



Trophic ecology of a migratory shorebird community at a globally important non-breeding site: combining DNA metabarcoding and conventional techniques

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ABSTRACT: Studies providing a detailed diet description of whole-bird communities are surprisingly scarce. Yet, these studies are pivotal to comprehend the mechanisms structuring communities and the persistence of each species in the ecosystem. We characterized the diet of a shorebird community in a key non-breeding area of the East Atlantic Flyway, the Bijagós Archipelago (West Africa), combining molecular and morphological prey identification based on 239 droppings from 15 species. Our results show that while relying upon a super-abundant prey (fiddler crab), shorebirds consumed a very high number of taxa. We stress the relevance of highly mobile prey (especially crustaceans but also fish), which typically appear to be of little importance in most shorebird studies. Our results suggest that by consuming a high diversity of prey, shorebirds may reduce competition. This may be critical in a site ranked as the second most important area in West Africa for migratory shorebirds but marked by low benthic invertebrate availability. We further compared the performance of DNA metabarcoding and morphological identification of prey. Overall, molecular and morphological methods combined delivered the most comprehensive results, although molecular methods largely surpassed morphological methods regarding taxonomic detail achieved and number of prey taxa found (4 times more). Taxonomic resolution in the identification of polychaetes and bivalves using the 16S primer was low (mostly to class), whereas this primer clearly performed better than mitochondrial cytochrome c oxidase in identifying more insects and fish. We emphasize the need to increase invertebrate representatives from West Africa in barcode databases, in order to enhance metabarcoding results.

KEY WORDS: Diet analysis · High-throughput sequencing · Waders · Guinea-Bissau · West Africa · East Atlantic Flyway

1. INTRODUCTION

The continued decline in migratory shorebird populations is a matter of concern worldwide, that has been mostly attributed to global changes driven by human activities (namely habitat loss and degradation) and human disturbance along their flyways

(e.g. Morrison et al. 2004, Amano et al. 2010, Catry et al. 2011, Watts et al. 2015, Piersma et al. 2016, Studts et al. 2017). Understanding the requirements of migratory populations in each stage of their annual cycle is fundamental to pinpoint the main causes of decline and to apply adequate management efforts. Despite the vast number of studies on the ecology

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and distribution of shorebird populations (as reviewed by van de Kam et al. 2004), there are still important gaps in knowledge concerning processes that take place during the non-breeding season in tropical regions (Delany et al. 2009, van Roomen et al. 2018, 2022). This is the case of the East Atlantic Flyway, where even baseline data on diet are still lacking for many species (but see Zwarts 1988, Zwarts et al. 1990, Ntiamoa-Baidu et al. 1998, Catry et al. 2016, Lourenço et al. 2016, 2017).

Diet studies at the community level may also provide key insights into the structure and function of an ecosystem (Estes et al. 2011, Catry et al. 2016). Shorebirds are pivotal predators of benthic invertebrates in coastal areas, having the potential to exert top-down regulation on prey populations whose effects can cascade down to multiple trophic levels and even affect ecosystem processes (e.g. Hamilton et al. 2006, Mathot et al. 2018). Thus, the ability to accurately describe shorebird diet may ultimately be central in understanding how current declines in bird populations will impact ecosystem functioning and resilience.

The accuracy of diet determination is to a large extent influenced by the methodologies used (Nielsen et al. 2018). The recent development of DNA metabarcoding techniques offers the possibility to potentially identify all prey consumed by an animal with a high taxonomic resolution (Wirta et al. 2015, Sousa et al. 2019, Ando et al. 2020), often allowing a more comprehensive prey identification over any other method, mainly for prey taxa well represented in DNA online databases. This development was particularly important in community studies, since estimates of diet overlap among species can be rather different depending on the taxonomic resolution achieved. When compared with morphological prey identification, DNA metabarcoding is particularly relevant in detecting soft-tissue prey (e.g. McInnes et al. 2017), which often leave no visible trace in faeces, pellets or digested stomach contents. Also, molecular techniques allow for the identification of the predator, which may be particularly relevant when samples consist of remains obtained non-invasively, i.e. sometime after prey consumption actually occurred. However, DNA metabarcoding techniques are unable to quantify prey abundance or size (Ando et al. 2020), but advances have been made in estimating the biomass of prey consumed through DNA metabarcoding techniques (Verkuil et al. 2022). Also, DNA metabarcoding results may be highly influenced by factors such as sample collection method, or the specific criteria used for taxonomic assignment

of prey (Pompanon et al. 2012, Alberdi et al. 2018). For example, primer selection is among the most relevant factors to ensure an adequate description of the diet (Elbrecht & Leese 2017, Esnaola et al. 2018, Sousa et al. 2019), and the use more than one primer is generally recommended to provide a more thorough insight into the diet (e.g. Alberdi et al. 2018, da Silva et al. 2019). As the different methods used to study diet have their own strengths and weaknesses, combining 2 or more complementary methods often delivers broader and more accurate results (e.g. Catry et al. 2019).

Diet studies of shorebird species have been mostly based on the morphological identification of prey found in faeces (e.g. Martins et al. 2013, Lourenço et al. 2015). Analysing faeces is particularly advantageous, because it is relatively simple to obtain a large number of samples with little disturbance to the birds, representing the most recent prey consumed. While DNA metabarcoding has become widely used to assess the diet in several groups (as reviewed by Sousa et al. 2019), this method has been mostly used in terrestrial ecosystems and is particularly underused in shorebirds. The few published studies using DNA metabarcoding to assess the diet of shorebird species allowed not only the identification of several new prey, but also the determination of unreported links between freshwater and terrestrial habitats (Wirta et al. 2015, Gerwing et al. 2016, Gerik 2018, Giatas et al. 2022, Huang et al. 2022).

The goals of this study were (1) to characterize the diet of the most common shorebird species of the Bijagós Archipelago, in Guinea-Bissau, West Africa (14 migratory and 1 resident species) using DNA metabarcoding techniques and morphological identification of prey from faecal samples, and (2) compare the relative performance and redundancy of these 2 methodological approaches. The Bijagós Archipelago is one of the most important non-breeding areas for shorebirds along the East Atlantic Flyway, holding up to several thousands of migratory shorebirds during the boreal winter months (Salvig et al. 1994, Delany et al. 2009, van Roomen et al. 2015). Surprisingly, the biomass of potential invertebrate prey (mostly molluscs and polychaetes) in the Bijagós mudflats is unusually low (Zwarts 1985, Lourenço et al. 2018), apart from the super-abundant West African fiddler crab *Afruca tangeri* that has been identified as an important prey for several species (Zwarts 1985, Lourenço et al. 2017).

For the molecular approach, we used 2 primer sets from 2 distinct genomic regions (16S and mitochondrial cytochrome c oxidase [COI] genes), and we

compared the performance of each primer, which enabled us to identify the main differences between morphological and molecular identification of prey in the diet of shorebirds. This is the first assessment of the diet of a non-breeding shorebird community along the East Atlantic Flyway using a DNA metabarcoding approach.

2. MATERIALS AND METHODS

2.1. Study area and sample collection

Fieldwork was carried out on 3 islands in the Bijagós Archipelago, Guinea-Bissau (West Africa): Orango (11° 02' 05.9" N, 16° 01' 53.2" W), Formosa (11° 27' 05.2" N, 15° 54' 24.8" W) and Bubaque (11° 18' 17.7" N, 15° 50' 29.8" W; Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m705p127_supp.pdf). The area comprises about 88 islands and islets off the coast of Guinea-Bissau, of which only 25% are permanently inhabited. The archipelago encompasses vast intertidal areas of mud- and sandflats, often bordered by mangrove forests, and it includes 3 protected sites covering about 20% of its area. The Bijagós Archipelago is classified as a Biosphere Reserve (UNESCO) and a Ramsar site (Campredon & Catry 2016).

We collected 239 droppings from 15 shorebird species during the boreal winters of 2017/2018, 2018/2019 and 2019/2020. The studied community includes 14 migratory species sampled during their non-breeding period (common sandpiper *Actitis hypoleucos*, ruddy turnstone *Arenaria interpres*, sanderling *Calidris alba*, dunlin *C. alpina*, red knot *C. canutus*, curlew sandpiper *C. ferruginea*, Kentish plover *Charadrius alexandrinus*, common ringed plover *Charadrius hiaticula*, Eurasian oystercatcher *Haematopus ostralegus*, bar-tailed godwit *Limosa lapponica*, whimbrel *Numenius phaeopus*, grey plover *Pluvialis squatarola*, common greenshank *Tringa nebularia*, common redshank *T. totanus*) and 1 resident species (white-fronted plover *Charadrius marginatus*). Fresh droppings were collected both in the field (by following foraging birds and also in roosting areas) as well as during bird capture and ringing sessions (with mist nets). Species identity could not always be assigned for droppings collected from foraging or roosting birds. The identities of the shorebird species producing the droppings were further confirmed by molecular analysis (see Section 2.2). We used pre-packaged sterilized spatulas and avoided collecting any surrounding sediment while taking the faecal sample. In

order to identify potential contamination sources of droppings from the soil, we collected 7 samples of intertidal sediment near some of the droppings. All samples were stored in absolute ethanol immediately after collection, and then stored at -20°C until further analysis.

In order to build a morphological and molecular reference collection of prey, benthic invertebrates were collected using 30 sediment cores (113.1 cm²) which were buried 30 cm deep, collected on 5 different days (between November 2019 and February 2020). Each core was sieved through a 0.5 mm mesh, and all invertebrates found were stored in absolute ethanol.

2.2. Laboratory analysis

DNA was extracted from all shorebird droppings using the Stool DNA Isolation Kit (Norgen Biotek) following the manufacturer's protocol and including batches of 23 samples plus a negative control to check for possible contamination during DNA extraction. Two DNA elutions of 50 µl were obtained and stored at -20°C until DNA amplification. The surplus sample that was not used in extraction and the tube after the first step of extraction were stored at -20°C for further morphological analysis of the dropping contents.

In order to identify the shorebird species from all samples collected from the ground, a first DNA amplification of the COI gene was performed using the primer set Leray-XT (mlCOIintF_XT: 5'-GGW ACW RGW TGR ACW ITI TAY CCY CC-3'; jgHCO2198: 5'-TAI ACY TCI GGR TGI CCR AAR AAY CA-3', Geller et al. 2013, Wangenstein et al. 2018), modified with Illumina adaptors. PCR reactions were carried out in a total volume of 10 µl, comprising 5 µl of Qiagen Multiplex PCR Master Mix with 2.5 µl of H₂O, 0.25 µl of each primer (10 pM) and 2 µl of DNA. Cycle amplification conditions consisted of an initial denaturing step at 95°C for 15 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 45°C for 40 s and extension at 72°C for 40 s, with a final extension step at 72°C for 10 min. Amplification was confirmed on a 2% agarose gel, and samples were sequenced in an Illumina Miseq system after library preparation following the manufacturer's protocol for metagenomics sequencing (Illumina). Briefly, successful PCR products were diluted 1:3 and 1:4 depending on band strength. After dilution, a second PCR was performed to incorporate P5 and P7 Illumina adaptors using custom primers tagged with 7 bp indexes, allowing the

individual identification of each amplified product. PCR reactions comprised 7.5 µl of KAPA HiFi HotStart ReadyMix (KAPA Biosystems), 1.5 µl of primers with indexes and 3 µl of PCR product. Cycling conditions included an initial denaturing step at 95°C for 3 min, followed by 10 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, followed by a final extension step at 72°C for 5 min. Indexed amplicon products were checked on a 2% agarose gel, purified using Agen-court AMPure XP beads (Beckman Coulter) and subsequently quantified using an Epoch Spectrophotometer System (BioTek Instruments). After quantification, all PCR products were pooled at equimolar concentrations (10 nM). The library was then quantified by qPCR (KAPA Library Quant Kit pPCR Mix, Bio-Rad iCycler), the fragment size was verified using the TapeStation System (Agilent) and diluted to 4 nM. The library was sequenced using part of a v3 run of a MiSeq desktop sequencer (Illumina; 6000 paired-end reads per sample).

In order to block the amplification of bird DNA during diet assessment, blocking primers for Leray-XT were designed (Table S1 in the Supplement; Leray et al. 2013) by preparing an alignment with all available COI sequences of each predator species obtained in this study or from reference databases (NCBI: <https://www.ncbi.nlm.nih.gov>; BOLD: <https://www.boldsystems.org>). The primers were further aligned with sequences of potential prey items, as well as blasted on NCBI to confirm their inability to block the amplification of dietary taxa.

Molecular prey identification was attained by targeting 2 mitochondrial gene regions in order to maximize the amplification of different prey taxa expected (Alberdi et al. 2018). The COI was amplified using the primer set Leray-XT (mlCOIintF_XT/jgHCO2198, to amplify a fragment of 313 bp, Geller et al. 2013, Wangensteen et al. 2018) and 16S was amplified using the primer set 16S (16S1F-degenerate: 5'-GAC GAK AAG ACC CTA-3', 16S2R-degenerate: 5'-CGC TGT TAT CCC TAD RGT AAC T-3', to amplify a fragment of 180–210 bp, Deagle et al. 2007, Elbrecht et al. 2016), both modified with Illumina adaptors. All PCRs performed for COI gene amplification were conducted with the same volumes and cycle conditions as described above but this time including 0.25 µl of a species-specific blocking primer (100 pM) depending on the identity of a sample. The PCRs performed for 16S gene amplification comprised 0.3 µl of each primer (10 pM), 5 µl of Qiagen Multiplex PCR Master Mix and 2 µl of DNA. No blocking primer was used for 16S since previous

studies did not report high/significant amplification levels of host DNA, including shorebird species, and because our 16S alignment of the primers with the bird sequences showed a mutation at the 3' end that usually prevents amplification (Gerwing et al. 2016, Gerik 2018). Cycle amplification conditions consisted of an initial denaturing step at 95°C for 15 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 60 s, with a final extension step at 72°C for 10 min. Amplification success was confirmed on a 2% agarose gel, and successfully amplified products underwent library preparation (all 239 samples for COI but only 195 for 16S). Preparation of diet libraries was done as for sample identification, except that PCR products were pooled per gene before qPCR quantification, and only then pooled equimolarly. Libraries were sequenced using a whole v3 run of a MiSeq Desktop Sequencer (Illumina; 28 000 paired-end reads per sample). After DNA extraction, droppings were further sorted using a stereomicroscope for morphological identification of prey remains to the lowest possible taxonomic level, based on available literature and compared to our own invertebrate reference collection (see below; Day 1967, Fauchald 1977, Carpenter & De Angelis 2014, 2016, Cosel & Gofas 2019).

Invertebrates collected in sediment cores during this study were identified to the lowest possible taxonomic level using a stereomicroscope (Day 1967, Fauchald 1977, Carpenter & De Angelis 2014, 2016, Cosel & Gofas 2019), and for those whose sequence was not present in online databases (BOLD and NCBI), DNA was amplified for the COI region and sequenced in a MiSeq desktop sequencer (Illumina; 1000 reads per sample; detailed information is provided in Text S1), to constitute a reference collection.

2.3. Bioinformatic analysis

Raw reads were processed using standard procedures for metabarcoding data. First, paired reads were merged using the command 'illumina-pairedend' of Obitools (Boyer et al. 2016), followed by primer removal and sample tagging of reads with 'ngsfilter'. Reads were then dereplicated per sample using 'obiuniq'. Afterwards, samples were merged and the reads denoised using the command '—cluster_unoise' in VSEARCH (Rognes et al. 2016). Resulting zero-radius operational taxonomic units (ZOTUs) were further inspected for chimeras using the command '—uchime3_denovo' and then clustered at

99% similarity using ‘—cluster_size’. Reads were then mapped back to the retained operational taxonomic units (OTUs) using the command ‘—usearch_global’ with an identity level of 99%. Finally, LULU (Frøslev et al. 2017) was used to merge similar OTUs (identity >84%) with high co-occurrence levels (>95% of samples) to reduce the number of retained PCR artifacts, sequencing errors, and nuclear copies of the mitochondria.

For each marker, prey items were identified by comparing the retained OTUs against online databases (BOLD and NCBI) using the BLAST algorithm, as well as unpublished sequences of 126 benthic invertebrates collected within the scope of this work in the case of COI.

Samples with <100 reads of diet items for both primers were eliminated. Moreover, within each sample, OTUs representing <1% of the total reads of that sample were also discarded.

2.4. Statistical analysis

To build a consensus in diet analysis, i.e. to reconcile prey identification obtained by using 2 molecular primers and by using morphological identification based on prey remains in droppings, we assumed that a given item recovered at lower taxonomic resolution (e.g. order or family) by one method was the same as all items of the same taxonomic group recovered at higher resolution by other method (e.g. genus or species). We only maintained lower taxonomic resolution identifications when they were exclusively from the morphological identification (e.g. if 2 bivalves were identified only morphologically in the same sample, one to species level and other to a higher taxonomic level, we kept both). For the purpose of methodological comparisons, we applied the same rationale within each method.

To describe the diet of each bird species, the frequency of occurrence (FO) of prey was calculated at class, order, family and species/genus taxonomic levels, as the ratio between the number of samples where the prey was present and the total number of samples analysed. In order to compare diet diversity among shorebird species, we used incidence-based Hill numbers, namely richness (q order = 0) and Shannon diversity (q order = 1), based on a dataset of FO of prey classified at the taxonomic level of order (Chao et al. 2014). Richness represents the number of prey orders found for each bird species regardless of their frequency, while the Shannon diversity accounts for the incidence frequency and can be

interpreted as the effective number of frequent prey orders in bird diet. Given that empirical estimates of Hill numbers tend to increase with increasing sampling effort, samples were standardized to an equal sample coverage (defined as the proportion of the total number of individuals in a community that belong to the species represented in the sample) in order to ensure comparable data (Chao et al. 2014, Hsieh et al. 2016). The standardization was performed using the lowest sample coverage recorded for the community that allowed the estimation of richness and Shannon diversity for all species (65%). We used the ‘estimateD’ function in the R package ‘iNEXT’ (Hsieh et al. 2016), which delivers 95% confidence intervals around Hill numbers using a bootstrap method. Species with non-overlapping confidence intervals were considered to have significantly different diversity. Species with small sample sizes ($n < 8$) were excluded from this analysis. To assess dietary overlap within the shorebird community, we calculated the Bray-Curtis index based on prey FO to the taxonomic level of order, excluding orders that occurred only once in the diet of all species combined, using the function ‘vegdist’ in the R package ‘vegan’ (Oksanen et al. 2022). The Bray-Curtis index ranges from 0 (no overlap) to 1 (total overlap), and values >0.6 are generally assumed to represent a significant overlap (Langton 1982).

The performance of each method used for prey identification (16S, COI, molecular, i.e. the 2 primers combined, and morphology) was assessed as the proportion of taxa identified by each method in relation to the total number of taxa identified per sample using the 3 methods simultaneously. Performance was assessed at the taxonomic level of both family and order. We tested for differences in the performance of methods using binomial generalized linear models (GLMs) with the sample as a random factor, followed by a pairwise comparison of the estimated marginal means calculated with the package ‘emmeans’ (Lenth 2019).

The similarity or redundancy between methods was calculated as the ratio between the number of prey taxa identified simultaneously by both methods and the total number of taxa identified in the sample using any of the methods in comparison. Again, similarity was assessed at family and order levels. When assessing both performance and similarity between approaches, we excluded samples without any prey identified to the taxonomic level used in the comparison between methods.

All analyses were run using R software version 4.1.3 (R Core Team 2022), and the packages ‘bipar-

tite' (Dormann et al. 2008) and 'ggplot2' (Wickham 2016) were used to create the figures.

3. RESULTS

We molecularly analysed 239 droppings of 15 shorebird species, of which 237 were also analysed morphologically (2 samples were discarded after molecular processing, and therefore not analysed macroscopically). All 239 samples were sequenced using the COI primer, and 195 of those samples were also sequenced with the 16S primer (see Section 2). In 5% of the samples, we were unable to identify any prey (delivering no results on diet items with either method); therefore, we obtained 226 samples with diet contents (Table 1). Within these, 31 and 16% of the samples delivered no prey identification with COI and 16S primers, respectively, and 28% were classified as empty (no diet contents) by the morphological approach. From the 7 sediment samples analysed, collected close to droppings, 4 did not provide any identifiable prey, and the remaining 3 contained only exclusive OTUs, i.e. OTUs that did not occur in any of the dropping samples. Therefore, we assumed that the sediment did not contaminate the droppings.

Concerning the invertebrate reference collection, we sequenced 126 individuals from 14 orders of 4 classes (Bivalvia, Gastropoda, Malacostraca and Polychaeta). Five percent of the COI OTUs found in droppings that had no match with online genetic databases (BOLD and NCBI) matched with sequences in our prey reference collection. In addition,

the use of our reference collection allowed for the identification of a further 14% COI OTUs to a lower taxonomic level as compared to the online genetic databases.

3.1. Diet composition of the shorebird community

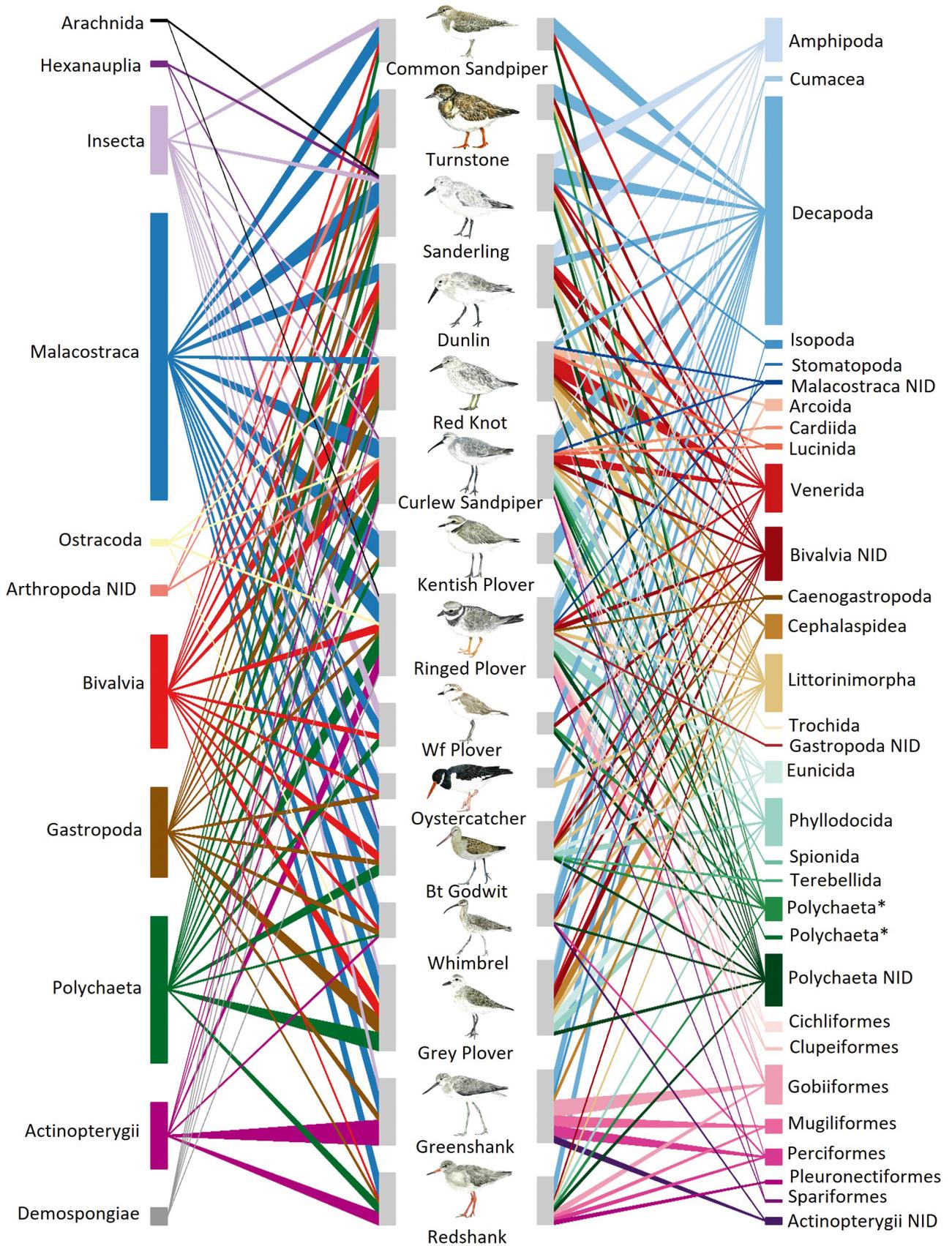
By combining the information from all identification methods, we found prey belonging to 14 classes from 10 phyla in the diet of the studied shorebirds, with the phyla Arthropoda and Mollusca occurring in the diet of all species (Fig. 1).

The class Malacostraca was consumed by all species, representing the most frequent prey item for the majority, with FO > 0.5 for all species but red knot. The class Bivalvia was also present in the diet of all shorebird species ($0.13 \leq FO \leq 0.97$) except in Kentish plover and common greenshank, while the class Gastropoda was present in all ($0.08 \leq FO \leq 0.73$) except common sandpiper and white-fronted plover. Within the studied community, only Eurasian oystercatcher and common greenshank did not consume the class Polychaeta, with the FO of this prey varying between 0.04 and 0.90 among the remaining species. Class Insecta was absent from the diet of 4 shorebird species, and was only somewhat frequent ($FO \geq 0.4$) in 3 species (white-fronted plover, grey plover and common greenshank). The orders Coleoptera and Hymenoptera were the most common insects found in droppings of white-fronted plover, and Diptera in common greenshank, while no order in particular dominated in grey plover (Table S2). Seven shorebird species consumed fish (class Actinopterygii), with frequent consumption by common ringed plover

Table 1. Number of droppings with diet contents ($N_{\text{total}} = 226$) analysed by species collected in the Bijagós Archipelago (Orango: N = 160, Bubaque: N = 62, Formosa: N = 4)

Species	N
Common sandpiper <i>Actitis hypoleucos</i>	4
Ruddy turnstone <i>Arenaria interpres</i>	3
Sanderling <i>Calidris alba</i>	26
Dunlin <i>Calidris alpina</i>	6
Red knot <i>Calidris canutus</i>	30
Curlew sandpiper <i>Calidris ferruginea</i>	28
Kentish plover <i>Charadrius alexandrinus</i>	12
Common ringed plover <i>Charadrius hiaticula</i>	29
White-fronted plover <i>Charadrius marginatus</i>	7
Eurasian oystercatcher <i>Haematopus ostralegus</i>	8
Bar-tailed godwit <i>Limosa lapponica</i>	23
Whimbrel <i>Numenius phaeopus</i>	28
Grey plover <i>Pluvialis squatarola</i>	10
Common greenshank <i>Tringa nebularia</i>	4
Common redshank <i>Tringa totanus</i>	8

Fig. 1. Bipartite network representing the trophic interactions between shorebird species from the Bijagós Archipelago, Guinea-Bissau, and their prey classes (left column) and prey orders from the classes Malacostraca, Bivalvia, Gastropoda, Polychaeta and Actinopterygii (right column). Prey orders grouped in a gradient of the same colour (e.g. blue) belong to the class matching the same colour (e.g. blue: class Malacostraca). The bar width of bird species and prey is proportional to the number of links of each, and the width of the links reaching each bird bar indicates the interaction intensity (i.e. prey frequency of occurrence). The classes Plidliophora, Sipunculidea, Enteropneusta and Hydrozoa, and the phylum Gastrotricha (class not assigned), occurred only in the diet of fewer than 4 bird species, always with frequency of occurrence ≤ 0.1 , and therefore were not included (detailed information in Table S2 in the Supplement). *Order not assigned. Some shorebird names are shortened for simplicity; Bt godwit: bar-tailed godwit; Wf plover: white-fronted plover. NID: Not identified



and common greenshank (FO = 0.76 and 1.00, respectively; Fig. 1). The remaining identified prey classes occurred in few species with low FOs (Fig. 1).

The class Malacostraca was not only the most represented in the diet of the shorebird community, but it also included the highest diversity in terms of the number of families identified (although some Polychaeta prey could not be assigned to a particular family; Fig. 2). Within the 5 Malacostraca orders identified (Amphipoda, Cumacea, Decapoda, Isopoda and Stomatopoda) we found 1 family each of isopods (Cymothoidae), mantis shrimp (Squillidae) and mud shrimps (Upogebiidae), 2 families of amphipods (Ampithoidae and Caprellidae), 3 families of shrimps (Alpheidae, Crangonidae and Penaeidae) and 6 families of crabs (Grapsidae, Ocypodidae, Panopeidae, Portunidae, Sesamidae, Varunidae). Despite this high diversity, a single species, the fiddler crab *Afruca tangeri*, was clearly the dominant malacostracan prey in the diet of the shorebird assemblage ($0.03 \leq \text{FO} \leq 1$), only absent from the diet of common greenshank. The caramote prawn *Penaeus kerathurus* ranked second ($0.07 \leq \text{FO} \leq 0.88$) and was present in the droppings of most species but particularly frequent in the diet of common greenshank and common redshank ($\text{FO} \geq 0.75$; Fig. 2).

Four orders of bivalves (Arcoida, Cardiida, Lucinida and Venerida) were recognized, with the clam *Pelecypora isocardia* (Veneridae) being the most frequent species, particularly in the diet of red knot ($\text{FO} = 0.87$; Fig. 2). We identified 4 orders of Gastropoda (Caenogastropoda, Cephalaspidea, Littorinimorpha and Trochida), among which the sea snails *Haminoea orbignyana* and *Assimineia* sp. were the most frequent species (Fig. 2). Interestingly, the 2 main gastropod consumers, red knot and grey plover, clearly showed different preferences regarding the choice of gastropod species (Fig. 2).

Polychaeta found in the diet of shorebirds comprised 4 orders (Eunicida, Phyllococida, Spionida and Terebellida), of which Eunicida and Phyllococida were the most frequent (Fig. 2). Again, the preferences for different worms seem to vary among the main consumers, with grey plover consuming more Eunicida than for instance curlew sandpiper and common ringed plover (Fig. 2).

Prey fish (Actinopterygii) comprised 7 orders (Cichliformes, Clupeiformes, Gobiiformes, Mugiliformes, Perciformes, Pleuronectiformes, Spariformes) and were frequent in the diet of common ringed plover, common greenshank and common redshank, within which the goby *Porogobius schlegelii*, the grooved mullet *Chelon dumerili* and the pigsnout

grunt *Pomadasys rogerii* were the more frequently consumed species (Fig. 2).

The diet overlap varied between 0.1 and 0.7 within the studied community (as assessed by the Bray-Curtis index). Common sandpiper, ruddy turnstone, red knot, white-fronted plover, grey plover and common greenshank showed no significant diet overlap (overlap < 0.6) with any other species of the bird community (Fig. 3). On the other hand, higher diet overlap was recorded for bar-tailed godwit (significant overlap with 5 species), Kentish plover (significant overlap with 5 species), common redshank (significant overlap with 4 species) and whimbrel (significant overlap with 4 species).

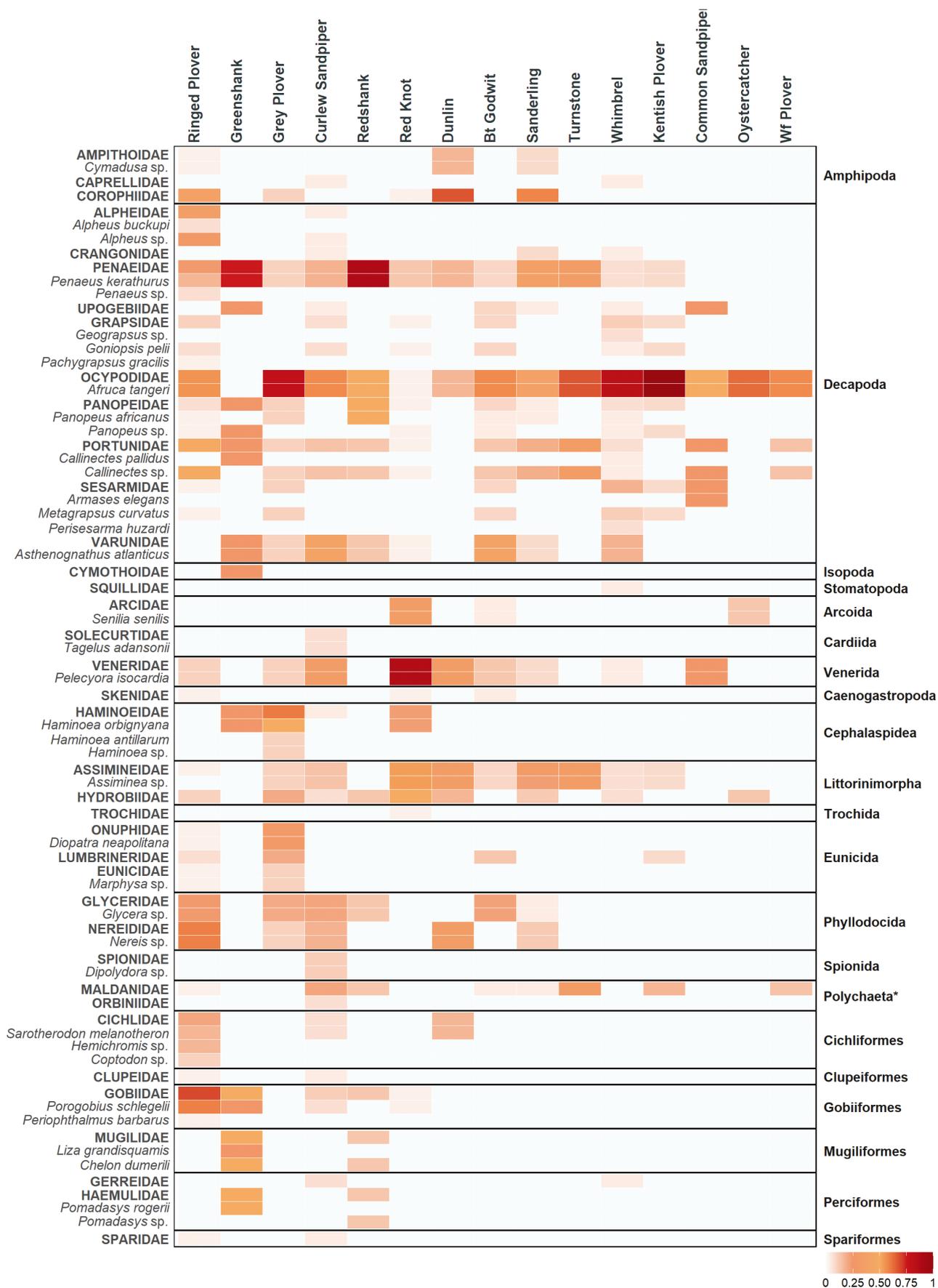
Diet richness (overall range 1.5 to 15.3) and diversity (overall range 1.5 to 10.1) were the highest for common redshank, curlew sandpiper, ringed plover, grey plover and bar-tailed godwit, and the lowest in whimbrel, Kentish plover and Eurasian oystercatcher (Table 2).

3.2. Comparison of the performance of molecular (COI and 16S primers) and morphological methodologies

From all the droppings collected, 193 were analysed using both morphological and molecular approaches (with both 16S and COI primers). Among these, 9 samples were considered empty (i.e. no diet contents) by all methods; therefore, we obtained 184 samples with diet contents. From those 184 samples, 13, 16 and 28% failed to deliver dietary results with 16S, COI and morphology, respectively.

The number of taxa identified per sample varied significantly among methods (Friedman test: $\chi^2 = 15.2$, $\text{df} = 2$, $N = 184$, $p < 0.001$), being significantly lower with morphological identification (mean \pm SD: 1.4 ± 1.3) than with 16S or COI (16S: 2.1 ± 1.9 ; COI: 1.8 ± 1.4 ; post hoc test, $p < 0.01$ and $p < 0.05$, respectively). The number of taxa identified was similar between COI and 16S primers (post hoc test, $p = 0.85$). Moreover, taxonomic resolution in prey identification differed between methods; with the morpho-

Fig. 2. Frequency of occurrence (FO) of prey families (in capital letters) and species of the classes Malacostraca, Bivalvia, Gastropoda, Polychaeta and Actinopterygii in the diet of 15 species of shorebirds from the Bijagós Archipelago, Guinea-Bissau. Species with $\text{FO} < 0.05$ were excluded. *Order not assigned (detailed information in Table S2 in the Supplement). Some shorebird names are shortened as in Fig. 1



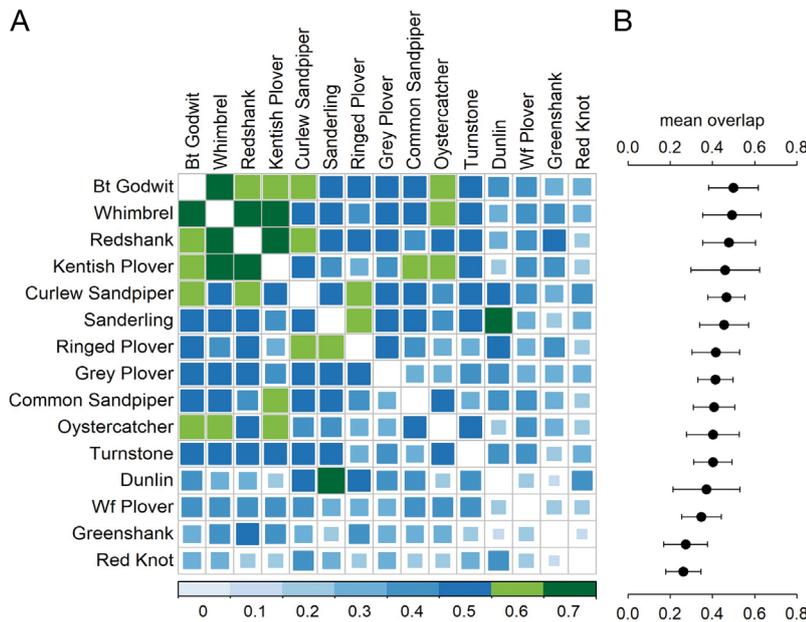


Fig. 3. (A) Pairwise and (B) mean diet overlap (calculated as 1 minus the Bray-Curtis dissimilarity index) for 15 shorebird species from the Bijagós Archipelago, Guinea-Bissau (prey classified to the taxonomic level of order). Some shorebird names are shortened as in Fig. 1. No significant diet overlap (<0.6) is presented in a gradient of blue and significant diet overlap (≥ 0.6) is presented in a gradient of green

Table 2. Estimates of coverage-standardized Hill diversity (richness and Shannon diversity) of shorebird diet for the studied community, calculated at the taxonomic level of order. Species with non-overlapping confidence intervals (CI) are considered to show significantly different richness and/or diversity. Species with small sample sizes ($n < 8$) were excluded from this analysis

Species	Richness		Shannon diversity	
	Estimate	95% CI	Estimate	95% CI
Sanderling	6.4	5.5–7.3	5.8	4.9–6.7
Red knot	4.4	3.8–5.1	4.2	3.6–4.8
Curlew sandpiper	12.0	10.2–13.8	10.1	8.4–11.8
Kentish plover	2.2	1.4–3.0	2.0	1.3–2.8
Common ringed plover	8.6	7.5–9.8	7.8	6.9–8.8
Eurasian oystercatcher	2.5	1.3–3.7	2.2	1.1–3.3
Bar-tailed godwit	8.0	6.1–10.0	6.1	4.6–7.6
Whimbrel	1.5	1.3–3.7	1.5	1.3–1.8
Grey plover	8.2	6.1–10.3	7.3	5.5–9.2
Common redshank	15.3	6.8–23.8	8.6	3.5–13.7

logical approach, the majority of prey were identified to the class and order levels, whereas prey identified molecularly (with 16S or COI primers) were frequently assigned to the species or genus levels (Fig. 4A).

The use of the COI primer allowed the identification of a higher diversity of taxa (at the phylum, class and order level) when compared with the 16S primer or the morphological identification (Fig. 4B). The 2

primers combined increased the number of species identified in comparison with COI and 16S alone by 36 and 42%, respectively. The morphological analysis added little information to that achieved by the molecular methodology. Nonetheless, Ostracoda (phylum Arthropoda) was exclusively identified morphologically, whereas 7 out of the 15 classes of prey (Hexanauplia, Demospongiae, Pilidiophora, Sipunculidea, Enteropneusta, Gastrotricha and Hydrozoa) were solely identified using the COI primer (Fig. 5). Taxonomic resolution in the identification of Polychaeta and Bivalvia using the 16S primer was rather low, with no taxa identified to order or lower taxonomic levels (Fig. 5). On the other hand, the 16S primer clearly performed better than COI for identifying insects and fishes (Fig. 5).

Prey-detection power varied among methods according to prey classes. Prey from the class Bivalvia were found more frequently using morphology than using the molecular approach (either COI or 16S primers; Fig. 6). COI delivered a higher detection rate of Polychaeta (especially compared to 16S), whereas 16S more frequently detected Malacostraca and particularly fishes (Actinopterygii; Fig. 6).

The performance of each method of prey identification to order level was significantly higher when using the 2 primers combined (mean \pm SD: 0.86 ± 0.25), as compared to 16S primer (0.56 ± 0.37), COI primer (0.61 ± 0.38) or morphology (0.48 ± 0.41 ; $p < 0.0001$ for all post hoc tests; Tables S3 & S4). COI performed significantly better than morphology ($p < 0.05$), whereas no differences were found between 16S and

COI or 16S and morphology ($p = 0.40$ and 0.65 , respectively, Table S4). Concerning prey identification to the family level, the performance of both primers combined (0.86 ± 0.25) was significantly higher than that of the 16S primer (0.54 ± 0.36), COI primer (0.51 ± 0.37) or morphology alone (0.20 ± 0.30 ; $p < 0.001$ for all post hoc tests; Tables S5 & S6). Furthermore, each primer (16S and COI) performed significantly better than morphology ($p < 0.001$),

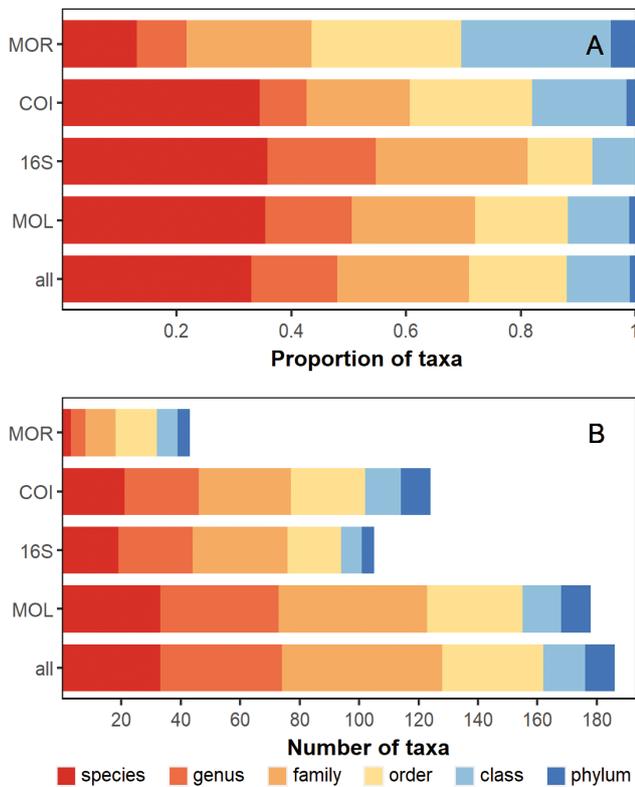


Fig. 4. (A) Taxonomic resolution in prey identification and (B) total number of prey taxa identified at different taxonomic levels in the droppings of the shorebird community using different identification methods (MOR: morphological identification, MOL: molecular 16S + COI primers; all = all methods combined; N = 184)

whereas the 2 primers performed similarly ($p = 0.88$, Table S6).

Similarity or redundancy in prey identification among methods was overall low. The similarity between COI and 16S primers in the identification of prey orders was on average 36%, whereas between morphology and COI or 16S was 37 and 29%, respectively. The similarity was even lower when assessed at the family level, as on average only 24% of the prey families were simultaneously identified by COI and 16S, while the redundancy level of morphology and molecular markers was 8 and 1% for COI and 16S, respectively (Fig. 4B).

4. DISCUSSION

4.1. Diet composition of the shorebird community

In this study, we were able to identify a very high number of prey taxa (38 orders from 14 classes belonging to 10 phyla; Fig. 2, Table S2), many of which

have not been previously detected in the diet of any studied shorebird species in West Africa (Engelmoer et al. 1984, Zwarts 1988, Zwarts et al. 1990, Ntiamao-Baidu et al. 1998, van Gils et al. 2013, Lourenço et al. 2016, 2017; studies based on morphology and focal foraging observations). These new taxa include a wide range of species and genera, but also prey belonging to higher taxonomic levels such as families, orders and even classes and phyla previously undescribed in shorebird diets.

Crustaceans (Malacostraca) have been previously shown to comprise an important fraction of the diet of many species within the shorebird community of the Bijagós Archipelago, with an unequivocal dominance of the fiddler crab *Afruca tangeri* (Zwarts 1985, Lourenço et al. 2017). Here, we confirm the fiddler crab as a key prey for this bird assemblage, but we also highlight a high diversity of other crustaceans, namely crabs (10 species/genera, 7 of which were previously unreported in shorebird diets in West Africa) and shrimps (including 2 species previously unreported). Shrimps (Penaeidae and Alpheidae) were present in at least one-third of the samples of common ringed plover, ruddy turnstone, sanderling, common redshank and common greenshank, being particularly relevant for the last 2 species for which FO reached 0.75 and 0.88, respectively. Other crustaceans, such as amphipods (*Caprella scaura*, *Cymadusa* sp. and Corophiidae, also not previously described in the diet of West African shorebirds), presented an FO > 0.5 in common ringed plover, while the FO reached 0.65 for sanderling and dunlin. In contrast, and based on morphological identifications, Lourenço et al. (2017) reported FO of amphipods no higher than 0.06 for any of the studied species.

We recorded a relatively low diversity of Polychaeta, which may partly result from the low taxonomic resolution of our identifications. Still, we were able to identify 1 previously undescribed order (Terebellida) and 3 new families (Onuphidae, Orbiniidae and Spionidae) in the diet of birds in the region. However, these new taxa had low FOs, with the families Nereidae and Glyceridae reaching higher FO and being present in a higher number of species (also in accordance with Lourenço et al. 2017).

All studied shorebirds consumed molluscs, with Veneridae clearly being the dominant bivalve family. Conversely, 3 families of gastropods were found to have FO ≥ 0.5 in the diet of red knot (Assimineidae and Hydrobiidae) and grey plover (Haminoeidae). Unsurprisingly, the highest consumption of molluscs was recorded in red knot, a well-known molluscivore throughout its distribution range (e.g. Prater 1972,

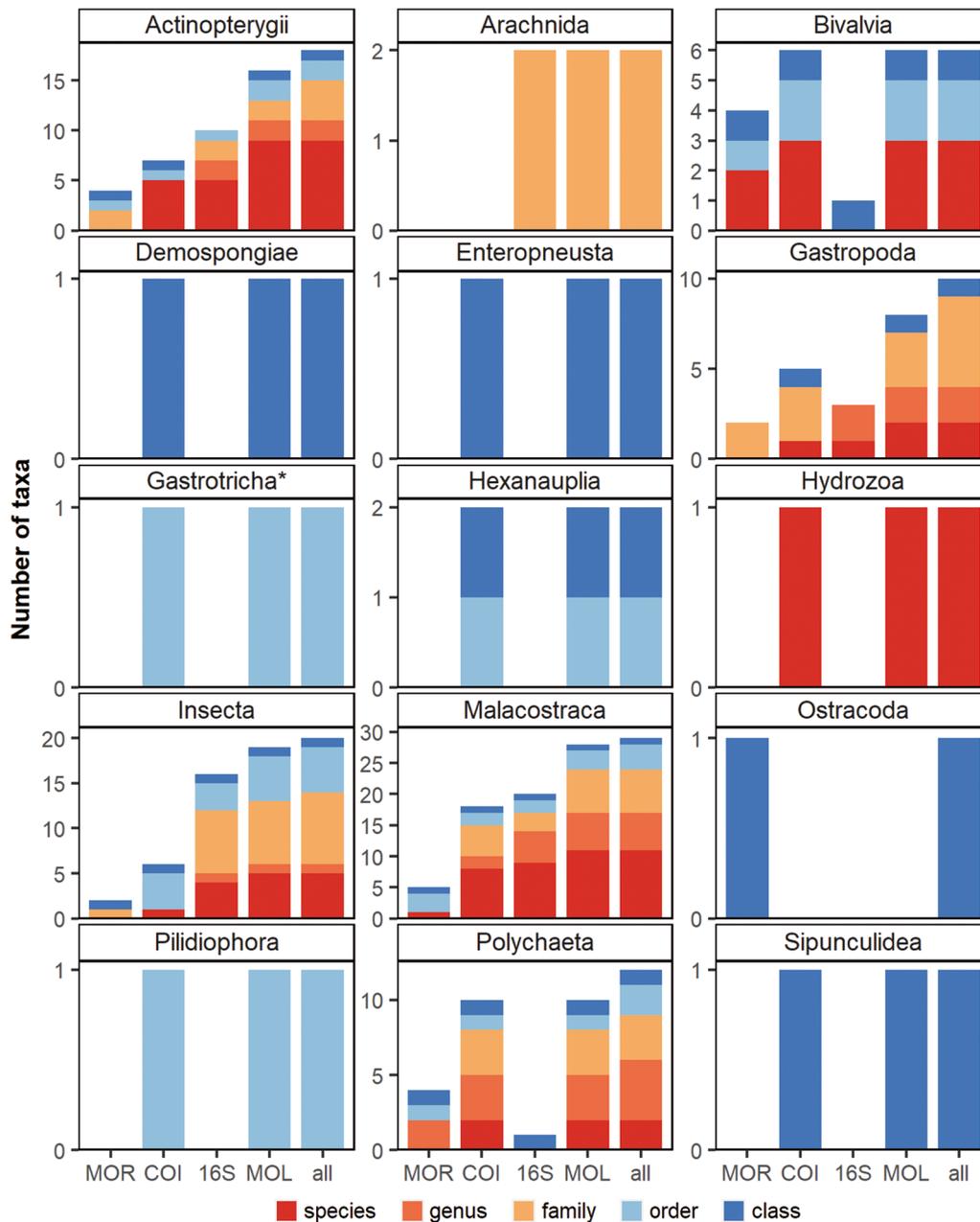


Fig. 5. Comparison of the number of taxa identified in the droppings of the shorebird community at the lowest taxonomic level by different methods among prey classes. *indicates class not assigned; other abbreviations as in Fig. 4. N = 184

Dekinga & Piersma 1993, Quaintenne et al. 2014, Lourenço et al. 2017).

Fish were important prey ($FO \geq 0.5$) for 3 species (common ringed plover, common greenshank, common redshank). Although several shorebird species in West Africa are known to feed on fish (e.g. whimbrel, common redshank, common greenshank; Engelmoer et al. 1984, Zwarts et al. 1990, Ntiamoa-Baidu et al. 1998), this prey is often missed in droppings

(Lourenço et al. 2016, 2017) and is rarely identified to a low taxonomic level, due to the small size and the difficulty in identifying the remains. We found bony fish (Actinopterygii) in the droppings of 7 out of the 15 studied species: these showed surprising diversity, with 7 orders identified, including 7 families and 11 species/genera (namely *Chelon dumerili*, *Porogobius schlegelii*, *Pomadasys rogerii* with $FO \geq 0.5$ in at least 1 species).

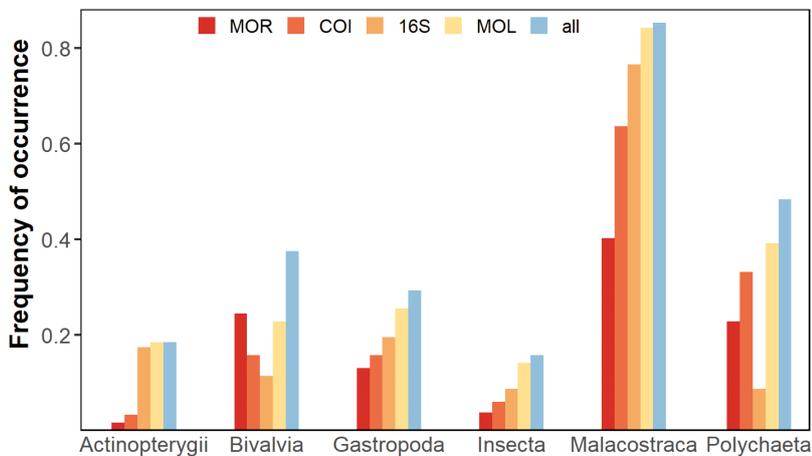


Fig. 6. Frequency of occurrence of prey identified by different methods for the 6 most frequent prey classes found in the droppings of the shorebird community. Abbreviations as in Fig. 4; N = 184

Insects were found to be an important prey for white-fronted plover (FO = 0.7) a species that is frequently found foraging in supratidal beaches, where benthic invertebrates are mostly absent. Insects were also a common prey for common greenshank (FO = 0.5) and grey plover (FO = 0.4), as found in other regions (e.g. Kalejta 1993, Perez-Hurtado et al. 1997). Again, the high diversity of insects found in our study (14 families, none previously described), contrasts with previous studies (e.g. Lourenço et al. 2016, 2017), highlighting the challenge in the identification of this group, particularly in poorly studied tropical regions.

Considering the remaining prey classes, which had mostly negligible contributions for the diet of the shorebird community, 1 species of Hydrozoa, 2 families of Arachnida, 1 order of Pilidiphora, the classes Hexanauplia, Sipunculidea, Demospongiae and Enteropneusta and the phylum Gastrotricha were new to the diet of the studied species.

Overall, common redshank, curlew sandpiper, ringed plover, grey plover and bar-tailed godwit had the more diverse diets within the studied assemblage, reflecting their generalist habits and opportunistic foraging. These species frequently preyed upon crustaceans, bivalves, gastropods and polychaetes as described for most of their range (Pienkowski 1982, Moreira 1996, Perez-Hurtado et al. 1997, Lourenço et al. 2015, 2017). As previously noted, however, the diversity and importance of crustaceans among these species is somehow singular. The frequent consumption of fish by common ringed plover is also noteworthy. Although diet diversity was not assessed for common greenshank (due to the small sample size),

this species consumed a large number of crustaceans and fishes from different orders. In fact, common greenshank seems to be highly specialized in the consumption of highly mobile prey such as small crabs, shrimps and fishes (Engelmoer et al. 1984, Kalejta 1993). On the other hand, Kentish plover, whimbrel and Eurasian oystercatcher showed the lowest richness and diversity in diet composition, reflected by a high degree of specialization in Malacostraca (mostly crabs). Despite the low sample size, the observed crab specialization by Eurasian oystercatcher is also somewhat surprising, as this species is more often described as a molluscivore and/or worm eater (Engelmoer et al. 1984, Boates & Goss-

Custard 1989, Tassie et al. 2011). At Banc d'Arguin in Mauritania, Eurasian oystercatcher feed mostly upon the bloody cockle *Senilia senilis* (Swennen 1990), which is also abundant in the Bijagós Archipelago (Lourenço et al. 2018). Whimbrel, common sandpiper, ruddy turnstone, Kentish plover, common ringed plover and grey plover have been considered to be fiddler crab *Afruca tangeri* specialists in Guinea-Bissau (Zwarts et al. 1990, Lourenço et al. 2017). However, these new findings contradict this idea for the last 2 species, as they presented among the most diverse diets.

Crabs (mainly fiddler crabs but also other species) also dominated the diet of bar-tailed godwit, a species usually referred as a worm specialist during the non-breeding period in the East Atlantic Flyway (e.g. Duijns et al. 2013), including in West Africa (Engelmoer et al. 1984, Lourenço et al. 2016, 2017). Lourenço et al. (2017) had already reported high FO of crustaceans (similar to the consumption of polychaetes) in the diet of this species in the Bijagós Archipelago, but our study reinforces the likely dietary shift of bar-tailed godwit when wintering in this tropical region. The consumption of fiddler crabs was low (FO < 0.5) in only 4 of the studied species: sanderling, dunlin, red knot and common greenshank. Nevertheless, Malacostraca was the most frequent class of prey in all but red knot.

White-fronted plover, the only resident species, foraged most frequently on insects as recorded in other locations, but also consumed crabs much more frequently (FO = 0.57) than previously recorded (McLachlan et al. 1980, van der Merwe et al. 1992, Kalejta 1993).

The high consumption of fiddler crabs recorded in our study is mostly likely explained by their high availability in the Bijagós Archipelago. Indeed, approximately 25% of the intertidal areas of the archipelago (corresponding approximately to 113 km²) is occupied by dense patches of fiddler crabs (Henriques et al. 2022). The distribution and availability of fiddler crabs is expected to be even larger since small individuals also occur outside typical patches (Paulino et al. 2021). On the other hand, the overall importance of crustaceans in the diet of the shorebirds in the Bijagós may also be due to the low abundance of other prey in the study area (Lourenço et al. 2018), as compared with similar communities from other non-breeding locations (e.g. Kalejta 1993, Moreira 1996, Lourenço et al. 2016, Faria et al. 2018).

Our results also highlight the importance of highly mobile prey (not only crabs, but also shrimps and fishes) for the shorebird community of the Bijagós Archipelago, drawing attention to the fact that accurate estimates of prey availability should account for this type of prey that is usually misrepresented, mostly when prey availability is only assessed with sediment core sampling (but see Yong & Lim 2019, Catry et al. 2022). More detailed studies of shorebird diet, preferentially using molecular methodologies, should reveal the relevance of these prey at different sites, and therefore inform the need for employing several prey-sampling techniques.

While describing diet based on FO of prey remains may overestimate the importance of infrequent prey, as well as prey with small importance in terms of biomass, it is acknowledged as a valid approach to describe diet (e.g. Baker et al. 2014). The overestimation of rare prey may be reduced by increasing the sample size. Recent studies using DNA metabarcoding to describe diet have successfully used the read abundance to estimate prey biomass (Verkuil et al. 2022). However, since the number of reads may be mostly influenced by unpredictable PCR and sequencing bias (Deagle et al. 2019, Jusino et al. 2019), presence-absence results may still be considered more reliable in most case studies (Elbrecht & Leese 2015). In our study, in order to minimize sequencing errors but also secondary predation and contamination, we removed every prey item representing <1% of the total reads in each sample within each primer, thus reducing the occurrence of non-target prey items in the diet (e.g. Deagle et al. 2019). Nonetheless, we recognize that for several of the studied species, increasing the sample size as well as the sampling sites within the Bijagós Archipelago could further help quantify the importance of prey described here.

4.2. Comparison of the performance of molecular (COI and 16S primers) and morphological methodologies

Overall, the combination of molecular and morphological methods increased the detail in describing diet composition as compared to any of the methods alone. However, molecular methods largely surpassed the morphological identification both in terms of the taxonomic detail achieved and the number of prey taxa found. Most of the orders and families of prey identified with the 2 primers used in the molecular approach (COI and 16S) were exclusive to each primer, as indicated by the low similarity between the identifications achieved by each (36 and 24% when comparing prey identified to the order and to the family levels, respectively). Therefore, the 2 primers present complementary outputs, rather than duplicating identifications. The difference in the performance between molecular markers was more striking for the classes Bivalvia, Actinopterygii, Polychaeta and Insecta. The COI primer was generally more efficient than the 16S primer in assigning worms and bivalves, but was not very effective in fish and insect identification. Regarding crustaceans, the primers showed somewhat similar efficiencies, but the 2 primers combined increased the number of prey identified. This result is in accordance with previous studies that advocate the use of multiple primers for diet studies (e.g. da Silva et al. 2019), particularly for species that forage on a broad range of taxa, as it is the case of shorebirds in the Bijagós Archipelago.

Despite the overall higher performance of molecular over morphological methodologies in our study, results obtained from DNA metabarcoding analysis may have some drawbacks. This approach tends to over-estimate taxa that are better represented in DNA online databases (BOLD and NCBI), potentially leading to a biased interpretation in diet studies (Sousa et al. 2019). This bias can be particularly striking in certain geographical regions where potential prey are poorly known and broadly misrepresented in those databases, which is the case of intertidal invertebrates in West Africa (e.g. Porter & Hajibabaei 2018, Jażdżewska et al. 2021). In our study, we tried to reduce such bias by sampling prey from the study area, therefore allowing higher match rates with dietary samples. Our sampling effort in the Bijagós improved the identification of 17% of the COI OTUs, but still many Polychaeta, Bivalvia or Gastropoda found in the diet could not be identified to a lower taxonomic level (e.g. species or genus). This suggests that species of those classes occurring in West Africa

remain underrepresented, and that more sampling is needed to obtain a complete representation of the shorebird prey species assemblage. On the other hand, Actinopterygii and Malacostraca were the classes with more taxa identified to the species level (using molecular techniques), most likely because they are more thoroughly represented in online databases (Porter & Hajibabaei 2018).

5. CONCLUSIONS

Studies providing a detailed description of the diet of shorebird communities are surprisingly rare (but see Kalejta 1993, Moreira 1996, Lourenço et al. 2016, Faria et al. 2018, Huang et al. 2022) despite the relevance they have to understand trophic relationships among species, which in turn is key to comprehending the underlying mechanisms structuring communities as well as the persistence of each species in the ecosystem. Our study stresses the high reliance of the Bijagós shorebird community on crustaceans, including not only fiddler crabs, but also shrimps, amphipods and other crabs. The importance of crustaceans may be a typical 'signature' of the Bijagós Archipelago, driven by the low availability of benthic invertebrates more often consumed by shorebirds (Lourenço et al. 2018). By including higher prey diversity in their diet, shorebirds may reduce inter- and intraspecific competition.

Our study is one of the few using DNA metabarcoding to describe the diet of shorebirds, and the results confirm it as a powerful tool, particularly with the combination of the primer sets chosen, adding new taxa and higher taxonomic resolution to the results obtained by traditional methods. Moreover, molecular techniques allow us to validate the bird species identification, which could very easily be misidentified in the field, mainly when samples are collected from multi-species roosts.

Our study further highlights the need for increasing representativeness of invertebrates from West Africa in online barcode databases in order to enhance DNA metabarcoding results. This would require substantial knowledge on the identification of these species and their taxonomy, which is usually limited and time-consuming.

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