during the day. The distal 1–2 mm of nail was clipped from 3 or 4 toes of each bird using sharp scissors and stored in individual plastic bags (sample sizes in Table S2 in the Supplement at www.int-res.com/articles/suppl/m563p219_supp.pdf). With the exception of 1 turnstone and 3 dunlin caught in the Tejo Estuary in late November, all sampling of shorebirds

took place between 12 December and 21 February, with a peak in January (48 % of all captures), to ensure that the isotopic signatures of the toenails only reflected the birds' local wintering diet (assuming that birds have been in their wintering grounds at least since early November but likely since September–October; the half-lives of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in toenails are roughly 1 mo; and that toenails reach isotopic equilibrium with a new diet after 100–120 d; Lourenço et al. 2015a).

Stable isotope analysis

All samples (except bird toenails) were ground into a homogeneous powder using a mortar and pestle. Shells of bivalves and gastropods and exoskeletons of crabs were discarded and only soft tissues were used to prepare the samples for analysis. Since the presence of lipids in tissues can bias the interpretation of diet reconstruction results (e.g. Tarroux et al. 2010), for macroinvertebrate samples, we performed lipid extraction by immersing each sample in a 2:1 chloroform:methanol solution with a solvent volume 3–5 times larger than the sample volume (Logan et al. 2008). Samples were mixed for 30 s, left undisturbed for approximately 30 min and centrifuged for 10 min at $1034 \times g$, after which the supernatant containing solvent and lipids was removed. This process was repeated at least 3 times until the supernatant was clear and colorless following centrifugation. Samples were re-dried at 60°C for 24 h to remove any remaining solvent.

Samples of sediment, bivalves, gastropods and crustaceans were tested for the presence of carbonates by adding several drops of 10% HCl to a subsample. Whenever a reaction (bubbling) was observed, more drops of acid were added; the sample was then centrifuged to remove excess acid and finally washed with distilled water. This process was repeated 3 times or until bubbling stopped (Vinagre et al. 2008). Given that acidification can affect $\delta^{15}\text{N}$ signatures, a non-acidified subsample was always kept for separate nitrogen isotope analysis (Carabel et al. 2006).

Toenails were washed in double baths of 0.25 N sodium hydroxide solution alternated with baths of double-distilled water to remove adherent contamination, and then dried at 50°C for 48 h (Catry et al. 2012).

Between 0.5 and 1.0 mg of each replicate from all sampled organisms in the study areas were stored in tin cups for stable carbon and nitrogen isotope assays.

Isotopic ratios were determined by continuous-flow isotope-ratio mass spectrometry. The standards used were IAEA-N1 and IAEA-600 for nitrogen isotope ratio, and IAEA-CH6 and IAEA-CH7 or IAEA-600 for carbon isotope ratio. Results are presented conventionally as δ values in parts per thousand (‰) relative to the Vienna PeeDee Belemnite for $\delta^{13}\text{C}$, and atmospheric nitrogen (N_2) for $\delta^{15}\text{N}$. Precision of the isotope ratio analysis, calculated using values from 6 to 9 replicates of laboratory standard material (casein) interspersed among samples in every batch analysis, was 0.2‰ for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$.

Data analysis

Potential food sources were divided into 5 groups, which were not all present in all study areas, as explained above. Saltpan macroinvertebrates included only Chironomidae larvae, biofilm included SOM and microalgae, and seagrass rhizomes included just rhizomes of *Z. noltii*. Intertidal macroinvertebrates were separated into 2 groups. In Banc d'Arguin and Bijagós there are Lucinidae bivalves that live in symbiosis with chemoautotrophic bacteria and therefore have a radically different isotopic signal from all other intertidal invertebrates (e.g. van der Geest et al. 2014, Catry et al. 2016b), while being important prey for some shorebird species (e.g. van Gils et al. 2013). Therefore, 1 group consisted of chemosymbiotic bivalves comprising only Lucinidae bivalves and another group consisted of all other intertidal macroinvertebrates consumed by shorebirds. For each study area, we calculated the mean and standard deviation of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for each group, using the average of all replicates of each source to avoid pseudo-replication when there were multiple sources in a group.

Dual-isotope Bayesian mixing models were used to estimate the contribution of carbon and nitrogen food sources to the different shorebird species in each area, using isotopic information from each group of potential food types and from bird toenails. We used the SIAR v.4.2 package running in R (Parnell & Jackson 2013) to produce all mixing models, including diet-tissue trophic discrimination factors of 3.4 ± 1.0 for $\delta^{15}\text{N}$ and 0.4 ± 1.3 for $\delta^{13}\text{C}$ (Post 2002). Each model ran 500 000 iterations, of which 50 000 were discarded as burn-in. In interpreting model results, we used the rule that a food type is considered a relevant part of the diet of a species when over 50% of model runs indicate it represents over 10% of the diet, i.e. when the median value was higher than 0.10.

Since mixing models may be sensitive to variation in discrimination factors (Bond & Diamond 2011), and since there is little published data on discrimination values for bird toenails (see Lourenço et al. 2015a), we conducted a sensitivity analysis by re-running all our models and calculating the median contribution of each source using all combinations of trophic discrimination values lying within the means used in our initial model ± 1 SD (-0.9 to 1.7 and 2.4 to 4.4 for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively), while still including the original standard variation for each mean value. Unlike Bond & Diamond (2011), this analysis indicated that even a substantial variation in trophic discrimination factors did not generate much variation in the contribution of food sources estimated by the SIAR models and, therefore, our main findings remain unchanged (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m563p219_supp.pdf).

RESULTS

Distinct food sources showed significantly different $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ signatures in Bijagós ($\delta^{15}\text{N}$: Kruskal-Wallis $H_{2,10} = 7.64$, $p < 0.05$; $\delta^{13}\text{C}$: $H_{2,10} = 7.64$, $p < 0.05$), Banc d'Arguin ($\delta^{15}\text{N}$: $H_{3,18} = 12.64$, $p < 0.01$; $\delta^{13}\text{C}$: $H_{3,18} = 14.29$, $p < 0.01$) and Sidi Moussa ($\delta^{15}\text{N}$: $H_{3,12} = 8.65$, $p < 0.05$; $\delta^{13}\text{C}$: $H_{3,12} = 10.27$, $p < 0.05$; Fig. 1). In the Tejo Estuary, $\delta^{13}\text{C}$ values were significantly different among food sources ($H_{2,8} = 6.25$, $p < 0.05$), while there was large variation and overlap for $\delta^{15}\text{N}$ ($H_{2,8} = 5.00$, $p = 0.079$; Fig. 1).

Clearly, and despite considerable uncertainty associated with mixing model results (see Fig. 2 and Table S3 in the Supplement at www.int-res.com/articles/suppl/m563p219_supp.pdf), intertidal macroinvertebrates were the main food source for the majority of shorebird species and study area; with maximum values recorded in the Bijagós Archipelago (median values range: 85.3–96.2%), minimum values in Sidi Moussa (median values range: 33.8–64.3%) and intermediate values in the other 2 study areas (Fig. 2 and Table S3). Intertidal macroinvertebrates comprised <40% of the diet only in little stint *Calidris minuta* and red knot *Calidris canutus* in the Tejo Estuary (Fig. 2), and little stint and redshank *Tringa totanus* at Sidi Moussa (Fig. 2). Intertidal bivalves with symbiotic chemoautotrophic bacteria appeared to be consumed only by red knots in the Banc d'Arguin, where they represented 26.4% of the red knot diet (Fig. 2 & Table S3).

Mixing model results also indicated other food sources could be a relevant part of the diet of several

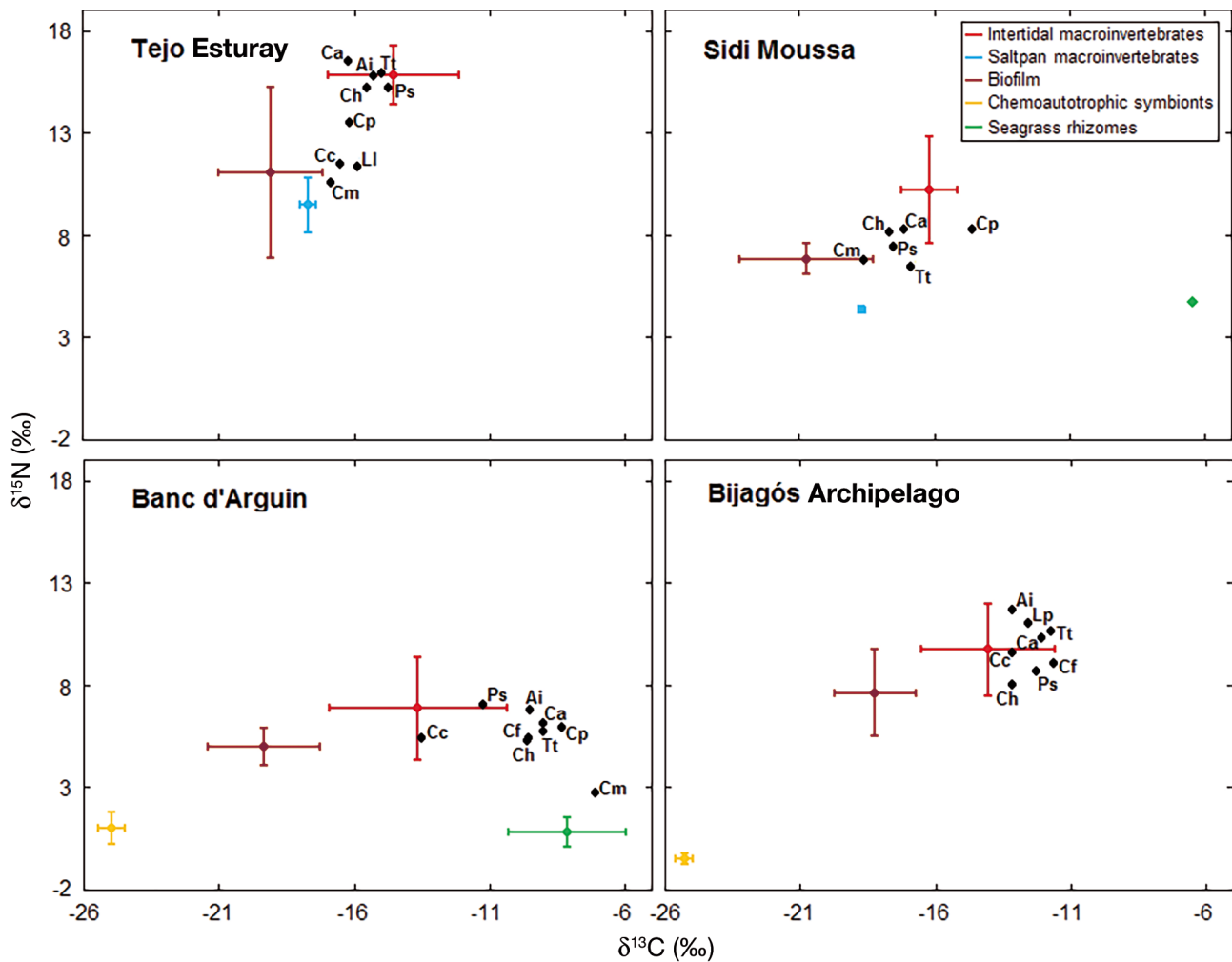


Fig. 1. Mean nitrogen ($\delta^{15}\text{N}$) and carbon ($\delta^{13}\text{C}$) isotopic values of food sources and shorebird species in the 4 study areas: Tejo estuary (Portugal); Sidi Moussa (Morocco); Banc d'Arguin (Mauritania); and Bijagós archipelago (Guinea-Bissau). Trophic discrimination factors (3.4‰ for $\delta^{15}\text{N}$ and 0.4‰ $\delta^{13}\text{C}$) were added to food source values for easier comparison with consumer signatures. For illustration purposes, standard deviations (bars) are presented only for food sources (for shorebird standard deviations see Table S2 in the Supplement at www.int-res.com/articles/suppl/m563p219_supp.pdf). Ai: ruddy turnstone *Arenaria interpres*, Ca: sanderling *Calidris alba*, Cp: dunlin *Calidris alpina*, Cc: red knot *Calidris canutus*, Cf: curlew sandpiper *Calidris ferruginea*, Cm: little stint *Calidris minuta*, Ch: ringed plover *Charadrius hiaticula*, Ll: black-tailed godwit *Limosa limosa*, Lp: bar-tailed godwit *Limosa lapponica*, Ps: grey plover *Pluvialis squatarola*, Tt: redshank *Tringa totanus*

species. In the Tejo Estuary, saltpan macroinvertebrates were particularly important for red knot (59.2%), black-tailed godwit (36.9%) and little stint (30.4%; Fig. 2 & Table S3 in the Supplement). In Sidi Moussa, saltpan invertebrates represented 13.1–45.9% of shorebird diets, with maximum values recorded for redshank (45.9%), sanderling *Calidris alba* (42.5%) and ringed plover *Charadrius hiaticula* (41.1%; Fig. 2 & Table S3). Seagrass rhizomes represented 1.3–17.3% of shorebird diets in Sidi Moussa, with values >10% for redshank (17.3%) and dunlin *Calidris alpina* (11.3%; Fig. 2) and >5% for 4 other

species (Table S3). In the Banc d'Arguin, seagrass rhizomes represented 5.7–28.4% of shorebird diets, with median values >10% for all species with the exception of ruddy turnstone *Arenaria interpres* (5.7%; Fig. 2 & Table S3).

Biofilm represented >10% of the diet of little stint at all sites where this species was studied, reaching 42.3% in the Tejo Estuary, 40.0% in Sidi Moussa, but only 11.7% in Banc d'Arguin. The presence of biofilm in the diet of little stints is also supported by our sensitivity analysis, which showed that even when isotope discrimination factors were changed within a

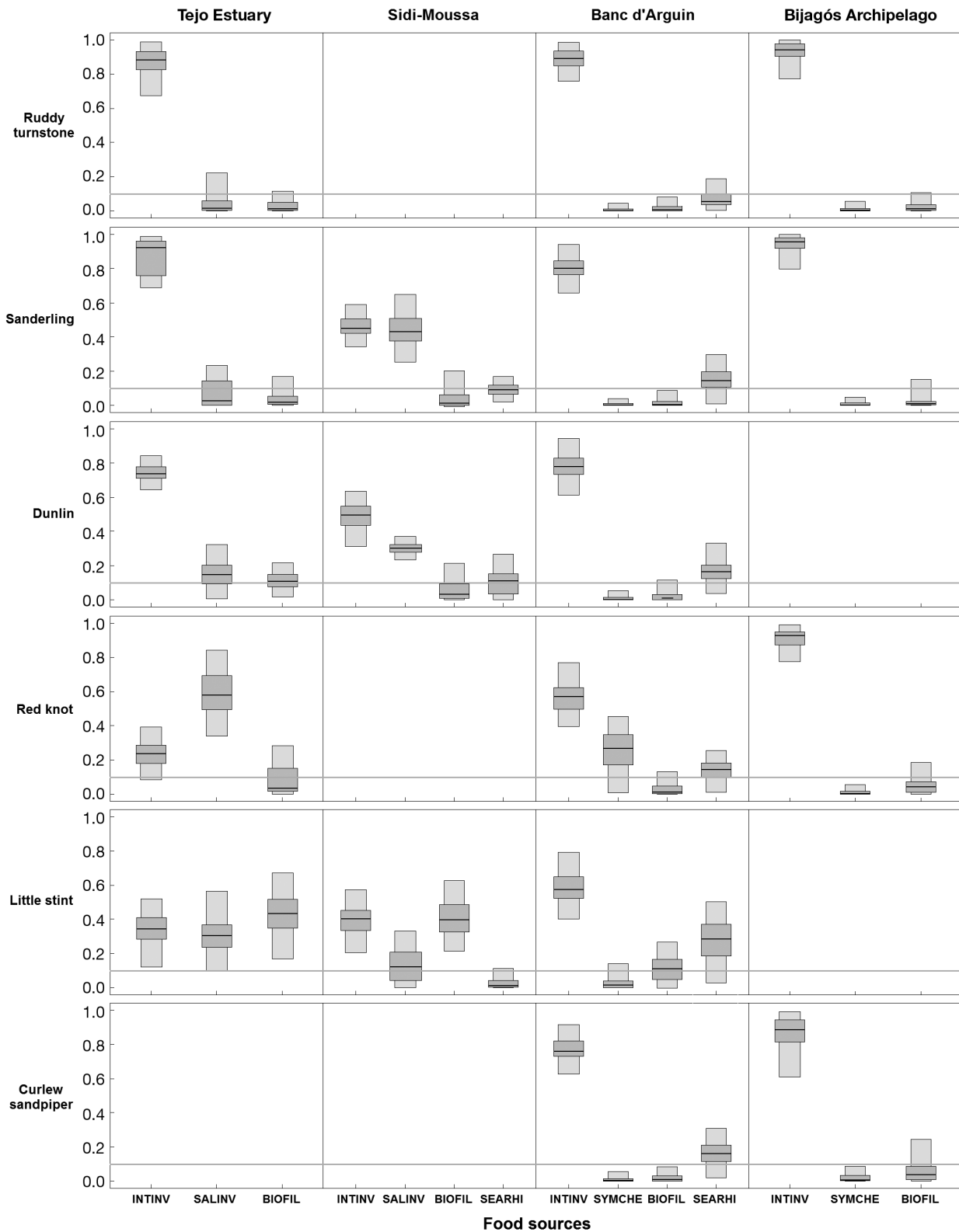


Fig. 2. Relative contribution of different food sources to the diet of shorebirds in the 4 study areas as estimated by Bayesian mixing models. For each case, we present the median and boxes for the 0.025–0.975 and 0.25–0.75 quantiles. The gray horizontal line indicates a proportion of 0.10 in the diet. Empty slots occur when a particular shorebird species was not sampled in a study area. For species taxonomic names, see Fig. 1. INTINV: intertidal macroinvertebrates, SALINV: saltpan macroinvertebrates, BIOFIL: biofilm, SEARHI: seagrass rhizomes, SYMCHE: intertidal bivalves with symbiotic chemoautotrophic bacteria

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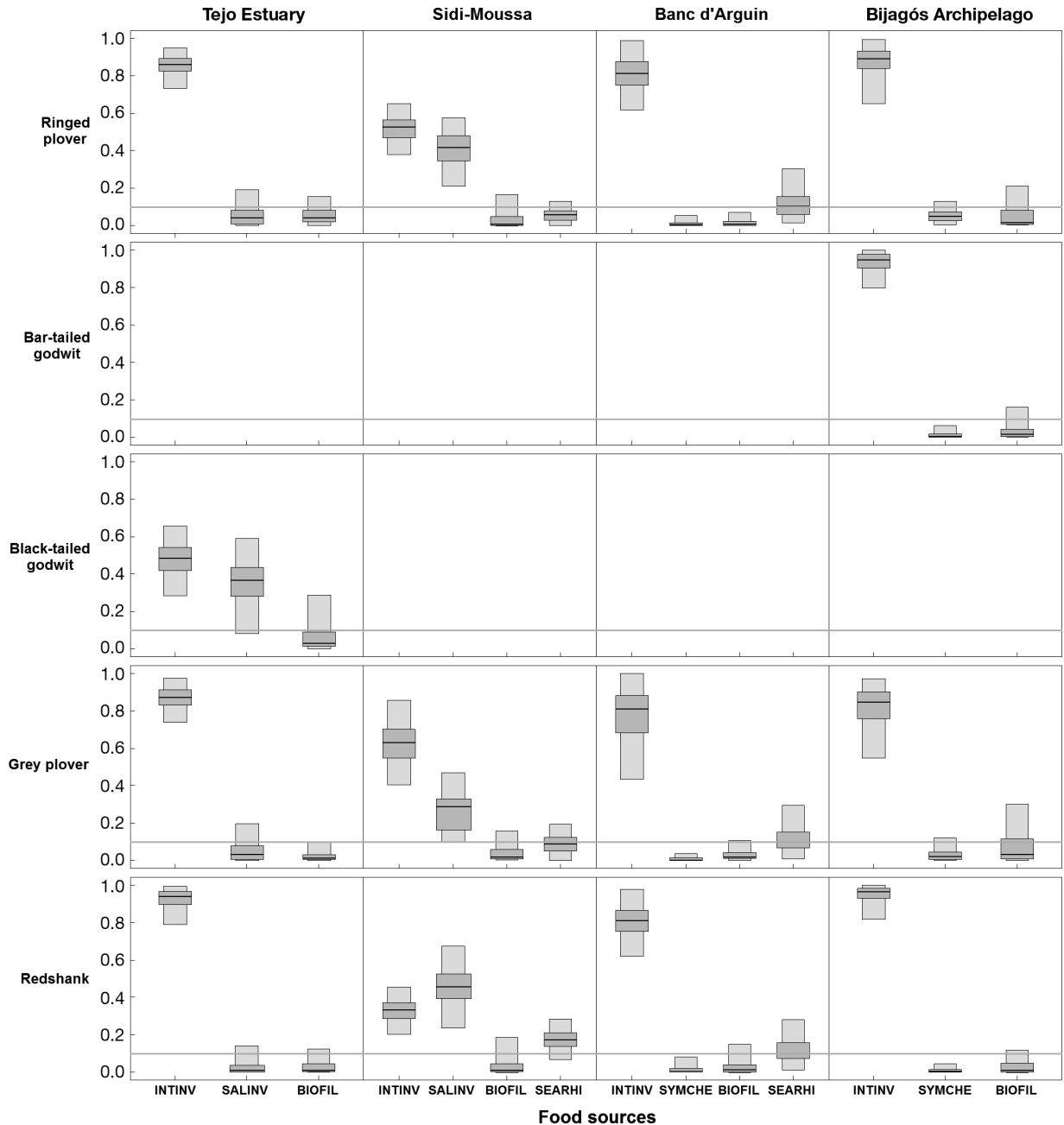


Fig. 2 (continued)

large range of potential values, the contribution of biofilm always remained >30% in the Tejo Estuary and Sidi Moussa, and >5% in the Banc d'Arguin (Fig. 3). In the Tejo Estuary, mixing models also indicated values >10% for the proportion of biofilm in the diets of red knot (17.6%) and dunlin (11.2%); however, as explained above, $\delta^{15}\text{N}$ values of biofilm and saltpan macroinvertebrates are very similar in the Tejo Estuary, so we should be cautious when interpreting these results.

DISCUSSION

Although this study confirms the general concept that wintering shorebirds rely mainly on intertidal macroinvertebrates for nourishment (e.g. Piersma 1987), the results also highlight the importance of other food sources for some species and wintering sites. In particular, when available, macroinvertebrates found in supratidal feeding areas such as saltpans can be an important trophic source for shorebirds.

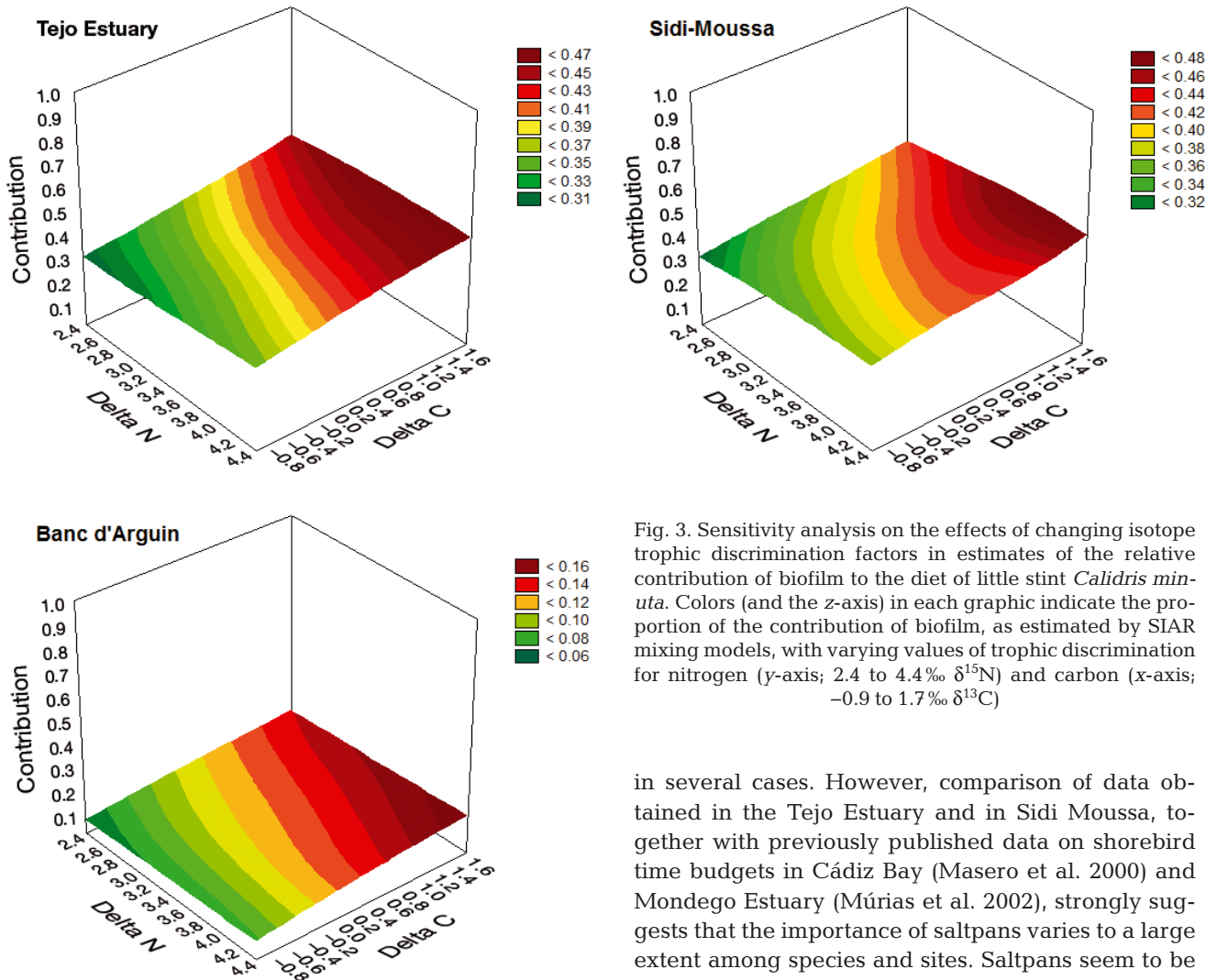


Fig. 3. Sensitivity analysis on the effects of changing isotope trophic discrimination factors in estimates of the relative contribution of biofilm to the diet of little stint *Calidris minuta*. Colors (and the z-axis) in each graphic indicate the proportion of the contribution of biofilm, as estimated by SIAR mixing models, with varying values of trophic discrimination for nitrogen (y-axis; 2.4 to 4.4‰ $\delta^{15}\text{N}$) and carbon (x-axis; -0.9 to 1.7‰ $\delta^{13}\text{C}$)

Previous studies have already evidenced the importance of salt pans as alternative feeding areas for shorebirds in a wide range of areas, including southern Europe (Masero et al. 2000, Múrias et al. 2002), southern Africa (Velasquez 1992), North America (Warnock et al. 2002) and East Asia (Li et al. 2013). A few studies have attempted to quantify the relevance of this foraging habitat based on bird counts and time budgets, based on the observation that some species may forage longer and in higher numbers in salt pans than in adjacent mudflats (Masero et al. 2000, Múrias et al. 2002). However, to our knowledge, only 1 study (on redshanks in Cádiz Bay) has managed to estimate the proportion of the diet represented by salt pan prey (23% in winter and 82% prior to northward migration departures; Masero & Pérez-Hurtado 2001). Our results allow a more precise evaluation of the true importance of salt pan prey in shorebird diets, suggesting that they can represent >30% of the diet

in several cases. However, comparison of data obtained in the Tejo Estuary and in Sidi Moussa, together with previously published data on shorebird time budgets in Cádiz Bay (Masero et al. 2000) and Mondego Estuary (Múrias et al. 2002), strongly suggests that the importance of salt pans varies to a large extent among species and sites. Salt pans seem to be important for little stint in Cádiz Bay, Tejo and Mondego Estuaries, but less so in Sidi Moussa; they seem important for dunlin, sanderling and redshank in Cádiz Bay, Sidi Moussa and Mondego Estuary, but less so in Tejo Estuary; they seem important for ringed plover in Sidi Moussa and Mondego Estuary, but less so in Tejo Estuary and Cádiz Bay. Of course, since salt pans are located outside the intertidal area, there is also a spatial component to their use as a trophic source. The different availability of salt pans in different wetlands, as well as their distance to intertidal feeding grounds (Dias et al. 2006), will influence the extent to which they are used by shorebirds.

Our result indicating that salt pan invertebrates represent 59% of the diet of red knots in the Tejo Estuary would seem inconsistent with the ecology of this molluscivore specialist (Piersma 2007, 2012). Indeed, since stable isotope signals in macroinvertebrates vary more among sites and environments than among taxa (e.g. Girard et al. 2011, Catry et al. 2016b), it is likely that the signature we obtained

from chironomid larvae will be similar to molluscs living in the same salt pans. In fact, cockles *Cerastoderma edule* can be abundant in salt pans (López et al. 2010) and this is also the case in at least some salt pans of the Tejo Estuary (A. D. Rocha pers. comm.), while other bivalves such as *Cerastoderma glaucum* and *Abra alba*, as well as *Hydrobia* sp. and other gastropods are also frequently found in salt pans (Evagelopoulos et al. 2008). The wintering population of knot in the Tejo Estuary is small (average 330 ± 205 individuals; Alves et al. 2011) and the only detailed study in this area suggests that they mainly feed on *Hydrobia ulvae* (Moreira 1994). This is likely due to the low availability of bivalves within harvestable sizes, leading to low intake rates in intertidal areas resulting from the low flesh/shell ratio of that prey (Moreira 1994). This could force red knots to resort heavily on salt pans as foraging grounds in the Tejo Estuary.

Although shorebirds are predominantly secondary consumers in all habitats and in all stages of their life cycle (van de Kam et al. 2004), increasing evidence shows that herbivory can be important for some populations. Hudsonian *Limosa haemastica* and marbled godwits *Limosa fedoa* rely mainly on *Potamogeton pectinatus* tubers in inland stopover sites in North America (Alexander et al. 1996), while the continental population of the black-tailed godwit *Limosa l. limosa* feeds mainly on rice seeds for most of its non-breeding season (Lourenço & Piersma 2008, Navedo et al. 2015), again in non-estuarine sites. Evidence of herbivory in estuarine areas is scarcer, but seeds have been recorded in the diet of several shorebirds at Cádiz Bay (Pérez-Hurtado et al. 1997) and, more recently, black-tailed godwits have been found to consume seagrass rhizomes in western France (Robin et al. 2013), a behavior also exhibited by red knots in Banc d'Arguin (van Gils et al. 2016). Our data suggests that rhizome consumption by shorebirds is possibly much more widespread than previously thought. In fact, with few exceptions, SIAR results suggest rhizomes represent >10% of the diet of most species in both Sidi Moussa and Banc d'Arguin, the 2 study sites where this food type was available. Isotopic data seem to be corroborated by the presence of plant fragments in a small proportion (10–12%) of faecal samples of sanderlings, dunlins, red knots and ringed plovers from Banc d'Arguin (Lourenço et al. 2016), while shorebird faecal samples from Tejo Estuary and Bijagós Archipelago, where seagrass is mostly absent, do not show any plant fragments (Lourenço et al. 2008, 2015b, authors' unpubl. data).

Biofilm is another lower-trophic-level food source known to be exploited by shorebirds (e.g. Elner et al. 2005, Kuwae et al. 2012). However, biofilm grazing has only been demonstrated for small *Calidris* sandpipers, and there is morphological evidence for smaller-bodied species having a more developed feeding apparatus adapted to consume biofilm (Kuwae et al. 2012). In addition, biofilm grazing by shorebirds has only been observed in the northern Pacific (both in Japan and Canada; Kuwae et al. 2012, Jardine et al. 2015) and on the Atlantic coast of Canada (Quinn & Hamilton 2012); but not in the Eastern Atlantic, arguably because there is competition with biofilm grazing mudsnails *Hydrobia*, which may prevent shorebirds from using this food source (Kuwae et al. 2012). Our data suggests that biofilm may be an important part of the diet of little stints in the Eastern Atlantic, representing 12–42% of their diet in all 3 sites where the species was sampled. As our sensitivity analysis shows, this result holds true even when discrimination values are pushed far from the average values used in the main analysis. The little stint is the smallest shorebird species in the Eastern Atlantic and, based on available morphological and phylogenetic data (Kuwae et al. 2012), it would be the most likely species to consume biofilm. The lower contribution of biofilm in the diet of little stints from the Banc d'Arguin could result from the much more extensive seagrass cover in this wetland, which may compete with the development of a good biofilm layer (Honkoop et al. 2008).

In the Tejo Estuary, biofilm also represented >10% of the diet of dunlin and red knot. Although this would agree with previous studies indicating that biofilm consumption is restricted to the Calidriinae, and dunlins are known to consume biofilm in the Pacific (Kuwae et al. 2012), the data on biofilm consumption in the Tejo Estuary must be interpreted with caution because at this site $\delta^{15}\text{N}$ signatures of biofilm were similar to those of saltpan invertebrates. In fact, a recent study on dunlin foraging behavior in the Tejo Estuary found no evidence of biofilm grazing in focal observations (Martins et al. 2013). Apart from little stints, it seems that this food resource is not relevant in the Eastern Atlantic, although we cannot completely rule out the possibility of biofilm consumption by dunlin and red knot in the Tejo Estuary.

These problems with the interpretation of biofilm feeding highlight some of the general issues of using stable isotopes and mixing models to infer diets. Some studies have shown evidence for differences in the relative importance of different food

sources in animal diets when using stable isotopes or more traditional methods such as faecal analysis (e.g. Alexander et al. 1996), which may be related to poor sampling of the isotopic variability of different food sources (Alexander et al. 1996, Phillips 2001, Phillips & Gregg 2003). On the other hand, the lack of inclusion of specific food sources will necessarily affect the results (Phillips & Gregg 2003). Modern Bayesian mixing models, such as those used here, are described as robust methods of dealing with variation in isotopic signals of both sources and consumers, with high similarity in the isotopic values of different prey types, and are also less affected by small variations in fractionation factors than linear models (Parnell et al. 2010). They are also able to deal with external sources of variation not connected to isotopic uncertainty, such as physiological differences or unidentified minor dietary sources (Parnell et al. 2010). Still, even if unlikely, we cannot exclude the possibility that important food sources may have been overlooked. For instance, small groups of shorebirds are occasionally seen foraging in flooded agricultural fields both in the Tejo Estuary and Sidi Moussa, especially after heavy rainfall (authors' pers. obs.). This is expected to influence the isotopic signals of these species. Also, and even though we sampled a large range of potential prey (see Table S1 in the Supplement), we did not sample all species in each study area and within each food source group. For instance, over 150 macroinvertebrate taxa have been recorded in the intertidal flats of the Banc d'Arguin (Wolff et al. 1993), many of which could potentially be consumed by shorebirds. We cannot rule out the possibility that sampling an even wider range of potential prey could change our mixing model results, but we must emphasize that the selection of prey taxa for stable isotope analysis was based on current knowledge of shorebird diets at each site (see 'Materials and methods').

Recent studies evidenced that mixing models may be sensitive to variation in trophic discrimination factors (Bond & Diamond 2011). Since there is little published information on discrimination factors for bird toenails (see Lourenço et al. 2015a), we decided to use values that are not specific to this tissue, which could affect the mixing model results. However, the sensitivity analysis we performed suggested that our main findings hold true even if trophic discrimination factors vary substantially from those used in our models (Fig. S1 in the Supplement). Another limitation of our method is the impossibility of determining the importance of indi-

vidual variation, i.e. when stating that a given source represents 30% of diet of a given species, this could result from 30% of individuals relying solely on that source, from all individuals obtaining roughly 30% of their nutrition from that source, or all cases in between. The number of samples used to obtain stable isotope signatures of some food sources was relatively small, which may increase the uncertainty of the mixing model results. In part, this resulted from logistic difficulties of working at such a wide geographic range, but the same wide scale of the study and the fact that the most relevant patterns discussed above are mostly consistent across species and across sites, would add to the robustness of our findings.

Empirical evidence for the generalized consumption of alternative food sources by intertidal avian predators is highly relevant for the interpretation of food-web structure and functioning in coastal wetlands. These areas are known to receive energy and nutrients from both autochthonous and allochthonous sources located both inland and in the ocean (Bouillon et al. 2011, Careddu et al. 2015), and such processes affect the structure and complexity of the local food webs (Olin et al. 2013, Catry et al. 2016b). The importance of salt pans as supratidal feeding areas highlights the need to integrate interconnection between intertidal and supratidal areas in trophic models, as shorebirds function as a mobile link that can harvest prey in both habitats and likewise input nutrients by producing faeces (Post et al. 1998, Hahn et al. 2007, Navedo et al. 2015). Shorebirds consuming lower-trophic-level food sources reveal a direct link between producers and secondary consumers (Colwell 2010, Kuwae et al. 2012), lowering the trophic position of these birds and showing greater food-web interconnection and complexity in intertidal habitats. This greater interconnection may increase the stability and resilience of these food webs (e.g. Dunne et al. 2002, Catry et al. 2016b) as the heterogeneity of distinct energy channels and their differential dynamic productivity favors the stability of complex ecosystems (Estes et al. 2011, Kuwae et al. 2012) while higher connectance delays the onset of rivet-like thresholds past which the food webs display extreme sensitivity to removal of highly connected species (Dunne et al. 2002). Although these trophic links between shorebirds and producers may thus strengthen coastal wetland ecosystems, they can also mean that the ongoing worldwide declines in shorebird populations (Stroud et al. 2006) will have further-reaching effects on the stability of whole ecosystems.

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